MECHANISMS REGULATING PHENOTYPIC CHANGE AND SURVIVAL OF VASCULAR CELLS: STUDIES OF THE EXPRESSION, SUBCELLULAR LOCALISATION AND REGULATION OF FOCAL ADHESION KINASE

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A thesis submitted for the degree of Doctor of Philosophy

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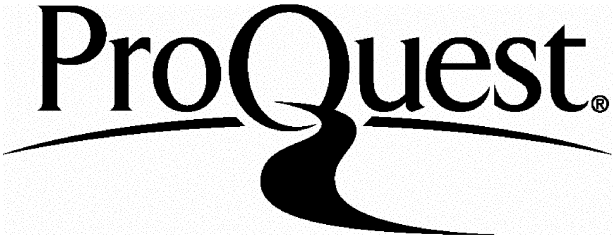

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DEDICATION

To my parents

Acknowledgements

I am most grateful to a number of individuals who have been involved in various aspects of this thesis. Firstly my supervisor, Dr Ian Zachary, whose guidance and advice has been invaluable. I have learnt a great deal from him, and in particular the importance of being critical. I am also indebted to Professor John Martin, my head of department, who has supported me throughout my time in the lab. John's relentless championing of science and his inspirational endeavours have continually innervated me over the years.

I also owe a big thank you to all the members of the Zachary lab who have helped in one way or another during the completion of the thesis. They include Husna Abedi, Barbara Herren, Claire Foley, Robin Abu-Ghazaleh, Tito Kabir, Alasdair Stewart, Haiyan Jia, Sylvie Jezequel, Georgia Gliki, and Spiros Servos.

Finally, a special mention for Dr Rosario Cospedal whose importance to me cannot be overestimated. Thank you for all your advice, support and encouragement, particularly when things were looking bleak.

ABSTRACT

Focal adhesion kinase (FAK) has been implicated in cellular processes linked to adhesion, migration and survival of animal cells. Evidence that FAK is involved in cellular locomotion suggested that it may also play a role in phenotypic transition of VSMC from a quiescent to a migratory state as seen in the development of neointimal hyperplasia. The expression of FAK and two other focal adhesion components, paxillin and $p130^{Cas}$, were upregulated in early aortic medial explant culture, leading to outgrowth and phenotypic modulation of VSMC. Explant treatment with PDGF-BB, IGF-I and bFGF also led to explant outgrowth and increased protein expression of FAK, paxillin and $p130^{Cas}$. These results indicate that increased expression of FAK may play a role in the early changes leading to phenotypic plasticity of VSMC.

Investigation of the subcellular localisation of FAK demonstrated nuclear expression of full length FAK ($p125^{FAK}$) in VSMC and HUVECs, in addition to its localisation to focal adhesions. A p50 NH_2-terminal domain FAK fragment (p50N-FAK) was exclusively localised in the nucleus in both cell types, whilst a p55 COOH-terminal FAK fragment (p55C-FAK) was found only in the cytosol. The findings of nuclear localisation of $p125^{FAK}$ and intracellular segregation of p50N-FAK and p55C-FAK suggest novel regulatory roles for FAK and FAK-related species in vascular cells.

Another major aim of this thesis was to examine the role of FAK in the endothelial cell apoptotic response. Apoptosis of HUVECs resulted in caspase-dependent $p125^{FAK}$ proteolysis and increased nuclear accumulation of p50N-FAK, which were preceded by rapid FAK tyrosine dephosphorylation at residues Y861, Y407 and Y397. Y861 phosphorylation was shown to be maintained via a PKC-dependent mechanism and regulated very early in the HUVEC apoptotic response. These findings provide novel insights into the apoptotic regulation of FAK in HUVECs and the signalling mechanisms important for maintaining FAK phosphorylation, which may have relevance for VSMC and other cell types.

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List of Abbreviations

AIF	apoptosis inducing factor
bFGF	basic fibroblast growth factor
CAKβ	cell adhesion kinase β
CAMP	cyclic adenosine 3'-5'-monophosphate
Cas	Crk-associated substrate
CHO	Chinese hamster ovary
$cPLA_2$	cytosolic phospholipase A_2
DCVC	dichlorovinylcysteine
DMEM	Dulbecco's modification of Eagle's medium
EC	endothelial cell
EBM	endothelial basal medium
ECM	extracellular matrix
EGF	epidermal growth factor
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
ET-1	endothelin-1
FA	focal adhesion
FAK	focal adhesion kinase
FADD	Fas-associated death domain protein

FAT	focal adhesion targetting
FBS	foetal calf serum
FITC	fluorescein isothiocyanate
FGFR	fibroblast growth factor receptor
FLICE	FADD-homologous ICE/Ced-3-like protease
FLIP	FLICE-inhibitory proteins
FRNK	FAK-related non-kinase
FSS	fluid shear stress
GPI	glycosylphosphatidylinositol
GRAF	GTPase regulator associated with FAK
GRB2	growth factor receptor-bound protein 2
HEF-1	human enhancer of filamentation-1
HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cell
IAP	inhibitor of apoptosis protein
ICE	interleukin-1 beta-converting enzyme
IGF-I	insulin-like growth factor-I
IGF-IR	IGF-I receptor
IRS-1	insulin receptor substrate-1
JNK/SAPK	Jun N-terminal kinases/stress-activated protein kinases
LDL	low density lipoprotein
LPA	lysophosphatidic acid
MAP kinase	mitogen-activated protein kinase
MHC	myosin heavy chain
MMP	matrix metalloproteinase
NLS	nuclear localisation sequence
NO	nitric oxide
NRPTK	non-receptor protein tyrosine kinase
ox-LDL	oxidised LDL
$p130^{Cas}$	p130 (Crk-associated substrate)
PA	plasminogen activator
PAF	platelet activating factor
PAI	plasminogen activator inhibitor
PBS	phosphate-buffered saline

PCD	programmed cell death
PCNA	proliferating cell nuclear antigen
PDGF	platelet-derived growth factor
PGI_2	prostacyclin
PI3-K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PLGF	placental growth factor
PRNK	PYK2-related non kinase
PTK	protein tyrosine kinase
PYK2	proline-rich tyrosine kinase 2
RAFTK	related adhesion focal tyrosine kinase
RGD	Arginine Glycine Asparagine
RPTK	receptor protein tyrosine kinase
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SH2	*src* homology 2
SH3	*src* homology 3
TGF-β	transforming growth factor-beta
TNF-α	tumour necrosis factor-alpha
TNFR1	tumour necrosis factor-alpha receptor 1
TPA	tissue-type plasminogen activator
TRADD	TNFR-associated death domain protein
TRAIL	TNF-related apoptosis-inducing ligand
TRITC	tetramethylrhodamine isothiocyanate
TUNEL	TdT-mediated dUTP nick end-labelling
UPA	urokinase-type plasminogen activator
UPAR	UPA receptor
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cell
ZVAD-fmk	benzyloxycarbonyl-valine-alanine-aspartate fluoromethylketone

CHAPTER 1
INTRODUCTION

The cardiovascular system in animals is composed of the heart and vasculature including the arterial and venous systems. The arterial and venous networks are connected by means of capillary vessels, which are responsible for delivering oxygen/nutrients to tissues. Many diseases of the cardiovascular system arise from abnormalities in one or more of its components and are responsible world-wide for an ever-increasing burden upon the health services. For instance in the Western Hemisphere approximately 50% of deaths are caused by cardiovascular disease, mostly accounted for by ischaemic heart disease. In other parts of the world, particularly developing countries, rheumatic heart disease is a major cause of hospital admissions. Globally speaking, hypertension is another major cause of morbidity and mortality and is a prime risk factor for atherosclerosis, which is the major cause of ischaemic heart disease. A better understanding of the pathophysiological mechanisms underlying the development of cardiovascular disease is essential to improve our ability to prevent and to treat these conditions.

1.1 ATHEROSCLEROSIS

Atherosclerosis is one of the most intensely studied human disease processes and has preoccupied researchers since the early 19th century. Atherosclerosis is a disease which affects arteries of all sizes including the aorta and coronary arteries but not vessels smaller than 3mm diameter (arterioles). In the initial stages of development, it is characterised by the 'fatty streak' in which lipid–rich macrophages and T lymphocytes accumulate in the intima (Stary, 1989; McGill, 1984). Some but not all of these fatty streaks go on to develop into intermediate lesions comprising macrophages and smooth muscle cells. These latter lesions preferentially arise at focal points in the arterial tree such as branch points or bifurcations. At a later stage, these lesions can develop into advanced lesions recognised macroscopically as the atherosclerotic plaque. This is composed of a core of lipid and /or necrotic debris covered by a dense connective tissue

cap containing vascular smooth muscle cells (Ross, 1993). It is the advanced lesion which is responsible for arterial luminal occlusion and restriction of blood supply leading to symptoms of ischaemic heart disease. In the worst case the plaque can rupture and the subsequent inflammatory/procoagulant response give rise to complete vascular occlusion, best recognised in the instance of coronary artery occlusion and subsequent myocardial infarction (Davies and Thomas, 1984).

Although fatty streaks have been recognised in the vasculature of children and teenagers (World Health Organisation, 1985; Stary, 1989; McGill, 1984), the intermediate and advanced lesions of atherosclerosis do not generally arise until much later in adult life (except in the case of youths with congenital abnormalities of lipid metabolism). The exact mechanisms by which fatty streaks arise or what predisposes them to develop into more advanced lesions remains unclear. Conventionally the lesion of atherosclerosis is thought of as a fibroproliferative or inflammatory response to various factors which cause the disease. In some situations the response may become exaggerated and thereby become part of and perpetuate the disease process. Previously researchers had identified the following components to be requisite for lesion formation: smooth muscle proliferation (with macrophage/lymphocyte involvement), elaboration of a connective tissue matrix, and the intracellular and extracellular accumulation of lipid (Fagiotto *et al.,* 1984a, 1984b; Mora *et al.,* 1987; Thomas *et al.,* 1985).

There are many theories which have been proposed to explain the pathogenesis of the atherosclerotic plaque. Perhaps the best known and most widely accepted is the response to injury theory (Ross and Glomset, 1973: Ross, 1986) in which it was postulated that endothelial cell dysfunction arising from various forms of insult (e.g. exposure to oxidised LDL), might predispose to increased adherence of leukocytes such as monocytes and T lymphocytes. Subsequently the attached white blood cells, under the influence of autocrine or paracrine factors, migrate into the arterial wall. Here they are able to accumulate lipid and form the lesion recognised as the fatty streak, followed later by the proliferation of smooth muscle cells and deposition of matrix leading to the development of the more advanced lesions.

Other theories of atherosclerosis include those proposing lesion development as a response to infection ("viral hypothesis", Fabricant *et al.,* 1983; "chlamydia and

atherosclerosis", Grayston, 1993), to chemical insult (Golubev *et al.,* 1996; "free radical theory" Dormandy, 1989), to hypoxia (Martin *et al.,* 1990). In these studies and in many others, it has become clear that lesion formation in atherosclerosis or post-angioplasty restenosis is characterised by the accumulation of cells within a 'neointima'. Common to all these studies is the notion that excessive cellular proliferation is the sole determinant of lesion cellularity. Recently, however, a growing body of evidence suggests that vascular structure and lesion formation is determined by a balance between cell growth and cell death by apoptosis. At this stage relatively little is known about the factors which regulate vascular cell survival, although clearly this is an area which could provide new therapeutic targets for the treatment of vascular disease.

VASCULAR CELLS IN ATHEROSCLEROSIS.

The normal arterial wall consists of three anatomically distinct layers – the intima, the media and the adventitia (see Fig 1.1). The intima is made up of a monolayer of endothelial cells surrounded by a sheet of elastic fibres known as the internal elastic lamina. The area between the internal elastic lamina and the endothelium is filled with extracellular matrix and beyond the lamina lies the medial layer. This consists of circumferentially oriented smooth muscle cells deposited in a meshwork of interstitial extracellular matrix. Separating the media from the outermost layer or adventitia is the external elastic lamina. The adventitia is composed of fibroblasts and smooth muscle cells loosely embedded in a matrix of collagen and proteoglycans.

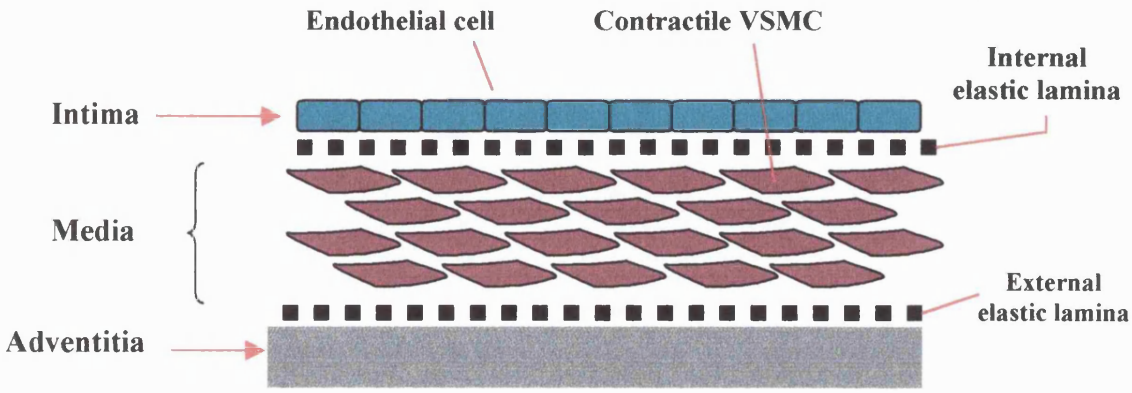


Fig. 1.1 Schematic representation of a normal artery.

1.2 VASCULAR SMOOTH MUSCLE CELLS.

1.2.1 Biological roles of VSMC

In the mature animal the vascular smooth muscle cell (VSMC) is a highly specialised cell whose principal function is contraction. During animal development, vascular smooth muscle cell precursors have an essential role in the maturation of the embryonic vasculature during which period they serve to proliferate and elaborate extracellular matrix (ECM) components of the vascular wall. Later, the fully differentiated VSMC ceases to proliferate and is specialised for the function of contraction and the maintenance of vascular tone. Despite this however, it is clear that mature VSMCs have considerable plasticity, because they retain the ability to divide. This is well illustrated by their roles in diverse biological and pathological processes - such as wound healing, atherosclerosis and restenosis – which are characterised by the migration and proliferation of VSMC (Ross 1993).

According to the response to injury models proposed by Ross, an excessive fibroproliferative response by VSMC is seen to contribute to the formation of atherosclerotic lesions. Such lesions of atherosclerosis predominantly affect the intimal layer of the vasculature, which in normal vessels is largely devoid of VSMC. Although the origins of the VSMC which accumulate in the neo-intima remain unclear, it is generally accepted that their involvement results from migration through the internal elastic lamina and proliferation within the sub-endothelium. A considerable body of evidence supports a role for certain growth factors in this process (Nilsson, 1986; Munro and Cotran, 1988; Ross and Raines, 1988).

1.2.2 VSMC proliferation

The mechanisms whereby growth factors may regulate VSMC proliferation in vascular disorders have been extensively examined and the role of mitogen-responsive transcription factors (proto-oncogenes: *c-myc, c-myb*) as well as other nuclear factors, including the retinoblastoma protein, is being elucidated (for a review see Gorski and Walsh, 1985). Further evidence for the importance of VSMC proliferation in the

formation of lesions of atherosclerosis comes from studies which utilised anti-proliferative modalities to effect a reduction in the development of neointimal lesions (Chang *et al.*, 1995a, 1995b; Bennett *et al.*, 1994; Shi *et al.*, 1994). Recently it has been proposed that cell cycle-dependent inhibition of VSMC proliferation by progesterone may represent a mechanism for the hormone's protective effect against atherosclerosis (Lee *et al.*, 1997).

1.2.3 Migration of VSMC

Studies of rates of VSMC proliferation indicate that indices of proliferation are raised in neointimal lesions, but not sufficiently to account for neointimal VSMC accumulation. In atherosclerosis, rates of proliferation derived from labelling indices are very low (Clowes *et al.*, 1983; Newby and George, 1993), but they are higher in models of restenosis and in post-coronary artery bypass grafts (Ip *et al.*, 1990). There is a large body of evidence deriving mostly from studies in the rat carotid artery balloon injury model, to support the role of VSMC migration in the pathogenesis of atherosclerotic lesions (Raines and Ross, 1993; Dartsch *et al.*, 1989; Jawien *et al.*, 1992). Additionally the expression of potent chemoattractants such as PDGF-BB is significantly upregulated in lesions of atherosclerosis and post-angioplasty restenosis in the rat carotid artery (Wilcox *et al.*, 1988; Majesky *et al.*, 1990; Golden *et al.*, 1991). Antibodies directed against PDGF have been shown to block neointima formation in the injured rat carotid by the inhibition of VSMC migration (Ferns *et al.*, 1991). There is evidence that VSMC migration also plays an important role in other pathophysiological situations including hypertension (Murphy *et al.*, 1981) and accelerated arteriopathy following cardiac transplantation (Billingham, 1987).

Migration and/or proliferation of VSMCs have clearly been shown to be affected by growth factors and polypeptide ligands for receptor tyrosine kinases. However, many other factors are thought to influence the behaviour of VSMCs in the vessel wall. Amongst these are cytokines such as TGF-β (Koyama *et al.*, 1990), and Interleukin-1, (Nomoto *et al.*, 1988), adhesion molecules, in particular the integrin family (Yue *et al.*, 1994) and components of the ECM (Naito *et al.*, 1991; Liaw *et al.*, 1994). Several integrins, including $\alpha_v\beta_3$ and β_1 integrins, are thought to play an important role in

VSMC migration *in vivo* (Clyman *et al.*, 1992; Yue *et al.*, 1994; Bilato *et al.*, 1997). In addition, recent interest has focused on the role of the ECM and enzymes responsible for the degradation of the matrix. The matrix metalloproteinases (MMPs) (Southgate *et al.*, 1992; Henney *et al.*, 1991) and serine proteases involved in the plasminogen activation system such as urokinase- type plasminogen activator (UPA), tissue-type plasminogen activator (TPA) (Jackson and Reidy, 1992) and thrombin (Walters *et al.*, 1993), are strongly implicated in the early events associated with neointima formation and VSMC migration. As well as its function in coagulation, thrombin is also well established to act through specific transmembrane receptors which mediate important actions on cells (Hung *et al.,* 1992; McNamarra *et al.* 1992). In addition, recent studies have demonstrated that UPA can act via its own cell surface receptor to stimulate early signalling events (Busso *et al.,* 1994; Resnati *et al.,* 1996).

1.2.4 Phenotypic modulation of VSMC

The neointimal accumulation of VSMC in atherosclerosis is associated with a striking change in the observed phenotype of "contractile" VSMC which populate the media, to a "synthetic" phenotype characteristic of VSMC found in the neointima (Campbell *et al.*, 1988). Adult contractile VSMC contain abundant myofilaments, poorly developed Golgi apparatus and minimal rough endoplasmic reticulum (Fig. 1.2). This constitutes the "contractile" phenotype (Chamley-Campbell and Campbell, 1981; Raines and Ross, 1993). Whilst contractile cells residing in the media are non-proliferating, fully differentiated and provide vascular tone, neointimal VSMC undergo a loss of contractile components, but are rich in synthetic organelles such as the Golgi apparatus and endoplasmic reticulum. It is these cells which are thought to be responsive to chemotactic and mitogenic factors and are able to synthesize ECM, whilst those residing in the media are capable only of responding to vasoactive agents causing contraction and relaxation (reviewed in Gorski and Walsh, 1995). Similarly diploid VSMC grown in culture in serum-free conditions retain some degree of their characteristic contractile phenotype and grow in the well recognised "hill and valley" pattern that distinguishes them clearly from endothelial cells or fibroblasts. However upon exposure to serum derived from whole blood, they begin to proliferate and redifferentiate to a synthetic phenotype (Campbell and Campbell, 1993).

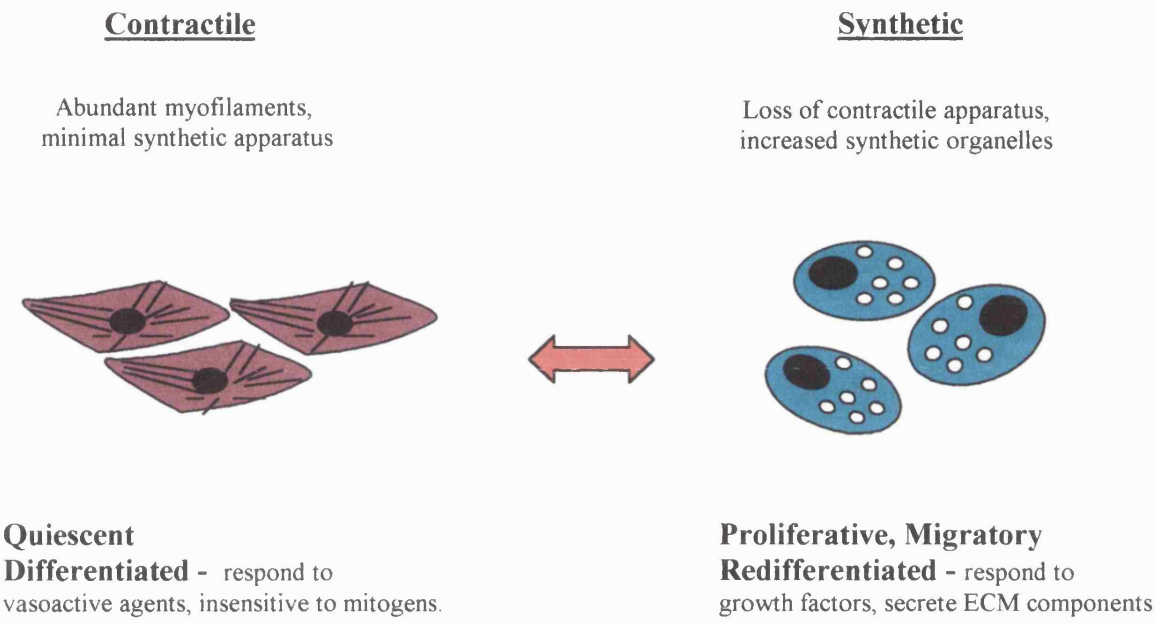


Fig. 1.2 Comparison of VSMC phenotypes

A change in VSMC phenotype from a contractile to a synthetic state in the development of neointimal hyperplasia is likely to involve changes in gene expression. Changes in gene expression are also well recognised in VSMCs during animal development (Katoh and Perisamy, 1996). VSMC are capable of expressing four different isoforms of actin including α-actin, β-actin, smooth muscle γ-actin and non-muscle γ-actin. In adult fully differentiated contractile VSMC, α-actin is the most abundant isoform whereas in the fetus and newborn animal, β-actin predominates (Owens and Thompson, 1986). Intimal ("synthetic" phenotype) VSMC display reduced expression of cytoskeletal and contractile proteins such as smooth muscle α-actin, smooth muscle myosin heavy chain (MHC), 150 kDa VSMC-specific isoform of

caldesmon, vinculin and desmin (Owens, 1995). In atherosclerotic plaques intimal VSMC express β-actin as the principal isoform (Gabbiani *et al.*, 1984) see also Table 1.1

VSMC PROTEIN	YOUNG/FETAL VSMC	ADULT VSMC	SYNTHETIC STATE CELLS IN CULTURE	REPLICATIVE RESPONSE TO INJURY
α-actin	+/-	++++	+/-	+/-
β-actin	++	++	++	++
γ-actin	+	+	+	+
Myosin	+/-	+++	+/-	+/-
Desmin	+/-	+++	+/-	+/-

Table 1.1 Phenotypic changes in cytoskeletal proteins in VSMC.

Studies of differential gene expression in contractile and proliferating rat VSMC indicate that some components of the contractile machinery are highly expressed in both types of VSMC, while expression of other components is markedly altered (Shanahan *et al.*, 1993; Gabbiani *et al.*, 1984). Examples of proteins that are upregulated in proliferating VSMC include osteopontin and matrix GLA protein (Shanahan *et al.*, 1993), and PDGF ligand and receptor (Wilcox *et al.*, 1988; Majesky *et al.*, 1990). Furthermore, rabbit VSMC in primary culture show a large upregulation in the number of cell surface receptors for basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) (Saltis *et al.*, 1995). Thus far, however, phenotype-specific changes in the expression of signalling molecules important for migration and proliferation of VSMC remain to be characterised.

The culture of VSMC by explant outgrowth is a potentially useful model for the study of such changes in gene expression (Campbell and Campbell, 1993). This model facilitates examination of the regulation of the very early stages of VSMC migration/proliferation *in vitro.* This is in contrast to studies of passaged VSMCs in primary culture where changes in phenotype may have already occurred. This model

also enables assessment of the effects of test factors (for instance growth factors) on phenotypic modulation of VSMC and accompanying molecular events.

1.2.5 VSMC and apoptosis

An accumulating body of evidence indicates a role for apoptosis in cardiovascular diseases such as atherosclerosis and restenosis. In the balloon-injured rat carotid artery, Clowes and others demonstrated that VSMC retained relatively high proliferative capacity even up until 12 weeks after injury. However at 12 weeks total arterial VSMC content was unchanged leading them to conclude that "cell death must account for our finding" (Clowes *et al.,* 1983). Subsequently, numerous studies have demonstrated apoptosis of neointimal VSMCs and foam cells to be a feature of human vascular pathology, especially restenotic lesions, and to a lesser degree, primary atherosclerotic lesions (Geng and Libby, 1995; Han *et al.*, 1995; Ball *et al.*, 1995; Bjorkerud and Bjorkerud, 1996; Isner *et al.*, 1995; Kearney *et al.*, 1997). It remains unclear whether apoptosis represents a harmful or beneficial mechanism in the context of vascular disease. Some investigators have hypothesised that a high apoptotic index of lesion VSMC may contribute to plaque instability and increased likelihood of clinical events (Geng and Libby, 1995; Han *et al.*, 1995; Bjorkerud and Bjorkerud, 1996). On the other hand it has been proposed that apoptosis may significantly reduce the cellularity of lesions, particularly those arising due to restenosis (Isner *et al.*, 1995). In such a situation, an enhanced apoptotic index might represent a useful therapeutic target to attenuate intimal hyperplasia.

1.3 ENDOTHELIAL CELLS

1.3.1 Biology of endothelial cells

Vascular endothelial cells are derived from angioblasts – cells of mesodermal origin defined as vascular endothelial cells that have not yet formed a lumen. Angioblasts are responsible for the formation of the embryonic primary vascular plexus by a process known as vasculogenesis. Endothelial cells (EC) constitute the endothelium, a monolayer forming the innermost surface of the vasculature or intimal layer, and perform several physiological functions. Among these are the provision of a non-thrombogenic surface, the maintenance of vascular tone through release of vasoactive agents (see Table 1.2) such as nitric oxide (NO) (Moncada and Higgs, 1991), prostacyclin (PGI_2) and endothelin (ET), and the regulation of leukocyte adhesion (reviewed in Cines *et al.*, 1998). EC are a rich source of growth regulatory molecules and cytokines such as TGF-β, γ-interferon, heparin/heparan sulphate, PDGF and bFGF and play an active role in maintaining the basement membrane which forms their substrate.

The endothelium acts as a gatekeeper controlling both the passage of substances across the vessel wall (Gimbrone, 1976) as well as the flow of blood cells along the vessel lumen. Whilst blood flow derives in part from the ability of quiescent endothelial cells to generate an active anti-thrombotic surface, it is also crucially dependent upon the secretion and uptake of vasoactive agents by the endothelium in response to various stimuli. The vasodilator NO acts by relaxing VSMC and, in addition, inhibits platelet adhesion, activation, secretion and aggregation (Mendelsohn *et al.*, 1990). Endothelial-derived NO also inhibits leukocyte adhesion to the endothelium (Kubes *et al.*, 1991; De Caterina *et al.*, 1995) and inhibits VSMC migration (Marks *et al.*, 1995) and proliferation (Garg and Hassid, 1989). These effects may be important in attenuating the vascular response to injury.

Molecule	Main effect	Other effects	Secretion	Compound
NO (Nitric Oxide)	Vasodilatation	Maintains basal vascular tone ↓ Platelet adhesion/activation, secretion and aggregation. ↑ Platelet disaggregation Inhibits VSMC migration/proliferation	Paracrine/constitutive and induced by thrombin, bradykinin, substance P, ADP, muscarinic agonists, FSS, cytokines	Heterodiatomic free radical derived from L-arginine.
PGI_2 (prostacyclin)	Vasodilatation	Inhibits platelet aggregation and deposition Inhibits VSMC proliferation	Paracrine/induced at sites of EC perturbation	Eicosanoid derived from Arachidonic acid
PAF (platelet activating factor)	Vasoconstriction	Promotes leukocyte adhesion at cell surface	Juxtracrine/induced	Phospholipid derived from Arachidonic acid
ET-1 (endothelin-1)	Vasoconstriction	Mitogen for VSMC: modulates effects of numerous compounds	Paracrine/induced by hypoxia, shear stress and ischaemia	21 Amino acid peptide derived from Preproendothelin-1 (203 amino acids)

Table 1.2. Vasoregulatory substances synthesised by the endothelium.
(Adapted from Cines *et al.*, 1998)

The natural anti-thrombotic properties of the endothelium derive from its ability to regulate the activity of thrombin (reviewed in Rosenberg and Rosenberg, 1984) and produce plasminogen activators (Hirsh *et al.*, 1994; Levin *et al.*, 1989; Cugno *et al.*, 1989). However, a pro-coagulant intima can arise following endothelial perturbation by physical forces (fluid shear stress) or specific factors which lead to the induction of tissue factor (Drake *et al.*, 1989) and plasminogen activator inhibitors (Sawdey *et al.*, 1991). Haemodynamic forces play an important role in the physiology and pathology of the arterial wall (Caro *et al.*, 1971; Dewey, 1984; Glagov, 1972). Fluid shear stress (FSS) is defined as the tractive force exerted upon the endothelial surface by flowing blood. FSS has been implicated in the development of atherosclerosis for some time following observations that a strong correlation existed between the location of developing arterial lesions and the regions where there are extremes of variations in FSS (Fry, 1968). Whilst earlier studies defined shear stress-induced alterations in EC morphology and cytoskeletal organisation (Dewey *et al.*, 1981; White *et al.*, 1983), more recently it has been shown that EC can sense FSS and trigger complex signalling pathways resulting in the induction of immediate early genes (Resnick and Gimbrone,

1995; Li *et al.*, 1996; Takahashi and Berk, 1997; Jo *et al*, 1997). At present the underlying mechanisms by which mechanotransduction serves to activate downstream signalling events remain unclear.

1.3.2 EC Dysfunction

Earlier observations in experimental animals showed that endothelial damage and loss – whether by catheter abrasion, chemical insult such as homocystinemia, or hypercholesterolaemia – led to a platelet response as well as neointimal formation (Ross and Glomset, 1976). Such observations were responsible in part for the 'response to injury' hypothesis of atherosclerosis proposed by Ross. Later it became clear that endothelial denudation is not a prerequisite for lesion development and currently it is loss of endothelial integrity or endothelial dysfunction which is thought to be an important factor in the initiation of the atherosclerotic lesion (Ross, 1997: McGorisk and Treasure, 1996).

Endothelial dysfunction can arise through several mechanisms, one of which - oxidant stress – has received considerable attention. Unsaturated lipids can be modified (in some cases by EC) to cytotoxic derivatives such as oxidised LDL (ox-LDL) and lipid peroxidation products including lysophosphatidylcholine, which have deleterious effects upon EC. ox-LDL can induce transcriptional activation of vascular cell adhesion molecule-1 (VCAM-1) promoting leukocyte adhesion, impair EC replication and angiogenesis and induce apoptosis (Henry, 1994).

It remains unclear how alterations in FSS are linked to EC dysfunction. Atherosclerotic lesions preferentially develop at focal sites in the arterial system such as at bifurcations and curved regions where flow is interrupted by flow separators (Glagov, 1988; Nerem *et al.*, 1993). In these lesion-prone areas FSS is low and unsteady and, in the instance of coronary branch points, associated with decreased vasodilator function (McLenachan *et al.*, 1990). The expression of diverse genes for proteins which regulate the functions of EC and VSMC, can be induced by means of shear stress response elements. These include genes whose products are involved in vasoregulation (NO, ET-1), control of VSMC (bFGF, PDGF, TGF-β), fibrinolysis (t-PA.), and cell adhesion

(VCAM-1, ICAM-1) (Malek and Izumo, 1995; Ando and Kamiya, 1996; Tsao *et al.*, 1996). Turbulent FSS has also been linked to increased DNA synthesis by EC *in vitro* (Davies *et al.*, 1986).

Endothelial dysfunction is reflected in abnormal vasodilator function in atherosclerotic arteries due to impaired release of NO and PGI_2 from EC (Ludmer *et al.*, 1986; Siegel *et al.*, 1993). Even patients lacking overt vessel stenosis but with risk factors for atherosclerosis (hypercholesterolaemia, diabetes, cigarette smokers), have impaired vasodilatation (Sorensen *et al.*, 1994; Steinberg *et al.*, 1996; Celermajer *et al.*, 1993). In some instances the reduction in NO is attributable in part to decreased expression of eNOS in diseased arteries (Buttery *et al.*, 1996), but the mechanism for this is unknown. However, it seems reasonable to hypothesise that perturbation of FSS may be implicated given the predilection of lesions for branch sites. Further evidence for the role of NO came from animal models in which inhibition of NO activity accelerates atherosclerosis (Naruse *et al.*, 1994), whereas supplementation with a NO precursor attenuates lesions (Cooke, 1996) and reverses endothelial dysfunction in hypercholesterolaemic human subjects (Creager *et al.*, 1992).

A healthy endothelium may contribute to the prevention of atherosclerosis by inhibiting platelet activation, limiting transendothelial passage of cells and lipids into the vessel wall and maintaining the intima in a quiescent state. Loss of these protective functions – for instance consequent upon oxidant, chemical or shear stress – appear to alter the biochemical profile of the endothelium in such a way as to promote inflammatory/fibroproliferative responses. *In vivo* endothelial cells retain their ability to regenerate and are responsible for new blood vessel development by the process of angiogenesis. Endothelial cells in culture grow in a strict monolayer and appear to be truly contact inhibited. Whether they are of arterial or venous origin they retain a differentiated phenotype (Ross and Glomset 1976) although it is accepted that cell culture does perturb them from a quiescent *in vivo* state to a more activated phenotype capable of rapid replication (Cines *et al.*, 1998).

1.3.3 Apoptosis of EC

A major cause of EC dysfunction may be programmed cell death or apoptosis. Apoptosis constitutes a systematic means of cell suicide within an organism during normal morphogenesis, tissue remodelling and in certain pathophysiological situations (Ueda and Shah, 1994; see also section 1.6). EC apoptosis has proven difficult to establish *in vivo* in either physiological or pathological situations. Reasons for this may be due to the extrusion of EC into the lumen of vessels where they are lost or due to the rapidity of the apoptotic process which is complete in a matter of hours. Indeed, the presence of apoptosis in instances where the dying cells form a small part of the overall tissue, is likely to be underestimated (Kerr *et al.*, 1995). However apoptosis of EC has been well documented *in vivo* in a variety of physiological circumstances including regression of the corpus luteum (Azmi and O'Shea, 1984), limb morphogenesis in chicken embryo (Feinberg *et al.*, 1986), renal regeneration (Gobe *et al.*, 1995), breast involution in mice (Walker *et al.*, 1989) and atrophy of the rat parotid gland (Walker and Gobe, 1987). More often it has been easier to demonstrate EC apoptosis in *in vitro* models (Araki *et al.*, 1990; Robaye *et al.*, 1991; Lefer and Ma, 1993; Pulonovsky *et al.*, 1994). Interestingly fluid shear stress was reported to inhibit EC apoptosis (Dimmeler *et al.*, 1996, 1997a; Kaiser *et al.*, 1997), which may go some way towards explaining the correlation between extremes of FSS and EC dysfunction (see 1.3.2).

Whether apoptosis of EC plays a role in the development of cardiovascular disease remains to be proven directly. However, recently, interesting reports have emerged suggesting that this particular mode of cell death may have been overlooked as a contributory factor in the pathogenesis of some vasculopathies. Gobe *et al.* (1997) found evidence of EC apoptosis in the development of microvascular rarefaction in hypertension using an established rodent model of hypertension. An important study of diseased human coronary arteries found apoptosis to be a feature of restenotic lesions and, to a lesser extent, primary atherosclerotic lesions (Isner *et al.*, 1995). The apoptotic cells were either positively identified as VSMC or macrophages but the identity of approximately 50% of cells could not be established, suggesting that EC apoptosis may have been underestimated. In a more recent study, apoptotic EC were detected on the luminal surface of atherosclerotic human coronary vessels but not in normal vessels (Alvarez Jr. *et al.*, 1997). A major caveat of this study is that the coronaries were

obtained from post-transplant patients with accelerated graft arteriosclerosis and therefore the finding of EC apoptosis may be restricted to this particular pathological entity rather than extended to all forms of atherosclerosis.

1.4 POLYPEPTIDE GROWTH FACTORS FOR VSMC AND ENDOTHELIAL CELLS

1.4.1 Platelet-derived growth factor (PDGF).

PDGF is a 30 kDa dimeric glycoprotein consisting of two different polypeptide chains - A and B - connected by disulphide bonds. In the original models of atherosclerosis proposed by Ross (Ross, 1986; Ross and Raines, 1988), PDGF of monocyte/platelet/endothelial-origin was suggested to be the initial stimulus for VSMC following endothelial injury. Thereafter chemotaxis and proliferation of VSMC was thought to give rise to the intimal proliferative lesion of early atherosclerosis. Subsequently it was shown that PDGF-BB is the most potent chemoattractant for cultured VSMC derived from a number of species (reviewed in Ross, 1993). The mRNA for PDGF ligand and receptors are all upregulated in human atherosclerotic plaques (Wilcox *et al.,* 1988), and in rat and baboon neointimal tissue induced by vascular injury or vascular grafting (Majesky *et al.,* 1990; Golden *et al.,* 1991). Furthermore, in rat balloon-injury models of atherosclerosis, PDGF-stimulated neointima formation involved predominantly migration of VSMC with little increase in proliferation (Jawien *et al.,* 1992). In addition, it was shown that an antibody to PDGF was able to inhibit neointimal VSMC accumulation in the rat carotid artery (Ferns *et al.*, 1991, Jawien *et al.,* 1992). These findings indicate a major role for PDGF-induced chemotaxis of VSMC in the development of neointimal lesions both in atherosclerosis and in post-angioplasty restenosis.

Given that PDGF has a half-life in circulation of only two minutes and has negligible levels in plasma under normal circumstances, this would suggest predominantly local synthesis and secretion for effective cellular responses. It has been reported that several cell types are indeed capable of providing a local source of PDGF to the vessel wall including circulating cells such as monocytes/macrophages (Shimokado *et al.*, 1985) in addition to platelets. Cultured megakaryocytes and endothelial cells (DiCorleto and Bowen-Pope, 1983), as well as VSMC (Walker *et al.*, 1986; Raines and Ross, 1993), were also demonstrated to produce PDGF.

The PDGF receptor is a transmembrane tyrosine kinase which becomes internalised and downregulated following ligand binding. Two isotypes of the PDGF receptor have been identified, the α and β receptor which exhibit different affinities for PDGF ligand isoforms. Whilst the α-receptor recognises all three isoforms of PDGF ligand (PDGF-AA, BB and AB), the β-receptor exhibits high-affinity binding of only the PDGF-B chain (Bowen-Pope *et al.,* 1989; Hart *et al.,* 1988; Gronwald *et al.,* 1988; Seifert *et al.,* 1989). An increasing body of evidence suggests that the different isoforms of PDGF ligand and receptor may mediate distinct cellular functions within the same cell-type. For example PDGF-BB is able to stimulate both cell migration and mitogenesis in VSMC from a number of different species. On the other hand, PDGF-AA, whilst able to stimulate DNA synthesis and cell proliferation in cultured VSMC (Majack *et al.,* 1990, Koyama *et al.,* 1994), appears to have no chemotactic activity in VSMC from a variety of species, and was shown to inhibit PDGF-BB-induced chemotaxis in baboon VSMC (Koyama *et al.,* 1992, 1994; Bornfeldt *et al.,* 1994b; Siegbahn *et al.,* 1990). In addition expression of PDGF-α and -β receptors was shown to be phenotype dependent in rat arterial smooth muscle cells with distinct upregulation of the PDGF-α receptor following phenotypic modulation of VSMC (Sjolund *et al.,* 1990). The PDGF-A chain gene is also specifically expressed in 'synthetic' VSMC (Sjolund *et al.,* 1988). Although PDGF-β receptor expression was not significantly altered in cultured primary rabbit VSMC undergoing phenotypic transition, the phenotypically modulated cells still demonstrated a marked proliferative response to PDGF-BB as determined by [^{3}H]thymidine incorporation (Saltis *et al.,* 1995).

Several PDGF-receptor mediated signalling events have been identified. These include phosphorylation of phospholipase C-γ, the p85α subunit of phosphatidylinositol 3'-kinase and GAP (Ullrich and Schlessinger, 1990; Cantley *et al.,* 1991). PDGF was also demonstrated to stimulate early activation of the mitogen-activated protein kinase (MAPK) pathway in human arterial smooth muscle cells (Graves *et al.,* 1993). The downstream signalling pathways which might specifically mediate the effects of PDGF on VSMC migration are undefined. It has been shown, however, that PDGF-BB stimulates tyrosine phosphorylation of focal adhesion kinase (FAK) and paxillin, two components of focal adhesions, in a manner which correlates closely with the chemotactic response of VSMC to PDGF-BB (Abedi *et al.*, 1995). Focal adhesions are

subcellular protein complexes which form at sites of cell-substrate contact and are thought to play a major role in regulating cell adhesion and movement (see section 1.5.1).

1.4.2 Insulin-like growth factor (IGF-I).

The ubiquitous polypeptide growth factor IGF-I, a key regulator of developmental growth, is thought to be an important paracrine/autocrine factor for VSMC (Pfeifle *et al.,* 1982; Clemmons and Van Wyk, 1985; King *et al.,* 1985; Bornfeldt *et al.,* 1991; Delafontaine *et al.,* 1991; Khorsandi *et al.,* 1992). IGF-I has been reported to have both mitogenic and chemotactic properties in a number of different cell types including VSMC in which it functions predominantly as a potent mitogen and survival factor (Bornfeldt *et al.,* 1994a; Banskota *et al.,* 1989). Inhibition of IGF-I binding to its cognate receptor (IGF-IR) decreased smooth muscle proliferation in response to balloon injury in the rat (Hayry *et al.,* 1995). Interestingly an anti-serum to IGF-I was also shown to inhibit PDGF- and angiotensin-induced proliferation in VSMC (Clemmons and Van Wyk, 1985; Delafontaine and Lou, 1993).

The actions of IGF-I are mediated through a receptor tyrosine kinase – IGF-IR – which has also been shown to be stimulated by IGF-II and weakly by insulin. IGF-IR is a heterotetrameric protein composed of two extracellular α-subunits linked by disulphide bonds to each other and to two membrane spanning β-subunits (Ullrich *et al.,* 1985; Ullrich *et al.,* 1986). Ligand binding to the α-subunit activates the tyrosine kinase activity of the β-subunit resulting in receptor autophosphorylation on distinct tyrosine residues (Hernandez-Sanchez *et al.,* 1995; Jiang, 1996). An insulin receptor substrate termed IRS-1 has been defined as the major intracellular substrate of both insulin and IGF-I receptors. One potential signal transduction mechanism (proposed by Baserga *et al.,* 1997), invoked a reported insulin-mediated association of IRS-1 with Grb-2 (Tobe *et al.,* 1993). The association of IRS-1 with Grb-2 would permit IGF-I to interact with the Ras–MAPK pathway thereby leading to cell proliferation. Indirect support for this came from experiments in ovine pituitary cells where it was reported that IGF-I could stimulate ERK activation (Hazlerigg *et al.,* 1996). In VSMC, however, IGF-I has been

reported either not to activate ERKs (Bornfeldt *et al.*, 1997), or to be a weak activator of ERKs compared with PDGF-BB (Cospedal *et al.*, 1999).

1.4.3 Basic fibroblast growth factor (bFGF).

Basic fibroblast growth factor (bFGF) is one member of a nine-member family of polypeptide growth factors – the fibroblast growth factors - known to have mitogenic effects in a number of different cells. Other biological effects of the FGF family include roles in cell motility, differentiation and survival (reviewed in Mason, 1994).

bFGF has been reported to stimulate the proliferation and migration of VSMC (Wang *et al.*, 1997; Gospodarowicz *et al.* 1988; Burgess and Maciag, 1989; Schmidt *et al.*, 1995) and HUVECs (Yoshida *et al.*, 1996), as well as having potent mitogenic effects in endothelial cells (Kang *et al.*, 1995). Basic fibroblast growth factor is known to be synthesised by VSMC (Schmidt *et al.*, 1995; Skaletz-Rorowski *et al.*, 1996), and is a powerful mitogen for VSMC during atherogenesis (Raines and Ross, 1993) and in the response to vascular injury (Ferns *et al.*, 1992). It was previously reported that bFGF released from arterial VSMC after injury is a powerful mitogen (Klagsbrun and Edelman, 1989), and that high bFGF expression and cell replication correlate in injured rat arteries (Lindner and Reidy, 1993). Neointimal proliferation in response to balloon injury of the rat carotid artery was significantly inhibited by an antibody to bFGF (Lindner and Reidy, 1991).

FGFs act through high affinity tyrosine kinase receptors (FGFRs), of which at least four have now been identified (FGFR-1, FGFR-2, FGFR-3 and FGFR-4). The expression of bFGF receptors was examined by Saltis *et al.* (1995) in primary cultures of enzyme-dispersed rabbit aortic VSMC during which time phenotypic modulation of cells took place. These workers reported a striking upregulation in the number of bFGF receptors after phenotypic modulation compared to the contractile phenotype, although the receptor subtype was not classified. The modulated VSMC also demonstrated rapid proliferation in response to bFGF as well as changes in cell morphology suggestive of additional functions other than enhanced replication (Saltis *et al.*, 1995).

It has been shown that FGFs must bind to heparan sulphate in order to activate their receptors and thus generate signal (Yayon *et al.,* 1991; Aviezer *et al.,* 1994). Although a number of intracellular signalling pathways have been defined which are responsible for propagating signal from receptor tyrosine kinases, the functional role of intracellular downstream signalling elements in bFGF-induced VSMC proliferation is not completely understood. A previous report has described activation of the MAPK pathway by bFGF in porcine thoracic aortic smooth muscle cells (Berrou *et al.,* 1996). In addition, it was recently shown that the MAPK cascade is required for bFGF-induced proliferation and that phorbol ester-sensitive PKC isoforms contribute to the bFGF-induced mitogenesis of bovine coronary artery smooth muscle cells (Skaletz-Rorowski *et al.,* 1999).

1.4.4 Vascular endothelial growth factor (VEGF)

The secreted heparin-binding polypeptide vascular endothelial growth factor (VEGF, also known as vascular permeability factor), acts through receptor protein tyrosine kinases (RPTKs) and is distantly related to the PDGF family of growth factors (Ferrara *et al.*, 1992). Five VEGF isoforms derived from alternatively spliced mRNA exist containing 121 ($VEGF_{121}$), 145 ($VEGF_{145}$), 165 ($VEGF_{165}$), 189 ($VEGF_{189}$) and 206 amino acids ($VEGF_{206}$). Of these, $VEGF_{121}$ $VEGF_{145}$ and $VEGF_{165}$ are secreted and biologically active isoforms. VEGF also belongs to a family of related polypeptide factors which includes to date placental growth factor (PLGF), VEGF-B, VEGF-C, VEGF-D and VEGF-E. Each of these ligands exhibits different affinities for VEGF receptor isotypes of which three have been identified: VEGF-R1 or Flt-1, VEGF-R2 or Flk-1/KDR and VEGF-R3 or Flt-4. VEGF exhibits high affinity binding to both Flt-1 and KDR receptors which belong to the PDGF family of receptor PTKs (de Vries *et al.*, 1992; Terman *et al.*, 1992; Matthews *et al.*, 1991). In addition, a non-tyrosine kinase receptor called neuropilin-1 has recently been identified for $VEGF_{165}$ (Soker et al., 1998).

VEGF is a hypoxia-inducible angiogenic factor (Shweiki *et al.*, 1992; Plate *et al.*, 1992) with potent mitogenic (Gospodarowicz *et al.*, 1989) and permeability-increasing activities (Conolly *et al.*, 1989) in EC. It is also a chemoattractant for

monocytes and acts to promote transendothelial migration of monocytes (Clauss *et al.*, 1990). Recent findings indicate that VEGF may have diverse effects in the cardiovascular system. Administration of VEGF protein and VEGF gene transfer inhibits intimal thickening after balloon angioplasty and improve blood flow in ischaemic limbs, effects mediated through EC regrowth and angiogenesis (Asahra *et al.*, 1995; Bauters *et al.*, 1995; Isner *et al.*, 1996). Impaired myocardial angiogenesis and ischaemic cardiomyopathy were found in mice generated to express solely $VEGF_{120}$ and therefore lacking the 164 and 188 isoforms (Carmeliet *et al.*, 1999a). Loss of a single VEGF allele results in embryonic lethality due to severe vascular defects (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). VEGF is upregulated in ischaemic myocardium (Banai *et al.*, 1994) and is proposed to play a role in neovascularisation of the advanced atherosclerotic plaque (Shweiki *et al.*, 1993; O'Brien *et al.*, 1994).

Although the key downstream targets for VEGF receptor-mediated signalling in ECs are incompletely understood, recent results have implicated VEGF as a major survival (anti-apoptotic) factor for the newly formed ECs found in immature, developing blood vessels (Alon *et al.*, 1995; Benjamin and Keshet, 1997; Jain *et al.*, 1998). This survival function of VEGF is thought to be highly important for its pro-angiogenic function and may help explain the observation of profound deficiency of vasculogenesis and angiogenesis in embryonic mice lacking just one VEGF allele (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). At present the mechanisms which could account for the anti-apoptotic function of VEGF have not been elucidated in detail, although some workers have found that VEGF can induce high levels of Bcl-2 in ECs (Nor *et al.*, 1999; Gerber *et al.*, 1998a). In addition, VEGF has been shown to activate the PI3-kinase/AKT signalling pathway (Carmeliet *et al.*, 1999b; Gerber *et al.*, 1998b) and also induce upregulation of at least two members of the Inhibitors of Apoptosis family (IAP – see section 1.67) in ECs (Tran *et al.*, 1999). It was also reported that VEGF stimulates tyrosine phosphorylation of FAK and paxillin and promotes recruitment of FAK to focal adhesions in HUVECs (Abedi and Zachary, 1997).

1.5 CELL MIGRATION

Migration of cells plays a central role in fundamental biological processes including embryonic development, wound healing and the immune response. The importance of VSMC migration and some of the polypeptide factors implicated in the regulation of VSMC migration were discussed in sections 1.2.3 and 1.4 respectively. Locomotion of cells is dependent upon several subcellular structures with specific roles in cell adhesion, and entails a complex interplay between them and the contractile apparatus of the cell. There are also cell type-specific prerequisites for locomotion: in VSMC, disruption of cell-matrix interactions is thought to be important. However in ECs, disruption of both intercellular adhesions and cell matrix interactions appear to be necessary.

The coupling of the cell cytoskeleton with the substratum on which the cell migrates is essential to the migratory process and is mediated by cell adhesion molecules. Integrins constitute the major family of cell surface receptors that mediate adhesion between the cell and the ECM and are also involved in cell-cell adhesion. Integrins are heterodimeric transmembrane glycoproteins composed of various combinations of α and β subunits (Hynes, 1992). They are comprised of an extracellular domain, a single transmembrane domain and a cytoplasmic tail. The extracellular domain binds specifically to components of the ECM. Integrins lack intrinsic enzymatic activity and at present the mechanism whereby ligand-engagement of integrins gives rise to intracellular signals is unclear. One possible mechanism is that ligand-binding to integrins results in conformational changes in the cytoplasmic domains which subsequently effect a change in cytoskeletal configuration. This might lead to alterations in cytoskeletal-associated signal transduction. Alternatively, integrins themselves may directly associate with and regulate components of the signalling machinery.

1.5.1 Focal adhesions.

The actin cytoskeleton communicates with the cell substratum through complex structures called focal adhesions (FAs). FAs are specialised juxtamembrane regions

which form at the termini of actin stress fibres and at sites of attachment of the cell to the ECM (reviewed in Burridge and Chrzanowska-Wodnicka, 1996). Numerous proteins have been associated with FAs including focal adhesion kinase (FAK- see 1.7), paxillin (1.8.1) and other non-structural cytoskeleton-associated components such as talin, vinculin and tensin. Several FA-associated proteins, and in particular FAK, have been strongly implicated in signalling pathways involved in cell migration (see sections 1.7.4 and 1.8.2). In addition, integrins are recognised to aggregate at FAs and are likely to interact with some of the other protein components. It is worth noting that interactions between integrins and the cytoskeleton are highly dynamic. For instance in non-motile cells, β_1 integrins are localised to focal adhesions at the ends of stress fibres, whilst in motile cells integrins are found in organised but dynamic macroaggregates that are in less intimate association with FAs.

In summary, cell locomotion begins with the formation of a leading edge, possibly through actin polymerisation, to form a lamellipodium that will attach to the substratum (Mitchison and Cramer, 1996). The attachments are made via integrins and through FAs. Establishment of the lamellipodium allows the cell to prepare for movement by utilising binding through points of lamellipodium-substrate attachment to generate the requisite traction. Movement is thereafter facilitated by release of adhesions at the rear of the cell with subsequent detachment and retraction (Lauffenburger and Horwitz, 1996).

1.6 APOPTOSIS

Apoptosis or programmed cell death is the name given to a genetically encoded cell suicide programme which has been observed in animal and non-animal cells including plants and some bacteria. It was first recognised many years ago that cell death played an important role in numerous aspects of animal development – an early example being amphibian metamorphosis (Vogt, 1842). Subsequently cell death has been observed to occur in many developing tissues of both vertebrates and invertebrates (reviewed in Glucksmann, 1951; Clarke and Clarke, 1996). In addition, apoptosis also participates in the maintenance of tissue homeostasis into adulthood. A striking example of this can be seen in the vertebrate haemopoietic system where vast numbers of neutrophils produced in the bone marrow die by apoptosis every day (Metcalf *et al.,* 1995). In recent years, evidence has accumulated suggesting that abnormal regulation of apoptosis plays a critical role in the genesis of a number of pathological disorders. Excessive apoptosis may give rise to organ atrophy or organ failure as seen in neurodegenerative diseases and viral hepatitis (Raff *et al.,* 1996; Martinou JC *et al.*; 1994). Conversely impaired apoptotic elimination of cells may lead to the development of neoplasia, congenital malformations and autoimmunity (Ellis *et al.,* 1996; Ameisan, 1994).

It was not until the seminal studies of Kerr, Wylie and Currie that apoptosis was recognised as a discreet mode of cellular death -"shrinkage necrosis"- and distinguished from necrotic cell death by a number of features (Kerr, 1971; Kerr, *et al.*, 1972). Necrosis is the mechanism by which pathological cell death takes place in the centre of acute lesions due to cellular injury arising from toxins, trauma or ischaemia. It is characterised by swelling of cells and their organelles and subsequent rupture leading to leakage of the cell contents. An inflammatory response and later scarring, such as the post myocardial infarction ventricular scar, often follow such a process. Cell death by necrosis, regarded as an uncontrolled or accidental process, is in marked contrast to apoptosis occurring in normal development, tissue homeostasis or at the periphery of acute lesions. During apoptosis cells shrink and condense, whilst retaining their membrane integrity, and are rapidly phagocytosed by macrophages (or other neighbouring cells) with no leakage of cell contents and thus no inflammatory response

is induced. Once inside the macrophage, the apoptotic cell is rapidly disassembled and its chemical constituents made available for re-use. The rapidity of the apoptotic process and removal of dead cells may itself be responsible for under-detection of this particular mode of cell death. Furthermore technical limitations in the currently used methods for detecting apoptosis may also underestimate its incidence.

	APOPTOSIS	NECROSIS
MORPHOLOGY	Volume loss, chromatin condensation, intact organelles	Increased volume, nucleus intact until late, disruption of organelles
PLASMA MEMBRANE	Remains intact	Early disruption
DNA BREAKAGE	Early, internucleosomal pattern	Later, random DNA breakdown
MECHANISM OF DNA BREAKAGE	Gene activated, endonuclease	Random injury, ATP depleted
TISSUE REACTION	No inflammation, phagocytosis	Inflammatory response
CALCIUM INFLUX	moderate	massive

TABLE 1.3. Distinguishing features of apoptosis and necrosis

Disposal of apoptotic cells appears to be dependent upon changes in the surface chemistry of the cells so that macrophages can recognise them. One of the earliest changes is the relocation of phosphatidylserine, a negatively charged phospholipid that is normally confined to the cytosolic leaflet of the plasma membrane lipid bilayer, to the extracellular leaflet (Martin *et al.,* 1995a; Bennett *et al.,* 1995a). Macrophages are also thought to recognise apoptotic cells by means of lectin-mediated binding of altered sugar groups on the cell surface or CD14- and CD36-mediated binding (Fadok *et al.,* 1992: Savill, 1998). In all these instances it is uncertain how activation of the cell suicide program leads to changes in surface chemistry.

1.6.1 Apoptosis in the cardiovascular system.

The investigation of apoptosis in the context of the cardiovascular system has gathered pace following the increasing number of recent reports suggesting a critical role for apoptosis in several cardiovascular diseases as well as in cardiac development. Although there is presently little direct evidence for apoptosis in cardiac organogenesis, it has been implicated in the formation of septal, valvular and vascular structures (Pexieder, 1975), suggesting that regulation of apoptosis may be important in the development of congenital heart disease. It has also been suggested that apoptotic cell death might also play a role in postnatal morphogenesis of cardiac conduction tissue and potential dysregulation of this process may give rise to cardiac rhythm disorders (James, 1994).

In the past several years, evidence has accumulated suggesting that myocyte death in cardiac disease may occur by programmed cell death rather than necrosis (Tanaka *et al.*, 1994; Gottlieb *et al.*, 1994). Subsequently cardiac muscle apoptosis has been documented in a variety of pathophysiological contexts including ischaemic heart disease (Saraste *et al.* 1997; Bialik *et al.*, 1997; Olivetti *et al.* 1996; Fliss and Gattinger, 1996), heart failure (Kajstura *et al.*, 1995; Weber and Brilla, 1991; Davies *et al.*, 1995), models of hypertension (Cheng *et al.*, 1995; Teiger *et al.*, 1996) and genetically determined hypertension (Hamet *et al.*, 1995). In addition to a role in the pathogenesis of atherosclerosis (see below), apoptosis has also been described in diverse cardiac pathologies such as myocarditis (Bachmaier *et al.*, 1997; Smith and Allen, 1992) and forms of cardiomyopathy (Narula *et al.*, 1996; Olivetti *et al.*, 1997).

Apoptosis is now a well documented, if variable, finding in atherosclerotic changes in the vasculature. Descriptions of the extent of apoptosis as detected by TdT-mediated dUTP nick end-labelling (TUNEL) staining in primary atherosclerotic lesions show that apoptosis is not always a consistent finding in all specimens (Kockx *et al.*, 1994; Bennett *et al.*, 1995b; Han *et al.*, 1995; Bjorkerud and Bjorkerud, 1996). In contrast, in atherectomy specimens from restenotic lesions, apoptosis was observed in nearly all cases and strongly correlated with the presence of intimal hyperplasia (Isner *et al.*, 1995). In rat balloon-injury models, apoptosis has been described in both neointimal smooth muscle cells (Han *et al.*, 1995), as well as medial VSMC (Perlman *et al.*, 1997).

It is worth noting that the exposure of phosphatidylserine on the surface of apoptotic cells has been shown to promote thrombin generation *in vitro* (Flynn *et al.,* 1997). Such a mechanism might putatively enhance thrombogenicity of atherosclerotic lesions *in vivo* and predispose to clinical consequences.

Thus far there is little in the way of direct evidence of endothelial cell apoptosis in atherosclerotic lesions (see section 1.3.3). Given the importance of endothelial integrity in protection of the vasculature, increasing attention is focussing on the role of oxidative mechanisms leading to endothelial cell apoptosis as a potential initiating factor in the pathogenesis of atherosclerosis (Dimmeler *et al.,* 1997b; Escargueil-Blanc *et al.,* 1997).

1.6.2 Mechanisms of apoptosis

In recent years great progress has been made in our understanding of the underlying mechanisms of programmed cell death. What has become increasingly apparent is the complexity of the pathways involved and their controls particularly with respect to the relationship between signal transduction and apoptosis. It is clear that all nucleated vertebrate cells constitutively express all of the proteins required to undergo apoptosis from the very earliest stage of development, the zygote, onwards (Weil *et al.,* 1998). In addition, experiments in both *C. elegans* and *Drosophila* (Steller, 1995; Shaham and Horwitz, 1996), indicate that the apoptotic machinery is also expressed constitutively in invertebrate cells implying that it is a basic feature of all animal cells.

1.6.3 Inducers of apoptosis

Although it is accepted that a number of physiological/pathological factors are able to trigger apoptosis, the mechanisms by which they do so have yet to be clarified. Diverse stimuli including heat, toxins, free radicals, radiation, glucocorticoids, nitric oxide and cytokines such as TGF-β, TNF-α have all been shown to initiate apoptosis. These stimuli act in concert with intrinsic cellular factors (e.g. cell line, cell phenotype),

which determine the cell's potential to undergo apoptosis. As an example of this, VSMC from normal arteries undergo apoptosis following withdrawal of growth factors such as PDGF and IGF-I. However, VSMC from atherosclerotic plaques proceed to apoptosis at a high rate even in the presence of growth factors and at an even higher rate without them (Bennett *et al.* 1995b).

Fas ligand, which acts through Fas, the prototypical death receptor (see below) and TNF-α, a pleiotropic cytokine, are two of the most studied apoptogens. Fas is believed, and TNF-α has been shown, to induce apoptosis – at least in part – via a sphingomyelin-mediated pathway resulting in ceramide production (Nagata and Goldstein, 1995; Dbaibo *et al.,* 1993; Xu, J. *et al.,* 1998; Cai, Z.Z. *et al.,* 1997). The same pathway is also implicated in ox-LDL-induced apoptosis of VSMC and HUVECS (Slowik *et al.,* 1996; Chatterjee, 1998). The importance of ceramide as a pro-apoptotic factor is also illustrated by a report in which production of sphingosine-1-phosphate (which inhibits ceramide-dependent apoptosis) by sphingosine kinase activation was shown to mediate resistance to TNF-α-induced apoptosis in HUVECs (Cuvillier *et al.,* 1996; Xia *et al.,* 1998).

1.6.4 Caspases

The caspases represent a novel family of cytoplasmic cysteine proteases that specifically cleave proteins after aspartic acid residues and are related to interleukin-1β-converting enzyme (Miura *et al.,* 1993). Originally identified as mammalian homologues of *C. elegans* cell death genes, more than ten family members are currently known to exist in humans. Caspases have been shown to participate in two distinct signalling pathways: (1) activation of pro-inflammatory cytokines, and (2) promotion of apoptotic cell death (for reviews see Nicholson, 1996; Zhivotovsky *et al.,* 1997). In common with other protease zymogens their activation is contingent upon limited proteolysis: cleavage of an interdomain linker segment results in generation of large and small subunits which reassociate to form an enzyme composed of two heterodimers.

Propagation of intracellular apoptotic signals is believed to arise by sequential caspase activation (Enari *et al.*, 1996), leading to one broad classification of caspases into upstream and downstream subgroups. Upstream caspases are characterised by long prodomains that appear to contain essential regulatory regions (e.g. caspases-1, -2, -4, -5). Their substrates, the downstream caspases (caspases-3, -6, -7), are ultimately responsible for cleavage of target proteins whose proteolysis is associated with apoptotic cell death (Nicholson *et al.*, 1993; Porter *et al.*, 1997; Cardone *et al.*, 1997; Sakahira *et al.*, 1998).

An increasing number of proteins have been identified as targets for caspases (see Table 1.4), most of which have been shown to be cleaved by downstream caspases-3 and -7 (Cardone *et al.*, 1997; Sakahira *et al.*, 1998; Kothakota *et al.*, 1997). Although many of these proteins have a nuclear localisation, there is mounting evidence that cytoskeletal and cytoskeleton-associated proteins are also key targets of caspases (Brancolini *et al.*, 1995; Cryns *et al.*, 1996; Martin *et al.*, 1995b; Herren *et al.*, 1998). In endothelial cells this is consistent with the finding that cytoskeletal organisation appeared to be a critical determinant of survival (Chen *et al.*, 1997).

Nuclear proteins	Regulatory proteins	Cytoskeletal and associated proteins
Lamin	MAPK/ERK kinase kinase 1 (MEKK 1)	Fodrin
Rb protein	Protein kinase Cδ	Gelsolin
DNA-dependent kinase	G4-GDI GDP dissociation inhibitor	Actin
70 kDa subunit of U1 small nuclear ribonucleoprotein	DNA fragmentation factor/inhibitor of caspase-activated DNAse	β-catenin
Mdm2	Sterol regulatory element binding protein	Plakoglobin
Poly (ADP)-ribosylating protein		Focal adhesion kinase

Table 1.4. Target proteins for caspases.

In vitro studies have also indicated that focal adhesions themselves are dismantled at an early stage in apoptosis and that some components of focal adhesions, including focal adhesion kinase (FAK), are targets for caspase-mediated cleavage (Levkau *et al.*, 1998;Gervais *et al.*, 1998; Wen *et al.*, 1997).

1.6.5 Mechanisms of caspase activation

At present two main pathways are recognised as being responsible for caspase activation: one utilising a cytoplasmic membrane receptor-dependent mechanism (death receptor pathway) and another implicating a role for the mitochondrion (see figure 1.3).

1). Death receptor pathway

Apoptosis can be induced following the engagement of extracellular death signal proteins (TNF-α, Fas ligand, TRAIL, Apo-3L) with their cell surface receptors (Zheng *et al.*, 1995; Suda *et al.*, 1993; Pitti *et al.*, 1996; Marsters *et al.*, 1998). The death receptors (such as TNFR1 or TNF-α receptor 1, and Fas) contain a unique cytoplasmic motif designated the "death domain" which is critical for the propagation of apoptotic signals (Itoh and Nagata, 1993; Tartaglia *et al.*, 1993). The mRNA for Fas has been identified in various organs including the heart as well as in atherosclerotic lesions (Geng *et al.*, 1997, Cai, W. *et al.*, 1997). Ligand binding induces death receptors to form homotrimeric complexes which recruit intracellular adaptor molecules to the cell membrane. Examples of such molecules include TNFR-associated death domain protein (TRADD) and Fas-associated death domain protein (FADD) (Chinnaiyan *et al.* , 1995; Hsu *et al.*, 1995). TRADD has been shown to interact with FADD which in turn is able to directly interact with caspase-8 thereby triggering the apoptotic proteolytic cascade (Muzio *et al.*, 1996; Boldin *et al.*, 1996; Fernandes-Alnemri *et al.*, 1996).

2). Mitochondrial pathway

Recent reports have implicated a role for at least two mitochondrial proteins in the induction of apoptosis. The mature haem-containing form of cytochrome c was

shown to be required for caspase-3 activation in a cell-free assay (Liu *et al.,* 1996). Cytochrome c is located in the mitochondrial intermembranous space under physiological conditions but its release into the cytosol appears to be critical for caspase activation (Yang *et al.,* 1997; Kluck *et al.,* 1997). Presently, the mechanism of cytochrome c release into the cytosol is unclear. Another mitochondrial protein termed apoptosis-inducing factor (AIF) has been shown to act as an apoptogenic protease whose proteolytic activity could be blocked by a broad-spectrum caspase inhibitor (Zamzami *et al.,* 1996; Susin *et al.,* 1996).

1.6.6 Non-caspase apoptotic proteases

Calpain is a non-lysosomal calcium-activated neutral protease found in almost all cells. At least two isoforms of the enzyme exist – calpain I and II – which differ mainly in their requirements for calcium for their activation *in vitro*. Calpain belongs to the family of cysteine proteases and consists of an 80 kDa catalytic subunit and a smaller 30 kDa subunit which is identical in all isoforms. Both subunits can bind calcium, but in addition calpain activity can also be regulated by autoproteolysis and the inhibitor protein calpastatin (Goll *et al.,* 1992). Although the exact functions of calpain are still unclear, a role for calpains has been proposed in a number of biological pathways including platelet aggregation, neuronal long-term potentiation and neutrophil activation (reviewed in Croall and DeMartino, 1991). A large body of evidence also implicates calpain in apoptosis (Sarin *et al.,* 1993; Squier *et al.,* 1994; Zhu *et al.,* 1995). Calpain has been shown to cleave a number of cytoskeletal components including actin (Villa *et al.,* 1998), fodrin (Brown *et al.,* 1999), transcription factors such as c-jun and c-fos (Hirai *et al.,* 1991; Watt and Molloy, 1993), as well as the p53 tumour suppressor protein (Kubbutat and Vousden, 1997). In addition, it was shown some time ago that calpain localised to focal adhesions and was responsible for cleavage of talin (Beckerle *et al.,* 1987). Subsequently, calpain-mediated proteolysis of FAK has been demonstrated in human platelets (Cooray *et al.,* 1996), and calpain-dependent cleavage of the cytoplasmic domain of the integrin β_3 subunit has been reported during apoptosis of HUVECs (Meredith Jr *et al.,* 1998).

Presently the precise interactions between caspases and calpain in apoptotic signalling are yet to be determined. During TGF-β-mediated apoptosis in lymphocytes, calpain appears to function in a caspase-independent manner (Wolff *et al.,* 1999), whilst drug-induced apoptosis of HL-60 cells was shown to require caspase-dependent activation of calpain (Wood and Newcomb, 1999).

1.6.7 Regulatory proteins

Bcl-2 family

Bcl-2 was first identified as a frequent translocation occurring in human B-cell follicular lymphoma and was found to function thereafter by promoting cell survival (Cleary *et al.,* 1986). Bcl-2 protein is a constituent of the mitochondrial membrane, nuclear envelope and endoplasmic reticulum, and is a potent inhibitor of apoptotic cell death. Overexpression of Bcl-2 was shown to inhibit apoptosis induced by diverse stimuli including radiation, anti-neoplastic agents, viral infection, growth factor withdrawal and cytotoxic lymphokines (reviewed in Reed, 1994). Interestingly, members of the mammalian Bcl-2 family have been shown to mediate both pro-apoptotic and anti-apoptotic signalling in gene-transfer experiments (Takayama *et al.,* 1994; Boise *et al.,* 1993). The proapoptotic Bcl-2 family protein Bad has been shown to be phosphorylated by the serine/threonine kinase AKT (Datta *et al.*, 1997), which requires PI 3-kinase activity for its activation by growth factor receptors (Burgering and Coffer, 1995). The findings of Datta *et al.* (1997) provided the first direct evidence linking a growth factor signal transduction pathway and apoptosis regulatory proteins.

Inhibitors Of Apoptosis Proteins

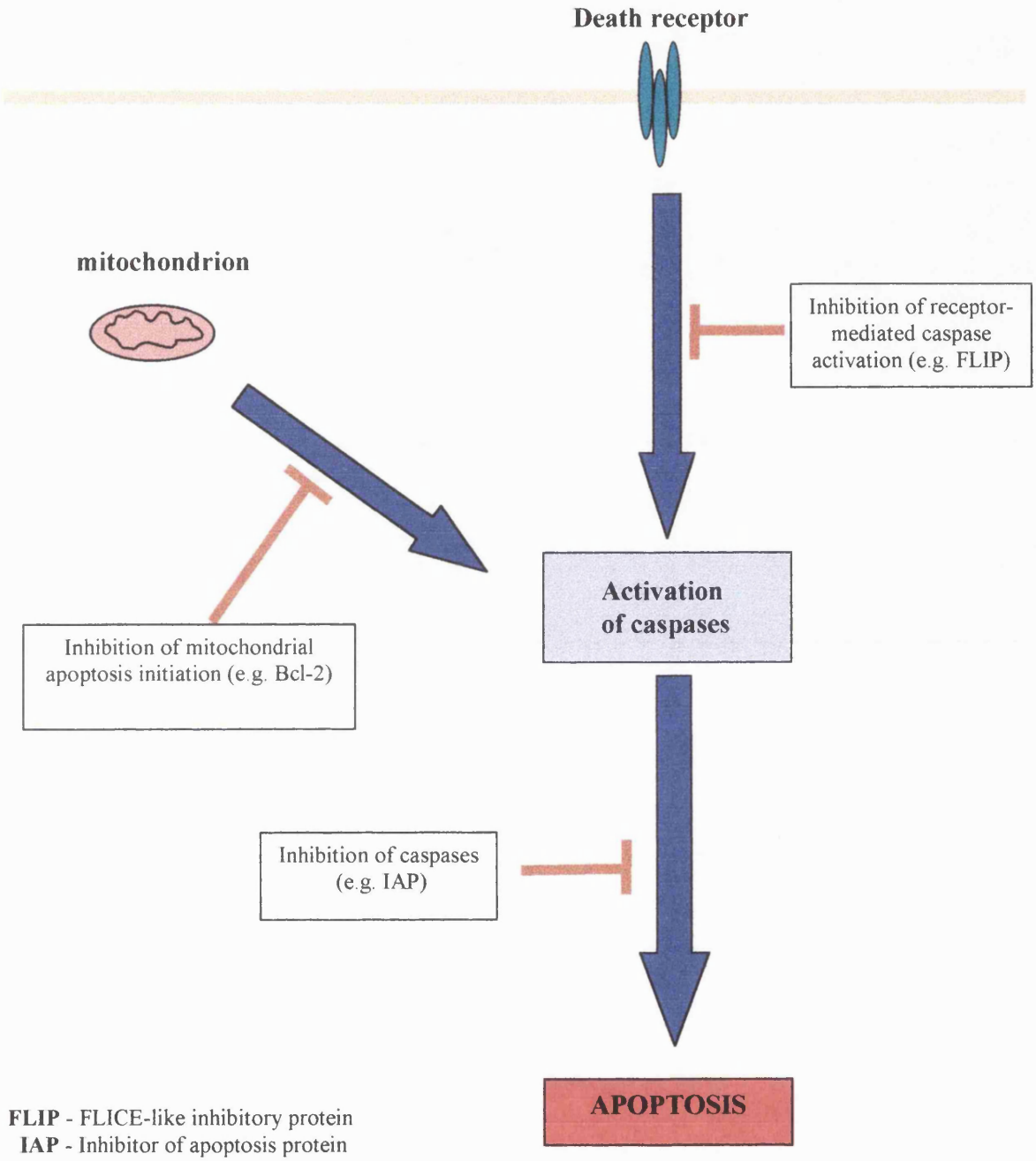
Inhibitors of apoptosis proteins (IAPs) were first identified as mammalian homologues of a baculovirus IAP (Birnbaum *et al.,* 1994; Uren *et al.,* 1996). Several mammalian IAPs prevent apoptosis triggered by ICE, serum withdrawal, or free radicals

(Liston *et al.,* 1996, Uren *et al.* 1996), and another is mutated in patients with spinal muscular atrophy, a neurodegenerative disease (Liston *et al.,* 1996).

Apoptotic pathways and the roles of IAP and caspases are summarised in Figure 1.3 overpage.

P53 and apoptosis

There is strong evidence that the tumour suppressor protein p53 mediates apoptosis in response to conditions that generate genomic instability (Ko and Prives, 1996; Levine, 1997). The proapoptotic effect of p53 has been linked to the p53-induced expression of Fas, bax and IGF binding protein 3 (Ko and Prives, 1996; Miyashita and Reed, 1995; Friedlander *et al.*, 1996; Ludwig *et al.*, 1996). Mutations in p53 are common in many malignant cell types which are no longer anchorage dependent for growth and survival. Of late, interest has focused on the role of survival signals from the extracellular matrix, transduced by integrins, in suppressing p53-mediated apoptosis (see section 1.7.6).



FLIP - FLICE-like inhibitory protein
IAP - Inhibitor of apoptosis protein

Fig. 1.3 Apoptotic signalling pathways.

1.7 FOCAL ADHESION KINASE

The process of cellular transformation, whereby cells undergo profound alterations in structure and loss of normal growth regulation, was recognised some time ago to arise following infection of cells with Rous sarcoma virus (Jove and Hanafusa, 1987; Parsons and Weber, 1989). Transformation required the efficient expression of the viral oncoprotein *v-src*, a protein-tyrosine kinase (PTK) whose activity in cells was accompanied by an increase in the tyrosine phosphorylation of a number of cellular proteins. The majority of these proteins were found in close association with the cytoskeleton (Kanner *et al.,* 1990). One of these proteins, a 125 kDa molecule, had intrinsic tyrosine kinase activity and became phosphorylated on tyrosine in response to both cell adhesion and oncogenic transformation (Guan and Shalloway, 1992). The molecule pp125 was designated Focal Adhesion Kinase (FAK) after immunofluorescent localisation studies had identified its presence in cellular focal adhesions and because it exhibited intrinsic tyrosine kinase activity (Schaller *et al.,* 1992). The deduced amino acid sequence of FAK indicated that it is a non-receptor PTK with a unique structural organisation. The intriguing observation that FAK is phosphorylated in response to the small neuropeptides bombesin, vasopressin and endothelin (Zachary *et al.,* 1992) stimulated further interest in this novel PTK which, in the light of existing evidence, was suggested to act as a point of convergence in the action of multiple signalling pathways (Zachary and Rozengurt, 1992).

Subsequently, the cDNAs encoding FAK have been isolated from a number of different species including chicken (Schaller *et al.,* 1992), mouse (Hanks *et al.,* 1992), human (André and Becker-André, 1993), and Xenopus (Zhang *et al.,* 1995)(see Table 1.5). The deduced amino acid sequences of these cDNAs showed sequence identity of over 90%. More recently, researchers have reported the cloning of a Drosophila gene – termed *Dfak56* - that is homologous to vertebrate FAK (Fujimoto *et al.,* 1999). The overall protein structure and deduced amino acid sequence of the putative *Dfak56* gene product showed significant similarity to vertebrate FAK. These findings suggest that FAK is highly conserved across species and is likely to have conserved roles in cell function.

Species	Cell Type	Size	Structure	Homology (% amino acid to chicken)
Chicken	Chicken embryo fibroblasts	116.5 kDa p41 FRNK	C-terminal non-catalytic domain	100
Mouse	Fibroblasts	119 kDa	25a.a. N-terminal extension	94
Human	T-Cell (Jurkat)	119 kDa	25a.a. N-terminal extension N-+C –terminus deletions and 88bp insertion in catalytic domain	95 where sequence overlaps.
	Brain	100 kDa		
Xenopus	Oocyte	--	---	~90
Drosophila		~130 kDa	Similar to vertebrate FAK	59% to human FAK in kinase domain

Table 1.5 Examples of focal adhesion kinases found in different species.

FAK is expressed throughout development (Furuta *et al.,* 1995), in many cell lines (Kornberg *et al.,* 1992; Matsumoto *et al.,* 1994; Zhang *et al.,* 1994), and in all adult tissues examined (André and Becker-André, 1993; Hanks *et al.,* 1992; Kanazawa *et al.,* 1995). The highest expression of FAK is seen in the brain, testes and in osteoclasts (André and Becker-André, 1993; Hanks *et al.,* 1992; Berry *et al.,* 1994). FAK was also found to be very highly expressed in metastatic human tumours of both epithelial and mesenchymal origin (Owens *et al.,* 1995). Truncated forms of FAK lacking focal adhesion targeting (FAT) sequences have been reported in human brain tissue (André and Becker-André, 1993), and alternatively spliced FAK variants were described in rat nervous tissue (Burgaya *et al.,* 1996, 1997).

In non-fibroblast cell lines FAK is not exclusively localised to focal adhesions. For instance in astroglia the majority of FAK is associated with the cytoskeleton, whilst in neurons FAK is distributed throughout the cell (Zhang *et al.,* 1994; Burgaya *et al.,* 1995, Grant *et al.,* 1995). Perinuclear and nuclear localisation of FAK was reported in HUVECs (Abedi and Zachary, 1997) and in Drosophila (Fujimoto *et al.,* 1999). These

results suggest that the functions of FAK may not necessarily be restricted to sites of focal adhesions.

1.7.1 The structural and functional domains of FAK

FAK is comprised of a central kinase domain flanked by amino- and carboxy-terminal domains of about 400 amino acids each (see Fig. 1.4). Unlike many other non-receptor protein tyrosine kinases (NRPTKs), it contains neither Src homology 2 or 3 (SH2 or SH3) domains which serve to mediate protein-protein interactions, nor a myristoylation site that anchors proteins to the membrane. Another unique feature of FAK is that the carboxyl terminal non-catalytic domain is independently expressed as a 41/43 kDa protein produced by alternative mRNA splicing (Schaller *et al.,* 1993 and see below).

The amino terminus.

The non-catalytic NH_2-terminal domain of FAK has been reported to associate with peptides corresponding to the cytoplasmic domains of β_1, β_2 and β_3 integrins (Schaller *et al.,* 1995). Additionally in a human keratinocyte-derived cell line, the integrin β_1 subunit was observed to associate with FAK in both adherent and non-adherent cells (Danker *et al.,* 1998). The precise location of the integrin-binding site in FAK remains to be determined and little else is known about the NH_2-terminal region.

The carboxyl terminus.

The FAK COOH-terminal non-catalytic domain contains a 159 amino acid sequence (the focal adhesion targeting domain or FAT) which is essential for FAK localisation to focal adhesions (Hildebrand *et al.,* 1993). The FAT sequence also mediates the association of FAK with paxillin, another focal adhesion-related protein which is also a putative FAK substrate (Tachibana *et al.,* 1995; Zachary *et al.,* 1993).

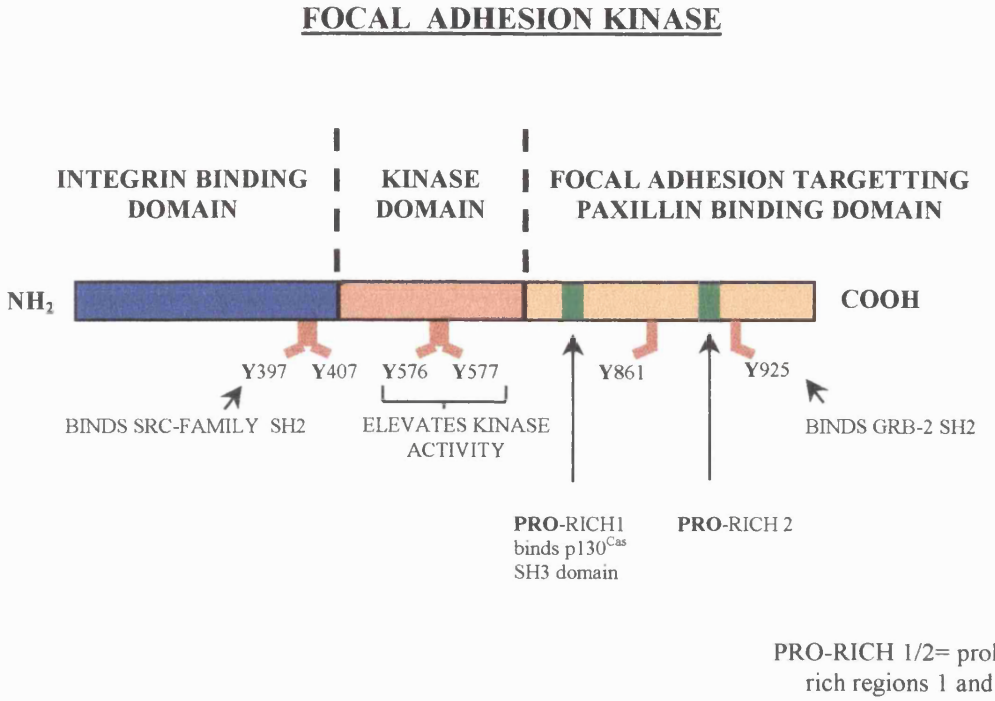


Fig. 1.4 Schematic representation of Focal Adhesion Kinase.

In some cells, alternative mRNA processing gives rise to the autonomous expression of the COOH-terminal domain, referred to as FRNK (Schaller *et al.*, 1993). FRNK is a p41/43 fragment which also contains the FAT sequence. It has been proposed that FRNK is a negative regulator of FAK localisation in focal adhesions (Richardson and Parsons, 1996); however FRNK has not been detected in all cells that express FAK (André and Becker-André, 1993; Ilić *et al.*, 1995a).

Also within the COOH-terminal domain of FAK are two proline–rich sequences (amino acids 861-882 and 711-741), which are able to associate with SH3 containing molecules such as $p130^{Cas}$, p105Cas-L or GRAF, the *G*TPase *r*egulator *a*ssociated with *F*AK (Polte and Hanks, 1995; Minegishi *et al.*, 1996; Parsons, 1996).

1.7.2 FAK-related proteins.

The second PTK to be identified as a FAK-related molecule was cell adhesion kinase (CAKβ), also known as the related adhesion focal tyrosine kinase (RAFTK) or

PYK2 (Avraham *et al.*, 1995; Lev *et al.*, 1995; Sasaki *et al.*, 1995). PYK2 has 61% sequence homology with FAK in the catalytic domain and 42% and 36% identity in the NH_2- and COOH-terminal domains respectively. The sequence marking the autophosphorylation site in FAK (Tyr397), Tyr-Ala-Glu-Ile, is conserved in PYK2 (Tyr404). The COOH-terminal of PYK2 also contains proline-rich regions as well as a region that is highly homologous (61% identity) with the FAT domain of FAK. Expression of PYK2 appears to be highest in brain tissues and in cells of haemopoetic lineage (Xiong *et al.*, 1998; Avraham *et al.*, 1997). It is noteworthy that splice variants of PYK2 have been identified including a PYK2-related non-kinase (PRNK), which may regulate PYK2 function in a fashion analogous to the negative regulation of FAK by FRNK (Xiong *et al.*, 1998).

PYK2 is phosphorylated in response to elevation of intracellular calcium, activation of PKC and stimulation with angiotensin II (Lev *et al.*, 1995; Yu *et al.*, 1996). Activation of PYK2 has been implicated in multiple signalling events including modulation of ion channels (Lev *et al.*, 1995), T- and B-cell receptor signalling (Berg and Ostergaard, 1997; Qian *et al.*, 1997; Manie *et al.*, 1997) and apoptotic cell death (Xiong and Parsons, 1997). Thus, whilst FAK and PYK2 are structurally similar, each has the capacity to mediate distinctly different signalling responses (Xiong and Parsons, 1997; Schaller and Sasaki, 1997).

Recently a new, putative FAK-related phosphoprotein termed fakB has been implicated in β_2 integrin-signalling in T lymphocytes (Kanner, 1996). Subsequently fakB was shown to undergo tyrosine phosphorylation in response to IL-2 stimulation in β_2-positive T cells (Brockdorff *et al.*, 1998). The pattern of fakB tyrosine phosphorylation in these cells was distinct from that of FAK and CAKβ: however detailed characterisation of fakB has not been reported and it remains to be seen whether fakB is itself a tyrosine kinase.

1.7.3 Activation and phosphorylation of FAK

It is now recognised that activation of FAK - resulting in its tyrosine phosphorylation – is thought to play a critical role in regulating its function in diverse adherent cell types, and is stimulated through multiple receptors and intracellular pathways. These include transformation by *v-src*, (Schaller *et al.,* 1992), integrin clustering (Kornberg *et al.,* 1992; Lipfert *et al.,* 1992), regulatory peptides acting through G –protein coupled receptors (Zachary *et al.,* 1992), lipid signalling molecules (Seufferlein and Rozengurt, 1994), and polypeptide growth factors acting through receptor tyrosine kinases (Rankin and Rozengurt, 1994; Abedi *et al.,* 1995; Abedi and Zachary, 1997). In addition, physiological factors such as fluid shear stress in ECs (Li *et al.,* 1997; Takahashi *et al.,* 1997) and pulsatile mechanical stretch in cardiomyocytes and endothelial cells (Seko *et al.,* 1999; Naruse *et al.,* 1998), are known to stimulate FAK tyrosine phosphorylation in HUVECs and bovine aortic ECs (reviewed in Lehoux and Tedgui, 1998). Regardless of the initiating stimulus, it is however clear that the integrity of the actin cytoskeleton is a prerequisite for FAK tyrosine phosphorylation (Seufferlein and Rozengurt, 1994; Rankin and Rozengurt, 1994; Sinnett-Smith *et al.,* 1993).

Although it has been widely studied, the mechanism of FAK activation is still unknown. For instance it remains to be established how integrin engagement with the ECM might regulate FAK activity. Some insights into FAK activation have been gained from studies of bioactive peptides (endothelin, bombesin) and the serum factor LPA which stimulate rapid tyrosine phosphorylation of FAK in Swiss 3T3 cells (Seufferlein and Rozengurt, 1994; Zachary *et al.,* 1992). Bombesin and LPA act through G-protein coupled receptors to stimulate the activation of the small GTP-binding protein Rho. Subsequently it was reported that the cellular effects of these molecules are mediated specifically by Rho in a study where both FAK activation and focal contact formation were inhibited by blocking activation of Rho (Rankin *et al.,* 1994). Although this would suggest that Rho might lie upstream of FAK activation, the relationship of Rho function to FAK activation remains controversial.

There are now 6 reported tyrosine phosphorylation sites in FAK (Fig. 1.4): Y397, Y407, Y576, Y577, Y861, Y925 (Calalb *et al.,* 1995, 1996; Schlaepfer *et al.,*

1996). Phosphorylation of FAK at Y397, the major autophosphorylation site, creates a high-affinity binding site for the SH2 domain of Src-family NRPTK (Cobb *et al.,* 1994; Schaller *et al.,* 1994; Eide *et al.,* 1995; Cary *et al.,* 1996). In addition, phosphorylation of FAK at Y397 was shown to be required for association of FAK with phosphatidylinositol 3-kinase (Chen *et al.,* 1996). FAK is also phosphorylated *in vitro* and *in vivo* by Src at tyrosine residues 407, 576, 577, 861 (Calalb *et al.,* 1995), and 925 (Schlaepfer and Hunter, 1996). Y925 is a binding site for the SH2 domain protein GRB-2 and therefore links FAK to the Ras/MAPK pathway (Schlaepfer *et al.,* 1994).

Other than Y397 and Y925, the functions of the remaining sites of tyrosine phosphorylation are currently unclear. Phosphorylation at Y576 and Y577 appears to maximise the kinase activity of FAK *in vitro* (Calalb *et al.,* 1995). Y861 has been identified as a site of FAK phosphorylation by Src-family kinases both *in vivo* and *in vitro*. It is thought that a likely function for this site is to mediate additional interactions between FAK and SH2 containing proteins. Similarly Y407 has also been recognised as an *in vivo* site of phosphorylation (Calalb *et al.,* 1995) and may be a preferred binding site for SH2 domains of c-Abl, c-Crk, Nck and phospholipase Cγ (Cantley and Songyang, 1994).

The evidence which has accrued to date suggests that FAK is at a crossroads for multiple signalling pathways. The documented associations of FAK with multiple proteins (see Fig. 1.5) indicate that FAK may act as a platform for the assembly of signalling cascades participating in the regulation of cytoskeletal organisation and modulation of growth factor function.

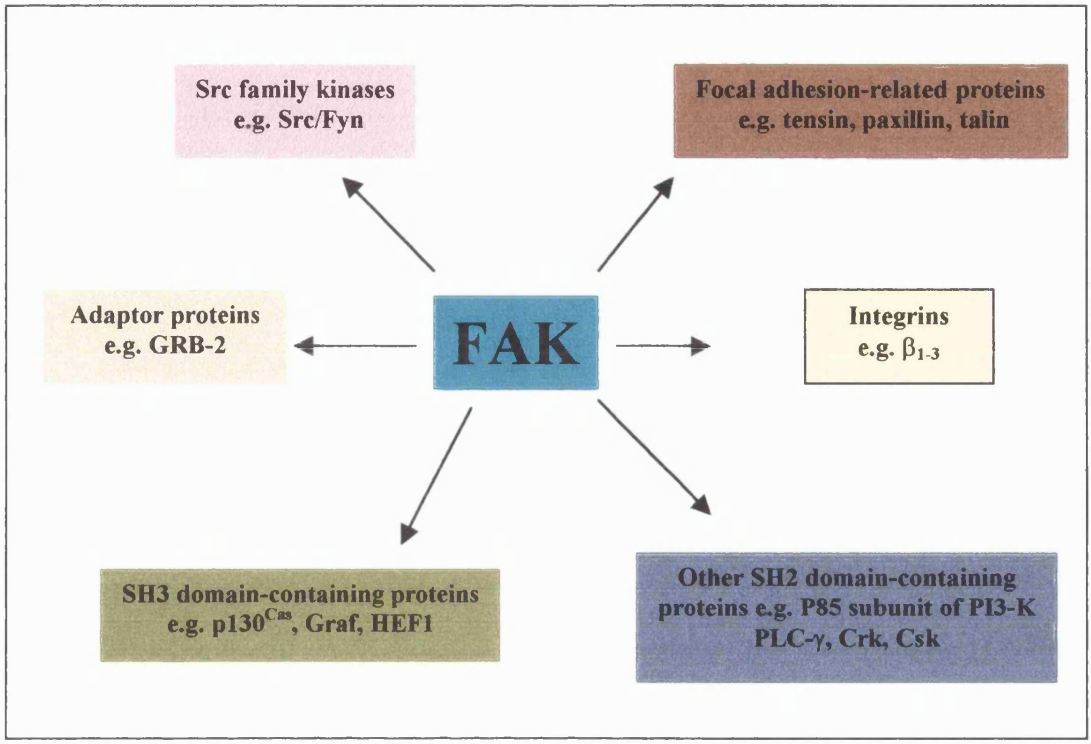


Fig. 1.5 FAK-interacting proteins.

1.7.4 FAK signalling in migration and development.

In light of its localisation to cell-substratum contact points and activation by integrin engagement, FAK was predicted to have a primary role in cell motility and migration. This question was addressed directly by targetted FAK-gene deletion in mouse embryonic stem cells and generation of FAK-deficient mice (Furuta *et al.*, 1995). FAK deletion was embryonic lethal (E8.0-E8.5) and associated with severe morphogenetic defects in axial mesodermal tissues and the cardiovascular system. Several possibilities exist to explain the mesodermal defects, including slower migration of FAK-null mesodermal cells. Additionally, reduced cell cycle length of cells entering the primitive streak in FAK-null embryos or reduced survival of mesodermal cells might plausibly contribute to the defects seen. It is of interest that the overall phenotype of FAK-null embryos resembles strongly that of fibronectin-deficient mice (George *et al.*, 1993). This data gives support to the concept of FAK playing a key role in integrin-

stimulated signalling. Further support for this notion comes from results showing that homozygous deletion of the murine β1 integrin subunit gene or the α5 integrin subunit gene, of the specific integrin fibronectin receptor α5β1, result in similar early embryonic-lethal phenotypes (Stephens *et al.,* 1995; Yang *et al.,* 1993).

FAK-null fibroblasts have been propagated from E8.0 embryos and these cells exhibit migration but not proliferative defects in cell culture (Ili *et al.,* 1995b). PYK2 expression was noted to be increased in these cells; however, transient PYK2 overexpression did not reverse the migration defects of these cells (Sieg *et al.,* 1998). Somewhat unexpectedly, FAK-null cells had increased numbers of focal adhesions suggesting that FAK might regulate focal adhesion turnover rather than assembly. Interestingly these cells were also noted to have normal phosphorylation levels of focal adhesion-related proteins such as $p130^{Cas}$ (Vuori *et al.,* 1996), paxillin and cortactin (Ili *et al.,* 1995a). This data suggests that other proteins are compensating for FAK in phosphorylating FAK-associated components, or that their phosphorylation is maintained via FAK-independent mechanisms.

Currently a good deal of evidence (in addition to FAK-knockout studies) has accumulated to implicate a role for FAK in cell migration. Stable FAK overexpression in Chinese hamster ovary (CHO) cells was shown to cause enhanced cell migration (Cary *et al.,* 1996, 1998). Reports of overexpression of FRNK leading to defective cell spreading and motility (Gilmore and Romer, 1996; Richardson and Parsons, 1996), and studies correlating elevated FAK expression with increased invasive potential of human tumours (Owens *et al.,* 1995), also point towards an important role for FAK in migration. In addition, enhanced FAK dephosphorylation achieved through overexpression of a protein tyrosine phosphatase, was shown to antagonise FAK-mediated migration events (Tamura *et al.,* 1999a, 1999b). Finally, it was recently shown that stable re-expression of FAK in FAK-null cells was able to rescue both the morphological and integrin stimulated migration defects of those cells (Sieg *et al.,* 1999).

1.7.5 Role of FAK in cell proliferation.

FAK has already been linked with an extensive network of signalling proteins that interface with growth regulation (reviewed in Clark and Brugge, 1995). These include Src (Schaller *et al.*, 1994; Xing *et al.*, 1994), the p85 subunit of PI-3 kinase (Chen and Guan, 1994; Guinebault *et al.*, 1995), the adaptor protein Grb-2 (Schlaepfer *et al.*, 1994), and Csk (Sabe *et al.*, 1994). These studies provided preliminary evidence suggesting a role for FAK in the regulation of cell proliferation. Interestingly studies of FAK-null mice had shown no evidence of effects on cell proliferation although it was remarked that FAK function may have been performed by other proteins in these cells (Ilic *et al.*, 1995a). Subsequently several studies have been reported which give more support to the notion that FAK is involved in cell cycle regulation. Microinjection of a glutathione-S-transferase fusion protein containing the COOH-terminal domain of FAK (GST-Cterm), was shown to decrease DNA synthesis in HUVECs as compared to control-injected or non-injected cells (Gilmore and Romer, 1996). Overexpression of wild-type FAK was reported to accelerate G1 to S phase transition in NIH3T3 cells, whilst conversely overexpression of a dominant-negative FAK mutant ΔC14 - which competes with endogenous FAK - inhibited cell cycle progression at G1 phase (Zhao *et al.*, 1998). Recently, utilising a dominant-negative strategy again, it was established that the activation of Jun NH_2-terminal kinase (JNK) by integrins is mediated by FAK and is necessary for cell cycle progression (Oktay *et al.*, 1999). The proposed mechanism by which integrin engagement with ECM activated JNK was shown to depend upon the association of FAK with Src and $p130^{Cas}$ and, subsequently, the recruitment of Crk.

1.7.6 FAK and the regulation of apoptosis.

A number of recent findings indicate a novel role for FAK in transducing survival signals in anchorage dependent cells (Frisch *et al.*, 1996; Hungerford *et al.*, 1996; Crouch *et al.*, 1996; Xu *et al.*, 1996; Xiong *et al.*, 1997; Tallett *et al.*, 1996). Expression of a constitutively activated FAK construct in MDCK cells or the keratinocyte HaCat cell line conferred resistance to anoikis, a subset of apoptosis arising when anchorage-dependent cells lose contact with the ECM (Frisch *et al.*, 1996).

Additionally, in chicken embryo fibroblasts, apoptosis was induced by microinjection of either an antibody to the FAT domain or a peptide corresponding to the FAK-binding site of the β1-integrin cytoplasmic domain (Hungerford *et al.,* 1996). Furthermore it was reported that attenuation of FAK expression using antisense oligonucleotides led to apoptosis in tumour cells (Xu, L.H. *et al.,* 1998).

Previously it was reported that proteolysis may be an important mechanism of the regulation of FAK activity (Cooray *et al.,* 1996). FAK proteolysis in thrombin- and collagen-activated platelets was shown to be mediated in part by the apoptotic protease calpain (Cooray *et al.,* 1996). More recently these observations were extended in human cell lines undergoing apoptosis, and the caspase family of apoptotic proteases were implicated directly in FAK cleavage (Wen *et al.*, 1997; Gervais *et al.*, 1998). Additionally, a comparison of caspase-mediated proteolysis of thirty-three signalling molecules during Jurkat cell apoptosis induced by Fas-L or etoposide, showed that FAK and two other molecules underwent cleavage with the same time course as for caspase activation (Widmann *et al.,* 1998). Furthermore, focal adhesion disassembly and caspase-mediated cleavage of FAK was reported during apoptosis of HUVECs induced by serum-deprivation (Levkau *et al.,* 1998). An insight into how proteolytic cleavage of FAK might be permissive for apoptotic signalling came from the studies of Gervais *et al.,* (1998). This group overexpressed chicken FAK in human cell lines and observed that FAK cleavage during apoptosis separates the tyrosine kinase domain from the FAT domain to create a FRNK-like polypeptide. These workers found that the carboxyl-terminal fragments which were generated were sufficient to suppress phosphorylation of endogenous FAK in human cell lines. In view of this, they proposed that the cleavage of FAK by caspases might play an important role in the execution of the suicide program by disabling the anti-apoptotic function of FAK (Gervais *et al.,* 1998).

At present the mechanism by which FAK might participate in anti-apoptotic signalling is unclear, as is the apoptotic pathway triggered when integrin-FAK signals are interrupted. A recent study of apoptosis in anchorage-dependent cells has provided some important insights into FAK-mediated survival signalling (Ili *et al.,* 1998). In this study multiple strategies were used to inactivate FAK and the p53 tumour suppressor protein in immortalised embryo-derived endothelial cells and fibroblasts.

The results showed that FAK transmitted survival signals from the ECM in both cell types and that in the absence of FAK, a p53-regulated apoptotic pathway was activated. p53-dependent apoptosis was suppressible by mutant p53 and the small pro-domain caspase inhibitor ZVAD-fmk, but not by the large pro-domain caspase inhibitor CrmA (Ili *et al.,* 1998).

In summary, the evidence to date suggests that FAK is an early, selective and important target for small pro-domain caspases (e.g. caspase-3), and may play a key role in mediating survival signalling in anchorage-dependent cells.

1.8 FAK-INTERACTING FOCAL ADHESION PROTEINS.

1.8.1 Paxillin

Paxillin was originally identified as a 68 kDa substrate for the *v-src* tyrosine kinase (Glenney and Zokas, 1989), and shown to be highly localised at focal adhesions in fibroblasts (Turner *et al.*, 1990). The paxillin cDNA initially isolated from fibroblasts was the α isoform of chicken (Turner and Miller, 1994), and human (Salgia *et al.*, 1995). Subsequently two further human isoforms, β and γ, were identified which are generated by the insertion of specific exons into the same site as the α isoform (Mazaki *et al.*, 1997). It has been shown that the mRNA of the α isoform is expressed in most normal human tissue, whereas mRNA of the β and γ isoforms are almost undetectable (Salgia *et al.*, 1995). In contrast to this, human cancer cells and monoblast cells cultured *in vitro* have been shown to express both these isoforms. Thus far it appears that each isoform exhibits different binding properties to focal adhesion proteins, suggesting distinct physiological roles for each isoform.

Paxillin is a prototypical member of a family of proteins including hic-5 and leupaxin (Lipsky *et al.*, 1998; Shibanuma *et al.*, 1994). These proteins contain four COOH-terminal LIM domains (Lipsky *et al.*, 1998; Shibanuma *et al.*, 1994; Turner and Miller, 1994), which are double-zinc finger motifs containing conserved amino acid residues that function to mediate protein-protein interactions (Schmeichel and Beckerle, 1994; Wu *et al.*, 1996). The LIM domains of paxillin and hic-5 target these proteins to focal adhesions (Brown *et al.*, 1996; Thomas, S.M. *et al.*, 1999; Fujita *et al.*, 1998). The amino-terminal regions of these proteins contain multiple copies of another interaction motif called the LD domain (Brown *et al.*, 1996). These motifs have been implicated in mediating binding to FAK, vinculin and bovine papillomavirus E6 protein (Turner *et al.*, 1999; Vande *et al.*, 1998). The amino-terminal of paxillin contains four residues that are sites of tyrosine phosphorylation (Schaller and Parsons, 1995; Bellis *et al.*, 1995). At present the role of tyrosine phosphorylation and the biological function of paxillin remain to be definitively established.

The available evidence points towards a role for paxillin in signal transduction through focal adhesions. Paxillin has been shown to interact directly with several focal adhesion proteins including vinculin, talin and the integrin β1 subunit (Turner *et al.*, 1990; Turner and Miller, 1994; Salgia *et al.*, 1995; Schaller *et al.*, 1995). Very recent evidence has shown that the tight association of paxillin with the α4 integrin tail was important in modifying cellular responses to integrin-mediated cell adhesion (Liu *et al.*, 1999). The phosphorylation state of paxillin has been shown to be regulated in several cell types by physiological stimuli including bombesin, PDGF, nerve growth factor and angiotensin II (Zachary *et al.*, 1993; Rankin and Rozengurt, 1994; Abedi *et al.*, 1995; Melamed *et al.*, 1995; Turner *et al.*, 1995; Abedi and Zachary, 1997). By means of its tyrosine phosphorylation upon cell-substratum adhesion (Burridge *et al.*, 1992), binding sites for the SH2 domains of Csk and Crk are created (Sabe *et al.*, 1994; 1995; Schaller and Parsons, 1995). Inhibition of this tyrosine phosphorylation blocks the formation of focal adhesions and cell cycle progression into S-phase (Burridge and Chrzanowska-Wodnicka, 1992).

The importance of the physical interaction between FAK and paxillin has not been firmly established. Given that the focal adhesion targetting sequence resides in the COOH-terminus of paxillin (whilst the FAK-binding site is in the NH_2-terminus), it is unlikely that this interaction is required for the correct localisation of paxillin to focal adhesions (Brown *et al.*, 1996). It has been proposed that FAK might target to focal adhesions via paxillin binding because a number of FAK mutants defective for paxillin binding were shown to mislocalise to focal adhesions (Tachibana *et al.*, 1995). Other workers have, however, demonstrated that there are several FAK mutants that are unable to bind paxillin yet target correctly to focal adhesions (Hildebrand *et al.*, 1995). It is also possible that the FAK-paxillin interaction is necessary to direct the tyrosine phosphorylation of paxillin. This hypothesis was examined in a recent study detailing the two FAK-binding sites in paxillin, followed by the characterisation of a paxillin mutant with lesions in each binding site (Thomas, J.W. *et al.*, 1999). Herein it was shown that mutations in both FAK-binding sites are required to significantly impair FAK binding *in vitro*. In addition, the results suggested that paxillin must bind FAK for maximal phosphorylation in response to cell adhesion and that FAK may serve to direct

tyrosine phosphorylation of paxillin in the process of *src*-induced cellular transformation (Thomas, J.W. *et al.*, 1999).

1.8.2 $p130^{Cas}$

$p130^{Cas}$ (Cas) was originally identified as a highly tyrosine-phosphorylated 130 kDa protein in cells transformed by $p47^{v\text{-}crk}$ (v-Crk), and was also shown to be tyrosine phosphorylated in *src*-transformed cells (Kanner *et al.*, 1991; Matsuda *et al.*, 1990; Reynolds *et al.*, 1989). Soon after, rat Cas was isolated and cloned by another group of workers (Sakai *et al.*, 1994a, 1994b). The predicted amino acid sequence of Cas revealed a unique structure comprising an SH3 region at the NH_2-terminus, 15 YXXP motif repeats in the trunk and several tyrosine residues and a proline-rich region near the COOH-terminus (Sakai *et al.*, 1994a and see Fig. 1.7).

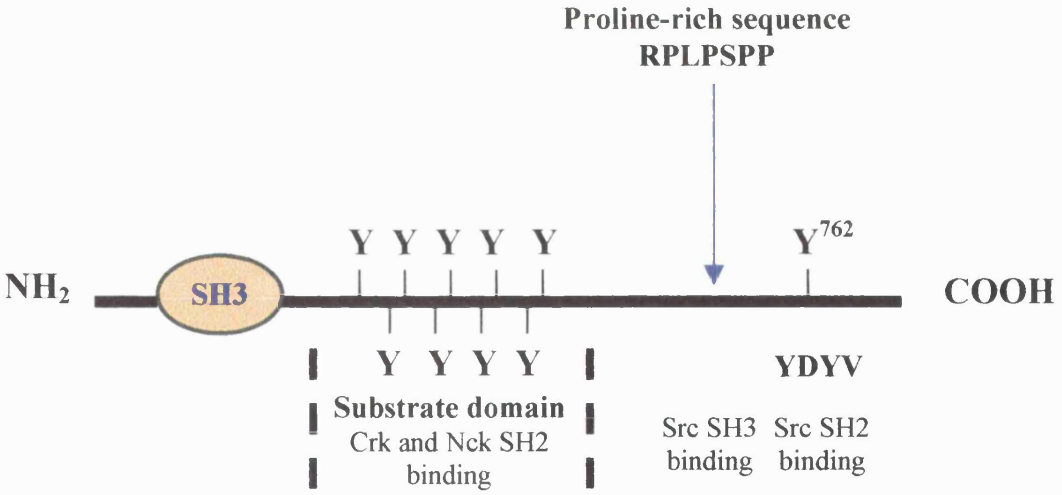


Fig. 1.6 Structural features of $p130^{Cas}$

The SH3 domain is known to bind to FAK and FRNK (Polte and Hanks, 1995; Harte *et al.*, 1996). The substrate domain binds to Crk and several other proteins

(Burnham *et al.*, 1996; Nakamoto *et al.*, 1996). The proline-rich region near the COOH-terminus and Y^{762} provide the binding sites for the SH2 and SH3 domains of Src respectively (Nakamoto *et al.*, 1996). In light of its potential interactions, Cas has been variously termed an adaptor or docking protein, which can assemble and transmit cellular signals through the SH2 and SH3 domains of a number of signalling molecules. Recently two novel proteins have been cloned and characterised: Efs (embryonal Fyn-associated substrate), or Sin (Src-interacting protein), and HEF1 (human enhancer of filamentation 1), also known as Cas-L (Law *et al.*, 1996; Minegishi *et al.*, 1996). These molecules have a similar primary structure to Cas and are thought to represent a new family of docking proteins.

Other than activation by oncogenic transformation, Cas has been shown to undergo tyrosine phosphorylation in response to various physiological stimuli. These include cell adhesion (Petch *et al.*, 1995; Vuori and Ruoslahti, 1995; Nojima *et al.*, 1995), cytokine receptor engagement (Ingham *et al.*, 1996; Schraw and Richmond 1995), and growth factor stimulation (Casamassima and Rozengurt, 1997; Ojaniemi and Vuori, 1997). However, Cas does not appear to be a tyrosine kinase itself. The *in vivo* roles of Cas were examined by Honda *et al.* (1998), who generated mice lacking Cas by homologous recombination. These workers demonstrated that Cas functions as an assembling molecule of actin filaments and plays essential roles in myofibril and Z-disk formation in the heart, and Src-induced morphological transformation and anchorage independent growth in soft agar (Honda *et al.*, 1998). Having established a role for Cas in embryonic development, cytoskeletal organisation and oncogenic transformation, they went on to investigate the roles of Cas in cellular function in a subsequent study. Here, they detailed the behaviour of Cas-null and Cas-null/Cas–re-expressing fibroblasts in response to several biological stimuli (Honda *et al.*, 1999). It was found that Cas-deficient fibroblasts exhibited significant defects in cell movement after mechanical wounding, and in cell migration toward fibronectin as compared to Cas-re-expressing cells. Interestingly, although Cas-deficient cells displayed delayed spreading on fibronectin-coated dishes, fibronectin-induced tyrosine phosphorylation of cellular proteins was similar in both Cas-deficient and Cas-re-expressing cells (Honda *et al.*, 1999).

At present, little is known about the signalling pathways in which Cas might be involved, and even its interactions with FAK are not well understood. Although Cas binds to FAK, and its tyrosine phosphorylation parallels that of FAK after integrin stimulation of fibroblasts, the PTK that phosphorylates Cas might not necessarily be FAK or PYK2 (Tachibana *et al.*, 1997; Schlaepfer and Hunter, 1998). Evidence is however accumulating that Src is involved in Cas phosphorylation (Vuori *et al.*, 1996; Nakamoto *et al.*, 1997; Schlaepfer *et al.*, 1997), consistent with previously recognised binding of Src SH2 and SH3 domains with Cas (Nakamoto *et al.*, 1996). FAK-Cas interactions are however likely to be important for certain cellular functions as was shown by Cary *et al.* (1998). In overexpression studies in CHO cells, these workers showed that a FAK mutant with defective Cas binding (due to a mutation in the Cas-binding proline-rich sequence of FAK), promoted cell migration less efficiently than wild-type FAK. In addition, Cas was able to associate with wild-type FAK and kinase-defective FAK and its levels of tyrosine phosphorylation were comparable with each, suggesting that Cas is not phosphorylated by FAK. It was notable that FAK-Cas association led to the subsequent binding of Cas to SH2 domain-containing proteins (of Src, Nck and the p85 subunit of PI 3-K), which may be downstream mediators of FAK-promoted cell migration. Finally overexpression of wild-type Cas in cells overexpressing wild-type FAK exhibited increased levels of migration compared to cells overexpressing wild-type FAK alone (Cary *et al.*, 1998).

1.9 OTHER SIGNAL TRANSDUCTION PROTEINS IMPLICATED IN VASCULAR CELL FUNCTION

1.9.1 The Mitogen-Activated Protein Kinase (MAPK) pathway

The MAPK pathway consists of several distinct members including extracellular signal-regulated kinases 1 and 2 (ERKs 1 and 2), Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPK) (Kyriakis *et al.*, 1994), and p38 kinases (Han *et al.*, 1994). In addition, other ERK-related enzymes have been identified such as ERKs 3 (Boulton *et al.*, 1991), and ERKs 5 and 6 (Abe *et al.*, 1997). The classical mitogen-activated protein kinase pathway (ERKs 1 and 2) is a multistep phosphorylation cascade that transmits signals from various cell surface receptors to cytosolic and nuclear targets in a number of different cell types (Seger and Krebs, 1995). Activation of ERKs 1 and 2 is initiated by ligand binding to the cell surface receptor which can be either a RTK (Blenis, 1993; Schlessinger, 1994), or a seven transmembrane domain receptor coupled to heterotrimeric G proteins (Alblas *et al.*, 1993; Wan *et al.*, 1996). In the case of RTKs, ligand binding to the receptor causes autophosphorylation of the receptor and subsequent recruitment of adaptor molecules (such as GRB2), whose SH2 domains bind to phosphotyrosine residues in the activated receptor. GRB2 is able to recruit the guanine nucleotide exchange factor Sos, which in turn promotes the association of GTP with Ras. Sequential phosphorylation leads to activation of the protein kinases Raf, MAP kinase kinase (MAPKK or MEK), and ERKs 1 and 2 (Morrison and Cutler, 1997). The two isoforms of MAPK, ERK1 (p44 MAPK), and ERK2 (p42 MAPK), are expressed in most cell types.

ERK 1 and 2 activation can arise through numerous stimuli including virtually all growth regulatory molecules. This fact, in addition to the reported striking homology of ERKs with two protein kinases, KSS-1 and FUS-3, which regulate cell cycle progression in yeast (Boulton *et al.*, 1990), pointed strongly towards a role for ERKs 1 and 2 in eukaryotic cell cycle progression. Subsequently, a number of other studies have provided further data consistent with the concept that ERKs 1 and 2 activities are directly correlated with cell proliferation (Cowley *et al.*, 1994; Seger *et al.*, 1994; Lai *et al.*, 1996; Mansour *et al.*, 1994). Although many of the identified substrates for ERKs 1

and 2 include nuclear transcription factors (and ERKs 1 and 2 can themselves translocate from the cytoplasm to the nucleus), other substrates have been reported. These include protein serine/threonine kinase $p90^{rsk}$, cytoskeletal proteins and cytosolic phospholipase A_2 ($cPLA_2$) (reviewed in Davis, 1993). It is now clear that in addition to regulating cell proliferation, the MAPK cascade is likely to be involved in diverse biological effects such as differentiation (Traverse *et al.*, 1992), cell attachment (Chen *et al.*, 1994), smooth muscle contraction (Adam *et al.*, 1995), prostanoid biosynthesis and protein synthesis (Servant *et al.*, 1996), depending on stimuli and cell type.

1.9.2 The Urokinase-type Plasminogen Activator Receptor (UPAR)

UPAR is the cell-surface receptor for urokinase-type plasminogen activator, a highly restricted serine protease that converts the zymogen plasminogen to plasmin (Kratzschmar *et al.*, 1993). UPAR has been identified in multiple cells as a highly glycosylated 55 kDa protein linked to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor (Kratzschmar *et al.*, 1993; Reuning *et al.*, 1993). Occupancy of UPAR by UPA has been implicated in a number of biological functions independent of the proteolytic activity of UPA. These include adhesion and chemotactic movement of myeloid cells (Waltz *et al.*, 1993; Gyetko *et al.*, 1994), migration of epithelial cells, endothelial cells and VSMC (Busso *et al.*, 1994; Odekon *et al.*, 1992; Okada *et al*, 1996), as well as promoting cell growth (Rabbani *et al.*, 1992).

Despite lacking the membrane-spanning and intracellular domains characteristic of other receptor subtypes (Ploug *et al.*, 1991), UPAR has been shown to mediate proteolysis-independent signalling events including protein tyrosine phosphorylation (Dumler *et al.*, 1993), diacylglycerol formation (Del Rosso *et al.*, 1993), and the activation of a serine kinase (Busso *et al.*, 1994). Importantly, a recent report has implicated the UPAR-associated activation of the Jak/Stat signalling cascade in the regulation of migration of human VSMC (Dumler *et al.*, 1998).

1.9.3 Protein Kinase C (PKC)

The protein kinase C family of enzymes is responsible for signal transduction promoting lipid hydrolysis. Diverse signalling mechanisms result in the generation of the second messenger diacylglycerol, which activates PKC: these include signalling through G protein-coupled receptors, RTKs and NRPTKs (Asaoka *et al.*, 1992; Nishizuka, 1992, 1995). PKC is regulated by two distinct mechanisms: by phosphorylation which regulates the active site and subcellular localisation of the enzyme, and by second messengers which promote PKC's membrane association and free the substrate binding site (reviewed in Newton, 1995). PKC has been implicated in mediating FAK tyrosine phosphorylation in response to extracellular factors such as VEGF (Abedi and Zachary, 1997), phorbol ester treatment of 3T3 fibroblasts (Sinnett-Smith *et al.*, 1993) and integrin-mediated muscle cell spreading (Disatnik and Rando, 1999). At present, however, the identity of the tyrosine phosphorylation sites that PKC might regulate and the importance of PKC-mediated signalling for the survival functions of FAK have yet to be established.

AIMS OF THE THESIS

At the outset of this thesis, work in our laboratory had established that FAK tyrosine phosphorylation was regulated by PDGF-BB in VSMC and that FAK was activated in PDGF-BB-stimulated VSMC chemotaxis (Abedi *et al.*, 1995). However, it was unknown whether changes in FAK expression were implicated in the phenotypic modulation of VSMC observed in lesions of intimal hyperplasia and also during primary culture of VSMC. It was also recognised that the survival factor VEGF promoted FAK tyrosine phosphorylation and increased FAK recruitment to focal adhesions in EC (Abedi and Zachary, 1997). At this time, the relationship between changes in FAK tyrosine phosphorylation status and EC survival was not known. The specific aims of this thesis were to examine the following questions:

1). The expression of FAK and other focal adhesion components during the phenotypic modulation of VSMC in explant culture.

2). The subcellular localisation of FAK in VSMC and HUVECs.

3). The apoptotic regulation of FAK tyrosine phosphorylation in HUVECs

CHAPTER 2

Experimental Procedures

2.1 CELL CULTURE

2.1.1 Animals

New Zealand White Male rabbits (NZWM) aged 9-12 weeks at a bodyweight of 3.0-3.5 kg were used. They were fed a standard pelleted chow (SD-1, SDS Ltd.) and allowed free access to water.

2.1.2 Tissue culture media

Media for VSMC

Dissecting medium consisted of Dulbecco's modification of Eagle's medium (DMEM) with 20 mM HEPES pH 7.4 (Sigma Chemical Co). Explant and cell cultures were maintained and propagated in tissue culture medium consisting of DMEM with 3.7 g/l sodium bicarbonate, 0.11 g/l sodium pyruvate and 1000 mg/l glucose supplemented with 20% fetal bovine serum (FBS), 8 mM L-glutamine, 0.1 mM non-essential amino acids, penicillin/streptomycin (10 units/ml and 100 μg/ml respectively) all obtained from GIBCO BRL.

Specific treatments of explants or VSMC were performed using culture medium prepared as above with the exception of FBS and supplemented with test factors. as indicated.

Media for HUVECs

Umbilical cord storage medium: Hanks balanced salt solution (Sigma), with 1.7 mg/ml sodium bicarbonate, 12.5 mM HEPES, and 100 μg/ml gentamicin sulphate. Freshly dispersed cord cells were seeded in M199 (GIBC0-BRL).

HUVEC culture medium: endothelial basal medium (EBM), supplemented with 50 μg/ml gentamicin sulphate, 50 ng/ml Amphotericin B, 10 ng/ml human epidermal growth factor (hEGF) and 12 μg/ml bovine brain extract (all from Clonetics).

2.1.3 Preparation of rabbit aortic medial explants.

Rabbits were sacrificed by pentobarbitone injection (500 mg Expiral, Animal Health Ltd.). Thoracic and descending aorta were harvested by gross dissection and kept in dissection medium. The periadventitial fat was removed and the endothelium denuded with a rubber policeman. After washing with dissection medium the inner media was dissected from the adventitia in 1-cm^2 segments. The medial segments were chopped into 1-mm^2 pieces using a McIlwain tissue chopper (Brinkman), according to the method of McMurray and colleagues (1991). Aortas from two rabbits were required for each time course and the medial explants from each timepoint (~50 mg wet weight) were collected into 50 ml Falcon tubes containing tissue culture medium as indicated in the text. Equivalent numbers of explants were seeded into 90-mm dishes, incubated at 37°C under humidified conditions (5% CO_2, 95% 0_2) and allowed to attach overnight. The explants were then fed with 10 ml of fresh tissue culture medium. Explants cultured in culture medium with FBS were not refed until day 7. Those explants maintained in medium supplemented with growth factors were refed on day 4 and day 7 with 5mls of medium.

Depending on the size of the rabbits, the two aortas provided enough media for experiments with 7 day or preferably 10 day time courses. Explants were examined daily for evidence of outgrowth onto the plastic culture surface. At the appropriate times, explants were harvested from the dishes after draining off residual medium by scraping in ice cold phosphate-buffered saline (PBS), and washed twice in PBS prior to storing at -70^{0}C. Only those explants which had attached to the tissue culture plastic successfully were used for experimental analysis. Explants for day zero timepoints were harvested immediately after dispersing the chopped media into equal quantities for each timepoint.

2.1.4 Rabbit aortic vascular smooth muscle cell culture

Cultured rabbit aortic VSMC were derived from aortic medial explants. The explants harvested from freshly dissected rabbit aorta were bathed in tissue culture medium, seeded into 25 ml vented flasks and incubated under humidified conditions. The following day the explants were fed using 5-7 ml of tissue culture medium, refed at 7 days and cells were left to grow for 10-14 days in total. When VSMC outgrowth from explants was confluent the cells were detached with 1% Trypsin in PBS for 3 minutes. Trypsin was neutralised with tissue culture medium and cell counting was performed using a haematocytometer. VSMC were plated onto tissue culture dishes or acid-etched glass coverslips (at a seeding density of $2.5x10^4$ cells/ml) as appropriate and allowed to grow to full confluence over 5-7 days. Prior to experimental use VSMC were routinely arrested in G_0 phase by incubating in serum free medium for 24 hours.

2.1.5 Human umbilical vein endothelial cell culture

Human umbilical cords were acquired from full term normal deliveries (maternity unit, University College Hospital) and placed in umbilical cord storage medium. The umbilical vein was located and all surrounding tissue was excised. The vein was cannulated and flushed several times with sterile PBS to remove blood clots and adherent material. Endothelial cells were detached by collagenase digestion: both ends of the vein were ligated and 0.5 mg/ml collagenase instilled for 10 minutes at 37°C. Cells suspended in collagenase were flushed out of the vein and pelleted at 200 *g* for 5 minutes and then resuspended in 5 ml M199 (supplemented with 20% FBS, 5 mM glutamine). The cells were seeded into a T25 flask coated with 1% gelatin and allowed to attach overnight. The following day the medium was aspirated and the cells rinsed once with PBS and then fed with 5 ml fresh HUVEC culture medium.

HUVECs were allowed to grow to full confluence and then cultures were expanded by detaching the cells (0.1% Trypsin) and reseeding at lower density. For experimental purposes cells were seeded onto 90-mm dishes or coverslips and allowed to grow to 100% confluence prior to treatments.

2.2 PROTEIN EXTRACTION, GEL ELECTROPHORESIS AND IMMUNOBLOTTING

2.2.1 Protein extraction from explants

Explants from each timepoint were prepared for protein analysis by grinding to a fine powder in liquid nitrogen using a ceramic pestle and mortar.* The powdered samples were lysed in modified RIPA buffer (50 mM Tris-HCL, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1 mM Na_3VO_4, 1% Triton, 0.5 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), 1 μg/ml aprotinin, 1 μg/ml leupeptin, 0.5% sodium deoxycholate, and 0.1% SDS). Lysis took place over four hours at 4^0C on a rotating carousel. Lysates were clarified by centrifugation for 15 minutes at 14000 RPM at 4^0C. Protein concentration was determined with the BCA protein measurement kit (Pierce) - using bovine serum albumin (Sigma) as a standard – according to the manufacturer's instructions. Thereafter aliquots were taken for immunoprecipitation and the remainder of the sample was extracted directly into 5X SDS-PAGE sample buffer (500 mM Tris-HCL pH 6.8, 15% SDS, 5 mM EDTA, 10% β-Mercaptoethanol, 25% Glycerol, 0.1% Bromphenol blue) adjusted to a final concentration of 1X SDS-PAGE sample buffer. Samples were heated to 95-100^0C for 5 min prior to Western Blotting.

2.2.2 Direct protein extraction from cultured cells

After treatment of cells with factors, the medium was aspirated and plates were washed once in ice cold PBS followed by the addition of 2X SDS-PAGE sample buffer. The samples were collected using a cell scraper and transferred to microcentrifuge tubes and heated to 95-100^0C for 5 minutes and thereafter stored at -20^0C prior to SDS-PAGE.

* In some experiments, the relative contribution of the component layers of the vessel wall to protein expression was compared by preparing lysates from whole aorta (with adventitia present), aorta which had undergone endothelial denudation (de-endothelialised aorta) and media alone.

2.2.3 Immunoprecipitation

Following treatment of VSMC or HUVECS, cells were washed once with ice cold PBS and lysed at 4°C in 1 ml lysis buffer (50 mM Tris-HCL, pH 7.4, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1 mM Na_3VO_4, 1% Triton, 0.5 mM AEBSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin) for 15 minutes. Cell lysates were clarified by centrifugation at 15000 *g* for 10 minutes at 4°C, the supernatant was used for immunoprecipitation and the pellet discarded. In some instances, the supernatant was extracted with 2X SDS-PAGE sample buffer and the pellet subjected to further extraction in concentrated SDS-PAGE sample buffer.

Cell lysates or explant lysates containing equal quantities of protein (30-50 μg), were precleared by incubation with Protein A/G+ agarose (Santa Cruz) and normal rabbit/mouse immunoglobulins for 30 min. The samples were then incubated with 2-10 μg of primary antibody at 4^0C for 2 hours, and immune complexes were precipitated with protein A/G+ agarose at 4^0C for a further 1 hour and washed three times with ice cold RIPA buffer. The precipitate was adjusted to SDS-PAGE sample buffer, heated to 95-100^0C for 5 min, and the proteins were separated by SDS-polyacrylamide gel electrophoresis on 8% gels followed by immunoblotting as described below.

2.2.4 Western blotting

(25μg)

Equal quantities of protein from individual timecourses were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in either 8% or 12% gels and electrophoretically transferred to pre-soaked polyvinylidene difluoride (PVDF) membranes (Millipore) for 1 hour at 100 V in a Mini Protean 11 Trans Blot apparatus (Bio-Rad) with cooling. The membranes were blocked with PBS/5% non-fat milk for 30 min with agitation at room temperature and then incubated for 1 hour at 37^0C with primary antibody diluted in blocking buffer. Thereafter membranes were serially washed in PBS/0.1% Tween 20 and then incubated with Horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at 37^0C. After further serial washes

immunoreactive bands were detected by chemiluminescence using the ECL system (Amersham).

2.2.5 Protein quantification and statistical analysis of data

Immunoreactive bands detected on western blots were semi-quantified by scanning densitometry using a flatbed scanner (Umax) and Quantiscan protein densitometry software (Biosoft, 1997) and values for immunoreactivity were expressed as means ± standard error of the mean (SEM).

2.3 SUBCELLULAR FRACTIONATION OF VASCULAR CELLS

A variety of different techniques were used to prepare subcellular fractions of vascular cells. Initially crude subcellular fractions were prepared using the non-ionic detergents Triton X100 or Nonidet-P40. Cells were lysed in either 1% Triton or 0.5% Nonidet-P40 for 15 minutes on ice and samples were collected into 15 ml falcon tubes and then subjected to centrifugation at 500 *g* for 10 minutes at 4^0C to pellet nuclei. The supernatant (designated the cytosolic fraction) was adjusted to 5X SDS-PAGE sample buffer and nuclei were extracted into 2X SDS-PAGE sample buffer. All samples were heated to 95-100^0C for 5 minutes prior to gel electrophoresis and western blotting as described above.

Subsequently a method of subcellular fractionation was used which has previously been reported to effectively separate nuclear and cytosolic proteins in cultured mammalian cells (Boyle *et al.*, 1984; Levkau *et al.*, 1998). This latter method makes use of the detergent digitonin which exhibits selectivity for membranes enriched in cholesterol (such as the plasma membrane lipid bilayer) without causing significant disruption of the nuclear membrane (Howard-Evans, Preparative centrifugation, in PA Series, 1990). Digitonin treatment of cells has been in use for a long time as a means of constituting a cytosol-free environment in the presence of intact nuclei for the purpose of studying nuclear transport mechanisms (Adam *et al.*, 1990). An alternative method of

subcellular fractionation was also employed, based upon hypotonic lysis of cells and separation of nuclei from other cellular constituents by isopycnic centrifugation through dense sucrose (Blobel and Potter, 1966).

2.3.1 Fractionation by selective plasma membrane permeabilisation

Nuclear and cytoplasmic fractions of cells were prepared using digitonin permeabilisation as previously described (Boyle *et al* 1984). Briefly, confluent cultures of cells were treated as described and treatments were terminated by transferring dishes onto ice. Both attached and floating cells were collected into 15 ml falcons and cells were pelleted by centrifuging at 500 *g* for 10 minutes at 4^0C and then washed twice by resuspending in 1ml ice cold PBS. Cells were pelleted again at 500 *g* and all supernatant was carefully aspirated using a fine tipped pipette. Thereafter the cell pellet was dispersed and lysed in 240 µl nuclear lysis buffer (150 mM NaCl, 150 mM sucrose, 20 mM Hepes pH 7.4, 5 mM KCl, 2 mM DTT, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 0.1% digitonin with AEBSF, aprotinin and leupeptin inhibitors as described earlier). The efficiency of plasma membrane permeabilisation was improved by pipetting samples gently on ice for 3-5 minutes: this action also improved the purity of the nuclear fraction by dissociating plasma membrane and cytoskeletal structures from nuclei.

Nuclei were harvested by centrifugation at 800 *g* for 10 minutes: the resulting pellet was designated the nuclear extract and the supernatant was designated the cytosolic fraction. Both the nuclear extract and the supernatant were adjusted to SDS-PAGE sample buffer and heated to 95-100^0C for 5 minutes prior to SDS-PAGE electrophoresis followed by immunoblotting as described above.

2.3.2 Fractionation by isopycnic centrifugation.

90-mm dishes of fully confluent HUVECS were washed twice in PBS at room temperature and cells were collected by scraping and centrifuging at 500 *g* for 10 minutes. The resulting pellet was washed once in hypotonic lysis buffer (10 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.1 mM AEBSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin). Cells were resuspended in 250 µl hypotonic lysis buffer

and allowed to swell on ice for 20 minutes. Cells were lysed in a 1ml Dounce homogeniser using 50-100 strokes. The efficiency of cell lysis was monitored using phase contrast microscopy. The homogenate was transferred to a 1.5 ml eppendorf and carefully underlayed with 500 µl sucrose cushion (10 mM HEPES, pH 7.5, 10 mM KCl., 40% sucrose, 1.5 mM $MgCl_2$, 1 mM DTT, 100 µM AEBSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin). Nuclei were harvested at 1500 *g* for 10 minutes in a swinging bucket rotor centrifuge. The nuclear pellet and supernatant (designated the cytosolic fraction) were adjusted to SDS-PAGE sample buffer and heated for 5 minutes at 100° prior to analysis by gel electrophoresis and western blotting.

2.4 IMMUNOFLUORESCENT STAINING AND MICROSCOPY OF CELLS

Freshly isolated VSMC or HUVECS were cultured on glass coverslips pre-etched in concentrated sulphuric acid for 24 hours. All procedures thereafter took place at room temperature. VSMC were washed in PBS and then fixed in 3% paraformaldehyde in PBS for 10 minutes. After washing again in PBS the cells were incubated in 50 mM ammonium chloride for 10 minutes and then washed again in PBS prior to permeabilising the cells in 0.2% Triton X-100 in PBS for 5 minutes. HUVECS were fixed and permeabilised by immersing in pre-cooled methanol for 10 minutes at -20°C and thereafter washed in PBS. Cells were incubated in blocking buffer with 2% FBS for 30 minutes, rinsed three times in PBS and then incubated in primary antibody/antibodies. The cells were washed three times again prior to incubation with fluorochrome-conjugated secondary antibody/antibodies, DAPI or Rhodamine Phalloidin. Primary and secondary antibodies were diluted in PBS/2% FBS and exposures lasted for 30 minutes each time. The coverslips were inverted onto microscope slides, mounted in Vectashield (Vector Labs, Inc.) and sealed using nail varnish. Specimens were studied on a Zeiss axiophot microscope using a 63X immersion objective with a numerical aperture of 1.25 and photographed using Kodak Gold or Ektachrome film. In all experiments utilising double-staining techniques, control stains were performed with each antibody separately to ensure that co-staining

was not as a result of antibody cross reactivity and further immunostaining using secondary antibodies alone demonstrated absence of fluorescence.

Peptide neutralisation studies: immunostaining was also performed after a one hour pre-incubation of primary antibody (either FAK-C or FAK-A) with a peptide corresponding to the immunogen. Blocking peptide was used at 5 and 10 fold excess by weight in order to verify specificity of immunostaining.

2.4.1 Scanning laser confocal microscopy

Cells were prepared for immunofluorescent staining as described already. Confocal microscopy was done as reported (Lakkakorpi *et al* 1993) using the Leica TCS NT and Bio-Rad MRC 600 or 1020 systems. Cells on coverslips were viewed with the confocal laser scanning microscope consisting of a Wild Aristoplan fluorescence microscope equipped with a multiline 750 mW air-cooled Omnichrome argon-crypton laser (Chino, California, USA). Cells were viewed with 40X and 63X immersion objectives with appropriately chosen pinholes using 1024 X 1024 image format. Fluorescent images were sequentially collected in 0.625-1.25 μm steps through VSMC for FITC and TRITC fluorochromes at 488-nm and 568-nm emission wavelengths respectively. Hard copy images were produced after computer processing of the data and generation of pseudocolour images.

2.5 ANALYSIS OF CELL DEATH

Endothelial cells were cultured on glass coverslips pre-etched in concentrated sulphuric acid. Following treatments to induce EC apoptosis, cells were fixed in 3.7% paraformaldehyde for 20 minutes and then permeabilised with 0.2% Triton X-100/PBS for 5 minutes on ice. After washing in PBS, they were subjected to terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) with the Apoptosis Detection System, Fluorescein Kit as recommended by the manufacturer (Promega Corp., Madison, Wisconsin). As a control for non-specific staining, the

reaction mixture was used without enzyme. The final wash was preceded by a 15 minute incubation in 1 μg/ml propidium iodide/PBS which stains both apoptotic and non-apoptotic cells red throughout the cytoplasm. Samples were mounted using Vectashield (Vector Laboratories, Burlingame, California). Fluorescin-12-dUTP incorporated into the ends of fragmented DNA, resulting in localised green fluorescence within the nucleus of apoptotic cells, was visualised with an epifluorescence microscope (as described in Section 2.4).

In the subsequent results chapters, each of the figures shown as representative examples are the result of at least three independent, replicate experiments and in some instances up to ten experiments were performed.

CHAPTER 3

Expression of focal adhesion components in early explant culture.

Introduction

Intimal VSMC in lesions of atherosclerosis undergo transition from a contractile phenotype - representing the form usually identified in the arterial medial layer - to a synthetic phenotype. VSMC in primary culture undergo a similar phenotypic modulation. The phenotypic transition of VSMC involves a loss of components of the contractile machinery, but the mechanisms are otherwise largely unknown.

A role for FAK in mesenchymal cell migration and proliferation has been suggested by several studies. In this chapter the expression of FAK and other focal adhesion components was examined in aortic medial tissue, and changes in the expression of these molecules were investigated in the early stages of explant culture of medial explant tissue prior to the onset of VSMC migration/proliferation. In addition, the regulation of focal adhesion component expression was investigated in response to defined growth factors.

3.1 Characterisation of VSMC in culture

Culture of explants derived from NZWM rabbit aortic medial tissue leads to outgrowth of VSMC from the explant and proliferation on the tissue culture dish. VSMC in primary culture were identified both on the basis of morphological appearance (Fig. 3.1A and see below) and by detection of VSMC-specific markers. In primary VSMC cultures, immunofluorescent detection of VSMC-specific α-actin (Fig. 3.1B) demonstrated that these cells were VSMC. This was corroborated by immunoblot analysis of VSMC lysates showing immunoreactivity for smooth muscle-specific myosin heavy chain (MHC) and α-actin (Fig. 3.3).

3.2 VSMC culture by explant outgrowth

Rabbit aortic medial explants cultured in 20% serum exhibited visible outgrowth of cells after 5 days at the earliest (Fig. 3.2A). Initially such outgrowth comprised one to several cells and involved small numbers of individual explants. It was not until later in the timecourse (after days 7-10) that most explants began to exhibit outgrowth but the extent of such outgrowth varied considerably between explants (Fig. 3.2B). With increasing time in culture, explants were observed to diminish in size consistent with the fact that cells were migrating from the tissue and not simply proliferating on the tissue culture dish (Fig. 3.2C-D).

Cells growing onto the tissue culture plastic from explants were identified as VSMC on the basis of morphology and by immunofluorescent detection of VSMC-specific α-actin. Initially cells migrating out of explants appeared to have extended processes and a 'fibroblast-like' appearance (Fig. 3.2A)– these cells are capable of proliferation. With further outgrowth and increasing cell growth around the explants the VSMC eventually become fully confluent by which point they have assumed a 'spindle-like' morphology, lacking the extended processes of subconfluent VSMC and exhibiting the characteristic 'hills and valleys' appearance of confluent VSMC (Fig. 3.1A).

3.3 Expression of VSMC-specific markers in rabbit aorta and during early explant culture

Tissue samples of rabbit aorta were analysed for expression of VSMC-specific proteins by direct protein extraction in RIPA buffer followed by immunoblotting of equal amounts of protein. Immunoblot analysis of extracts prepared from fresh whole aorta, de-endothelialised aorta and aortic media showed that smooth muscle-specific myosin heavy chain (MHC) expression was readily detectable in all extracts but was relatively most abundant in fresh media (Fig. 3.3A). As Fig.3.3A also indicates, western blot analysis of VSMC-specific α-actin showed high levels of expression.

Assessment of protein expression of the VSMC specific marker MHC in serum-fed explants showed that MHC immunoreactivity decreased markedly in early explant culture (Fig. 3.3B). MHC protein expression remained unchanged during days 0 to 2 in culture and declined rapidly thereafter in contrast to increased expression of focal adhesion components with time (Fig. 3.5). The onset of reduction in MHC expression occurred prior to visible outgrowth of cells from any explants. The expression of MHC continued to decrease after later times in culture up to 7-9 days. Levels of expression of VSMC-specific α-actin protein did not appear to vary significantly during the same timecourse until after several days in explant culture (days 5 to 7) when there was a noticeable decrease in expression (Fig. 3.3B).

3.4 Expression of p125^{FAK} and paxillin in rabbit aorta

Tissue samples of rabbit aorta were then analysed for expression of focal adhesion components by direct protein extraction in RIPA buffer as described. Immunoblot analysis of extracts prepared from fresh whole aorta, de-endothelialised aorta and aortic media showed that p125FAK and paxillin were expressed in the intact aorta and their expression was not significantly altered after endothelial denudation (Fig. 3.4). In contrast FAK expression was detectable at a relatively lower level in isolated media. Expression of paxillin in isolated media was readily detectable but its expression was reduced compared to the whole aorta.

3.5 Changes in expression of p125^{FAK} and paxillin in the early stage of medial explant culture.

Next the protein expression of the focal adhesion components FAK and paxillin was determined in extracts of medial explants cultured for different times from 0 to 10 days. Although visible outgrowth from most explants was not observed until between 7 to 10 days after placing explants in culture, FAK immunoblots showed that the expression of the 125 kDa form of FAK increased during explant culture with a

detectable increase as early as 3 days (Fig. 3.5A). FAK expression was virtually undetectable over days 0-2 but thereafter increased throughout the timecourse.

Paxillin was expressed at significant levels from the outset of the timecourse in fresh uncultured medial tissue. During explant culture, significant increases in paxillin protein expression took place prior to the development of visible VSMC outgrowth, from approximately day 3, and continued to increase throughout explant culture (Fig. 3.5A). Semi-quantification of immunoreactive bands showed that $p125^{FAK}$ immunoreactivity in explants increased by 2.1 ±0.6 fold (n=4) and 3.7 ±0.5 fold (n=4) after 3 and 5 days in culture respectively (Fig. 3.4B). Corresponding increases in expression of paxillin obtained from quantification of parallel samples were 3.2 ±0.5 fold (n=3) and 4.2 ±1.3 fold (n=3) respectively.

3.6 Detectable expression of $p125^{FAK}$ in uncultured explants.

In some timecourses it was noted that FAK expression transiently declined over days 1-2, before increasing again over the remainder of the timecourse (Fig. 3.6). The aortas from which these samples were derived did not appear morphologically different to those in which $p125^{FAK}$ expression was not observed at day 0.

3.7 Changes in the expression of $p130^{Cas}$ in early explant culture

There was also a striking increase in expression of the FAK-associated protein $p130^{Cas}$ during explant culture. Increased expression of $p130^{Cas}$, which was first detected at day 3 in explant culture, continued up to and including day 10 (Fig. 3.7A). Consistent with previous reports, immunoreactive $p130^{Cas}$ bands characteristically migrated as a doublet comprising isoforms of 130 and 125 kDa of which the former was predominant. FAK is known to associate with several focal adhesion components including $p130^{Cas}$ and paxillin. In order to examine whether FAK association with $p130^{Cas}$ increased during explant culture, FAK immunoprecipitates of explant lysates were prepared. Using a polyclonal antibody to the carboxy-terminus of FAK (FAK-C, Santa Cruz),

immunoprecipitates of explant lysates were prepared and the resultant immune complexes were immunoblotted with antibody to $p130^{Cas}$ (Fig. 3.7A upper). Over a 10 day timecourse there was a significant increase in the association of $p130^{Cas}$ with FAK which became apparent by day 2 and was sustained thereafter (Fig. 3.7B lower). $p130^{Cas}$ antibody characteristically recognised doublet protein bands in FAK immunoprecipitates similar to results obtained by direct western blot of protein extracts with this antibody.

3.8 Regulation of expression of focal adhesion components by growth factors

It was next examined whether defined growth factors known to simulate proliferation and/or migration of VSMC could also increase expression of FAK, paxillin and $p130^{Cas}$. Medial explants were cultured for various times in the presence of 25 ng/ml PDGF-BB, 25 ng/ml IGF-1 and 10 ng/ml bFGF in serum-free tissue culture medium. VSMC outgrowth from these explants did not arise until much later (8-10 days) and was much less marked compared to outgrowth from explants cultured in 20% serum. In addition the cells appeared less healthy with ragged processes and numerous vesicles; on no occasion did such outgrowth progress to a fully confluent culture despite prolonged culture (exceeding 20 days) and replenishment of test factors.

Protein expression of FAK, paxillin and $p130^{Cas}$ increased markedly during explant culture with growth factors, but in all cases the time-course of this increase was slower compared to changes observed in serum-cultured explants (Fig. 3.8A). As noted with serum-fed explants however, the increase in expression of these focal adhesion components occurred several days prior to the onset of VSMC outgrowth. FAK association with $p130^{Cas}$ also increased during explant culture with growth factors in a manner paralleling the results obtained in serum-fed explants (Fig. 3.8B).

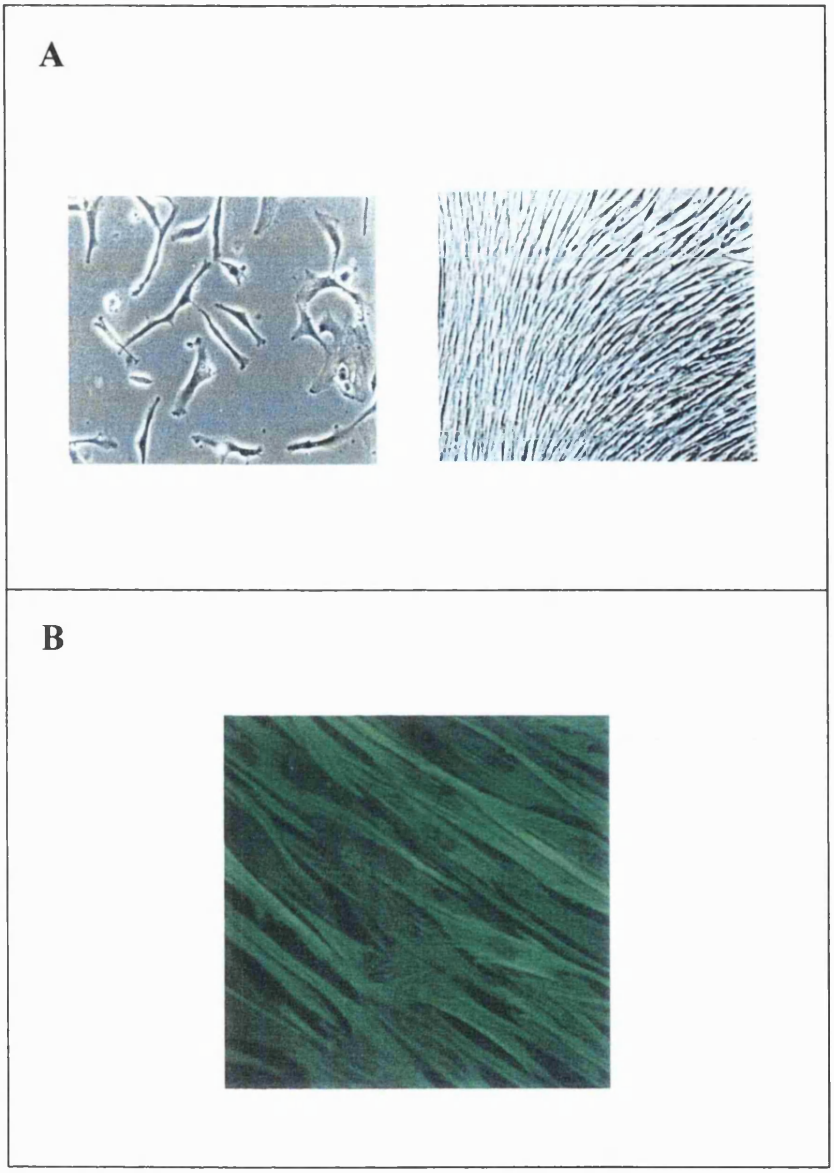


Fig 3.1 Characterisation of VSMC in primary culture.
A. Morphological appearance of subconfluent (left, day 7) and confluent (right, day 12) explant culture of VSMC as observed using phase contrast light microscopy.
B. Immunofluorescent staining of VSMC with an antibody to the VSMC-specific marker α-actin conjugated to FITC. All photographs were taken with a Nikon camera fitted to a Nikon Optiphot microscope

(Scale bars needed)

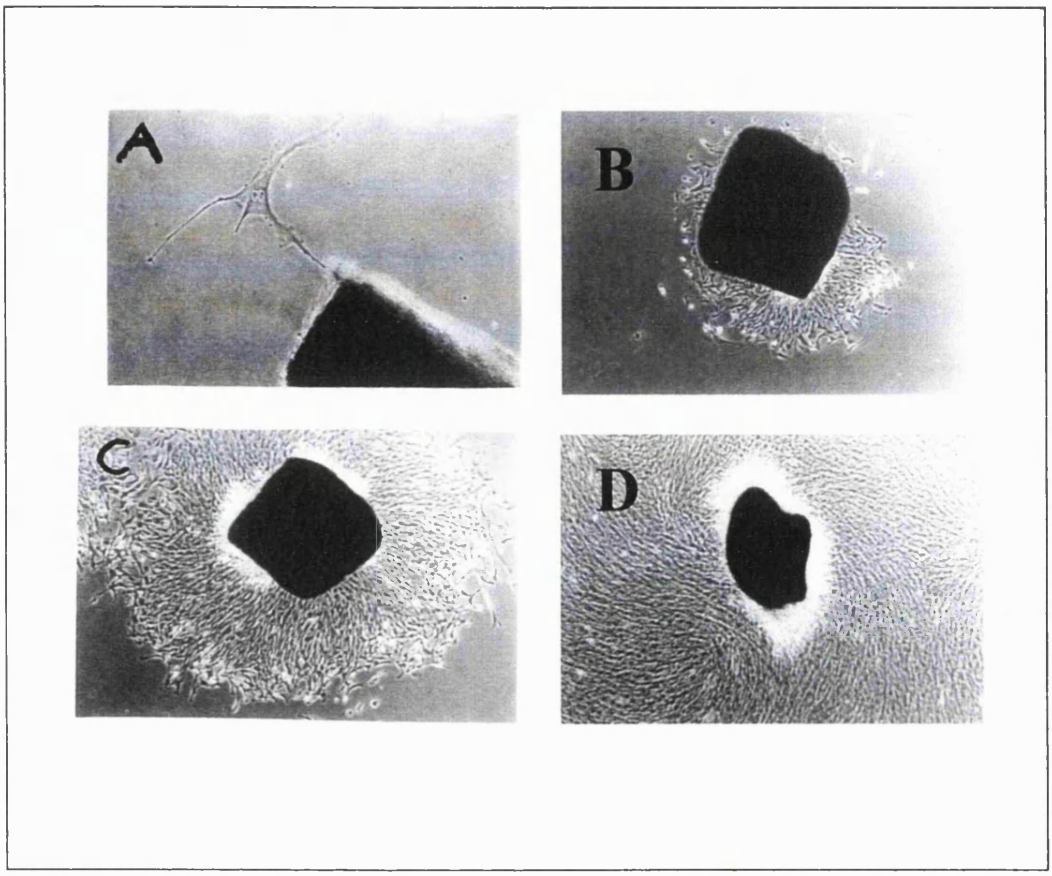


Fig. 3.2 Outgrowth of VSMC from explants of rabbit aortic media.
New Zealand white male rabbits were sacrificed by pentobarbitone injection and aortae were dissected and periadventitial fat was removed. The endothelium was denuded using a rubber policeman and the inner medial layer was dissected in 1-cm^2 segments. The segments were chopped into 1-mm^2 explants and seeded into 25 ml flasks and fed the following day with 20% FBS. Outgrowth was first observed after 5 days (**A**) at the earliest and VSMC outgrowth was generally established by day 10 (**B**). After 14 days (**C**) more cells were apparent and nearly all attached explants exhibited some degree of VSMC outgrowth. By day 18 the cells were fully confluent (**D**) and explants had concomitantly diminished in size.

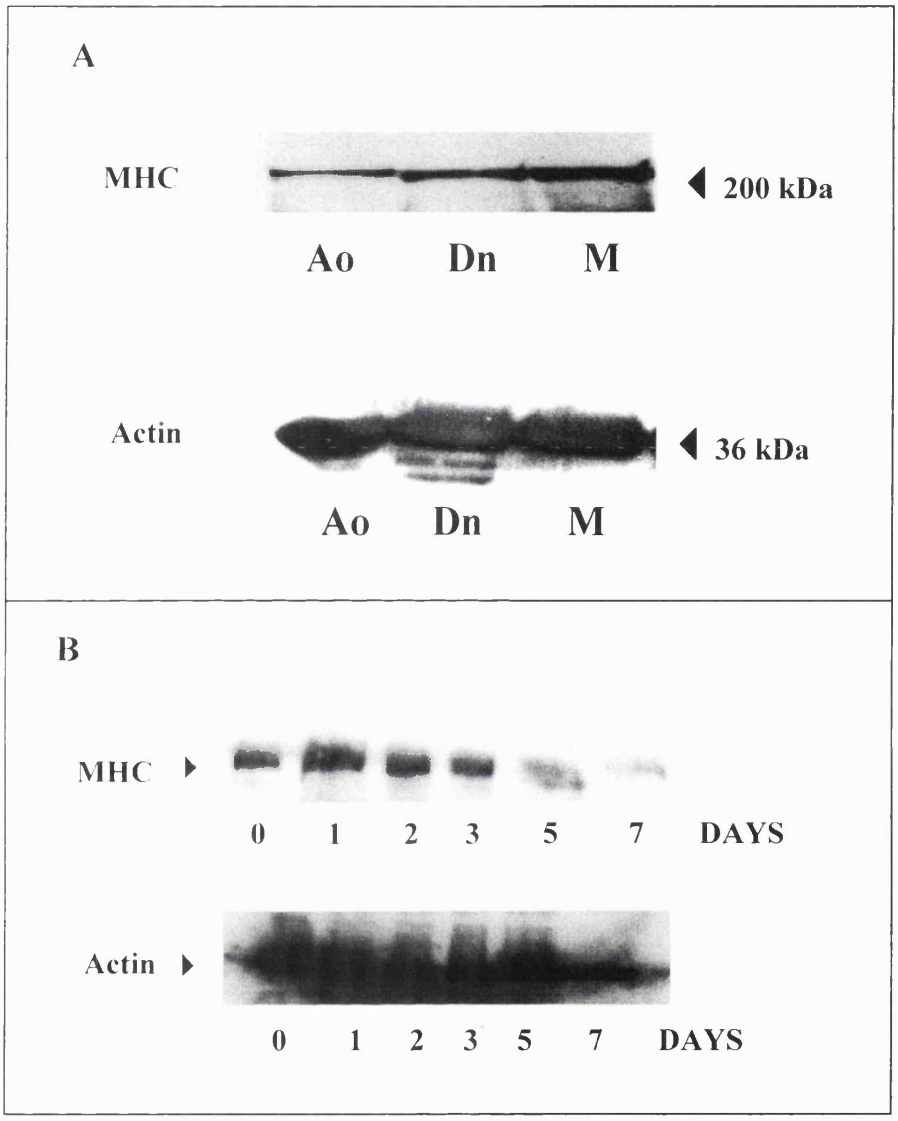


Fig. 3.3 Protein expression of VSMC-specific markers in rabbit aorta and in early explant culture.
A: Protein extracts were prepared from whole aorta (**Ao**), deendothelialised aorta (**Dn**) and media (**M**) and equal amounts of protein were immunoblotted with antibodies to the smooth muscle-specific proteins myosin heavy chain (MHC) and α-actin (Actin).
B: Extracts were prepared from explants cultured for the times indicated in days and equal amounts of protein were directly immunoblotted with antibodies to the VSMC-specific proteins MHC and α-actin (Actin).

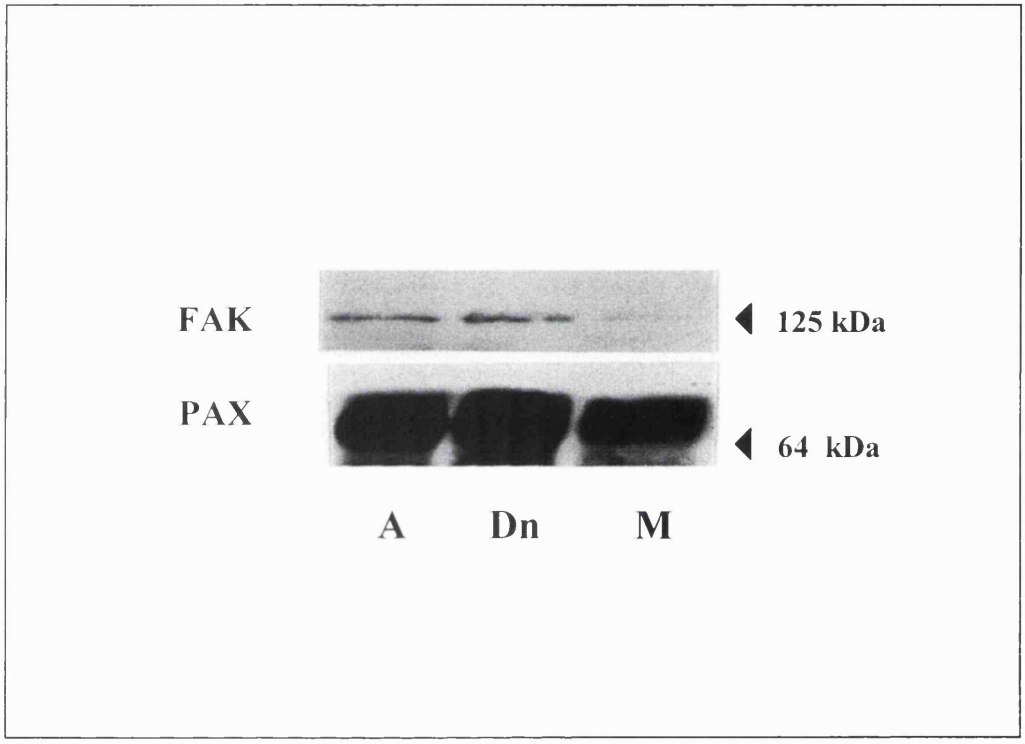


Fig. 3.4 Expression of FAK and paxillin in the rabbit aorta.
Protein extracts were prepared from whole aorta (**A**), deendothelialised aorta (**Dn**) and media (**M**) and equal amounts of protein were immunoblotted with antibodies to FAK and paxillin (PAX)

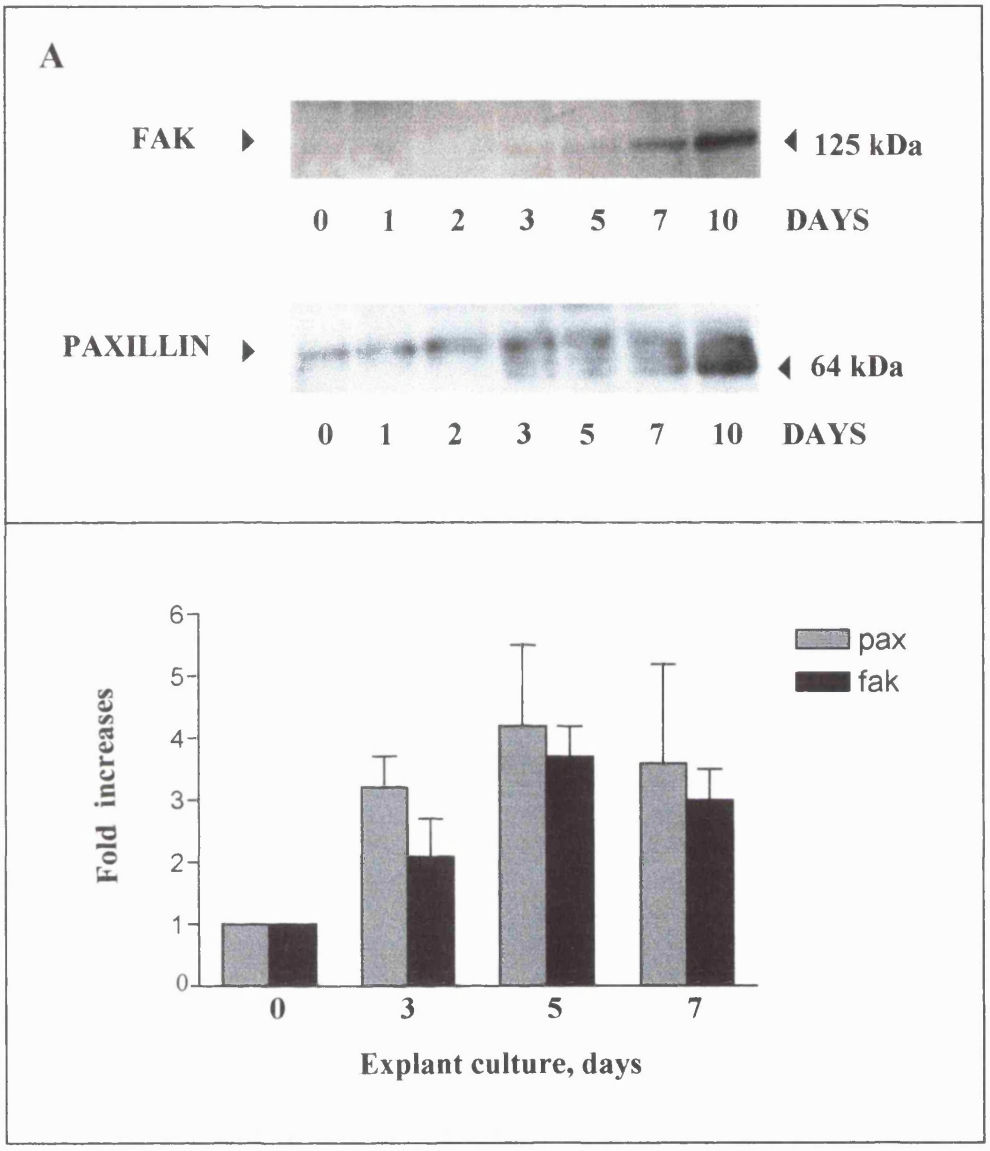


Fig 3.5 Changes in FAK and paxillin expression during early VSMC explant culture.

Explants cultured for the indicated time were lysed in modified RIPA buffer and after clarification and protein quantification, lysates containing equal amounts of protein were subjected to SDS-PAGE followed by transfer of proteins to PVDF membranes. **A:** Immunoblotting with antibody to FAK-C and paxillin was then performed. The blots shown are representative of at least four similar independent experiments. **B**: Mean fold increases in FAK and paxillin expression. Error bars represent standard error of the mean.

The fold increases shown are representative of the results of four independent experiments.

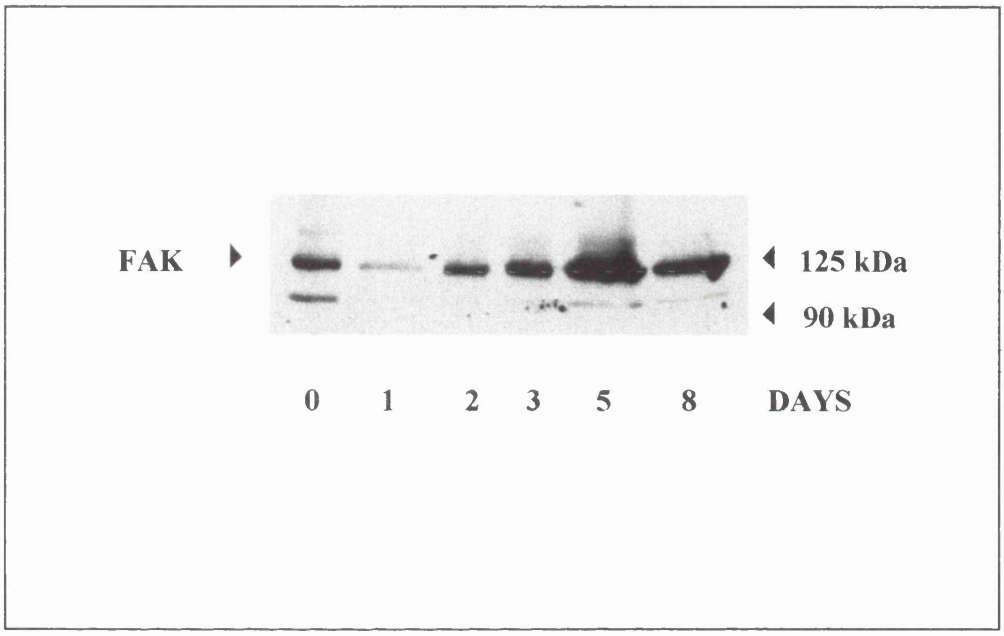


Fig 3.6 Elevated basal expression of FAK in uncultured medial tissue. Explants cultured for the indicated time were lysed in modified RIPA buffer and after clarification and protein quantification, lysates containing equal amounts of protein were subjected to SDS-PAGE followed by transfer of proteins to PVDF membranes. Immunoblotting with antibody to FAK-C revealed detectable expression of immunoreactive FAK in uncultured explants in some time courses.

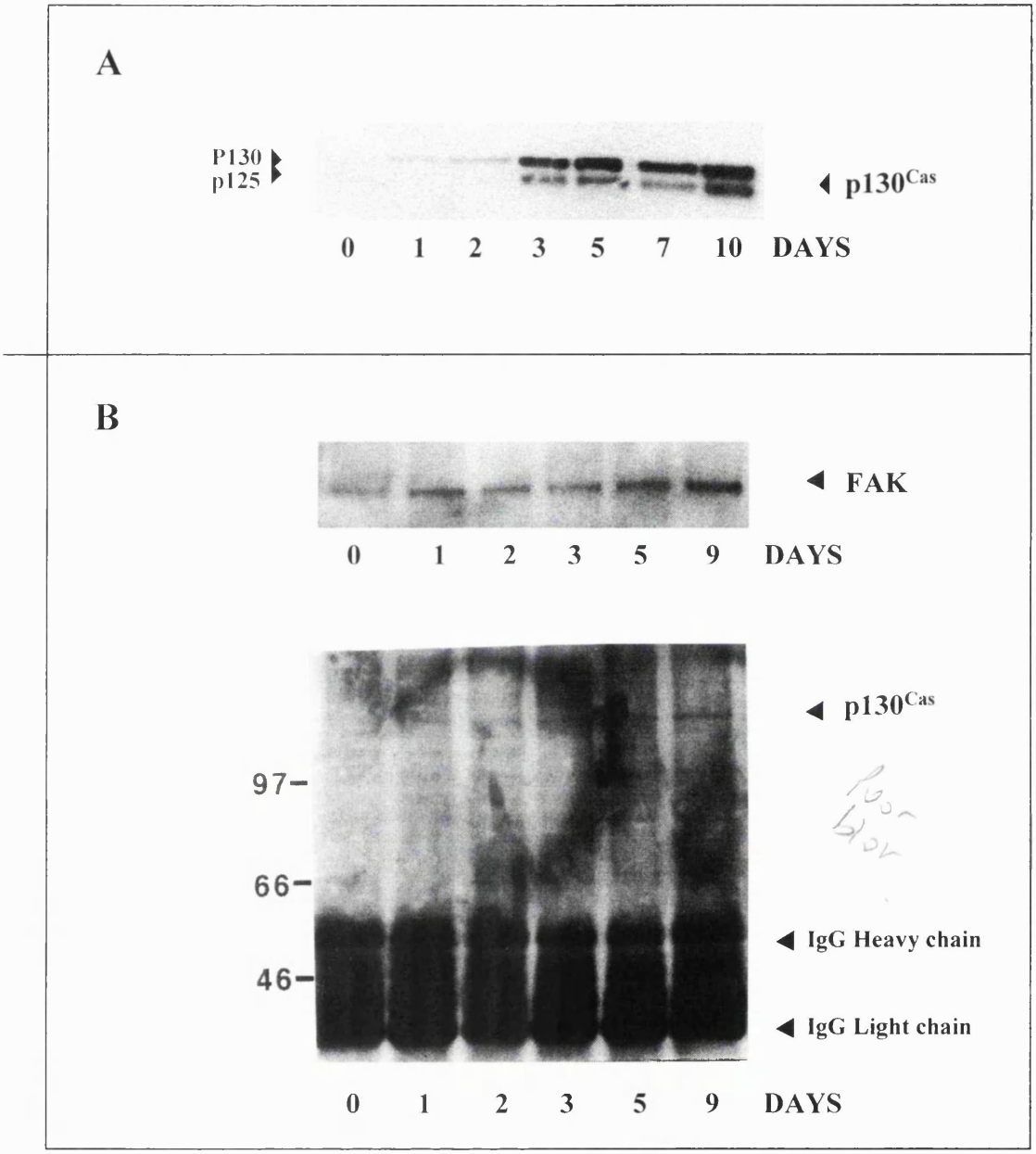


Fig 3.7 Increased expression and FAK association of p130^{Cas} during early VSMC explant culture. Explants cultured in 20% serum for the indicated times were lysed in modified RIPA buffer and clarified at 14000 RPM for 20 min at 4^{0}C. Equal amounts of protein from each timepoint were either extracted directly as described above and immunoblotted with p130^{Cas} antibody (panel **A**) or immunoprecipitated using FAK-C antibody (panel **B**). Immunoprecipitates were collected using Protein A/G+ agarose and after washing, proteins were separated by SDS-PAGE, transferred to membranes and subsequently immunoblotted with antibody to FAK (panel **B**, upper) or to p130^{Cas} (panel **B**, lower). In panel B, the positions of IgG heavy and light chains are indicated. The results shown are representative of at least 3 independent experiments.

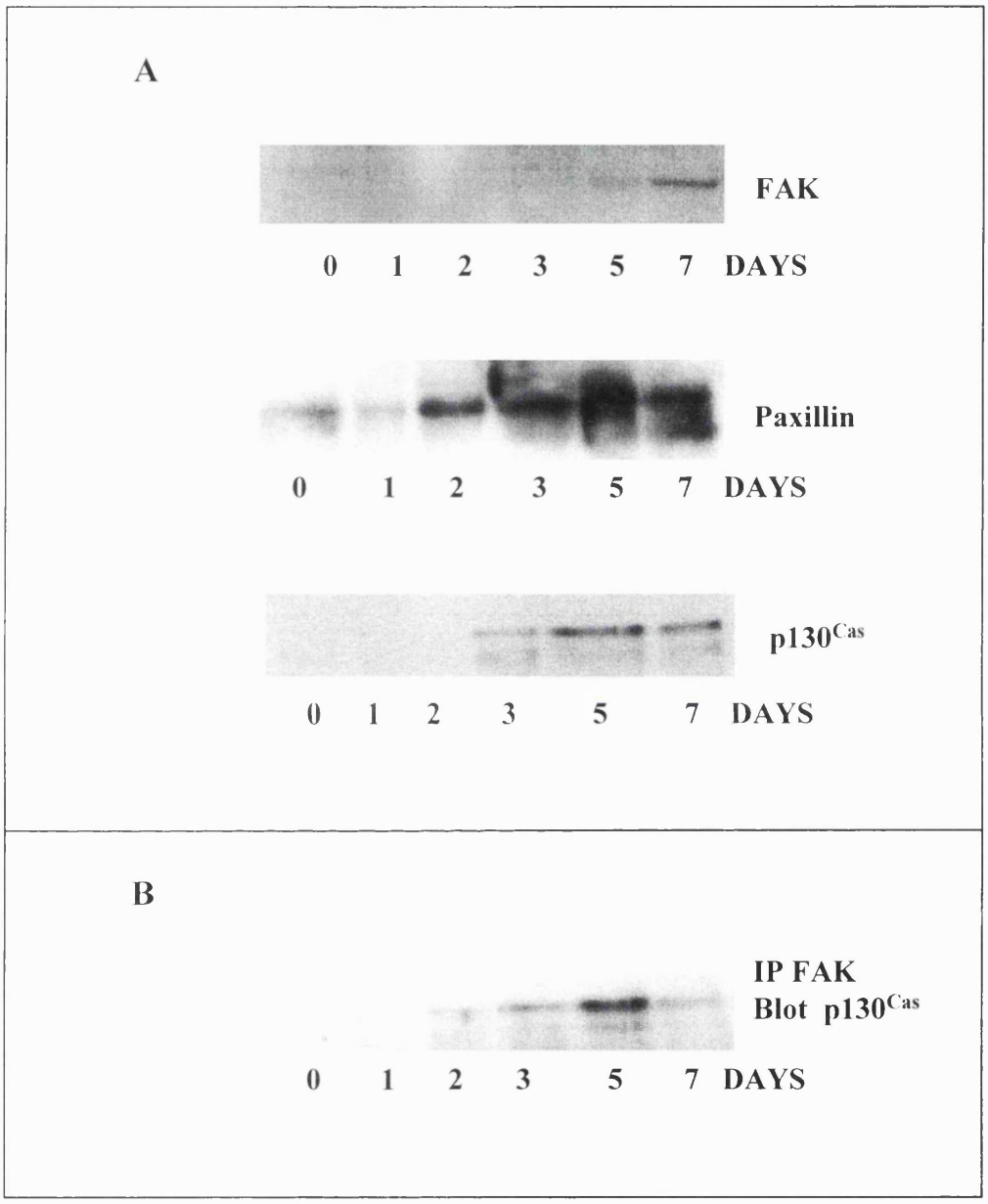


Fig 3.8 Growth factors induce expression of FAK, paxillin and $p130^{Cas}$ and increase FAK/$p130^{Cas}$ association in medial explant culture.
Extracts were prepared from explants cultured with 25 ng/ml PDGF-BB, 25 ng/ml IGF-1 and 10 ng/ml bFGF for the times indicated in days and equal amounts of protein were either directly immunoblotted with FAK, paxillin or $p130^{Cas}$ (**A**) or were used for immunoprecipitation of FAK and subsequent immunoblot with $p130^{Cas}$ (**B**).

SUMMARY

The explant model of VSMC outgrowth from medial explants was used to examine changes in the cellular constituents of focal adhesions during the early stages of phenotypic modulation of VSMC from a contractile phenotype to a more 'synthetic' phenotype characterised by the acquisition of the ability of VSMC to proliferate and migrate.

The results presented in this chapter show that the focal adhesion components FAK, paxillin and $p130^{Cas}$ are upregulated in cultured rabbit aortic medial explants. The evidence indicates that there is upregulation of expression of these focal adhesion components in the early stages of explant culture prior to the onset of visible outgrowth of cells from the explants. This increased expression may be a prerequisite for the transition of VSMC from a contractile to a synthetic form. Concomitant with increased expression of focal adhesion components, expression of VSMC-specific MHC declined throughout explant culture, consistent with a transition from a contractile to a synthetic phenotype.

It was also demonstrated that increased protein expression of FAK and $p130^{Cas}$ during explant culture was associated with an increase in association of these components in immunoprecipitable complexes. Treatment of explants with the defined growth factors PDGF-BB, IGF-1 and bFGF also led to explant outgrowth and marked increases in the protein expression of FAK, paxillin and $p130^{Cas}$.

CHAPTER 4

Expression of other signalling molecules in early explant culture.

Introduction

The development of a neointima in vivo requires the migration and proliferation of VSMC originating from the arterial media. Similarly in the explant model of VSMC outgrowth, both migration of cells from the explants as well as proliferation of VSMC takes place. It was therefore of interest to define the expression of proteins which take part in the signal transduction cascades leading to mitogenesis and cell migration, and compare and contrast these with expression of focal adhesion components.

Mitogen activated protein kinases (ERKs 1 and 2) are well established to play a role in mitogenic signalling in many cell types including VSMC. Upstream of ERKs 1 and 2 lie adaptor proteins linking ligand-engagement of cell-surface receptors to the ERKs 1 and 2 cascade. One such protein is GRB-2 (growth factor receptor-bound protein 2) which is implicated in ras-dependent activation of ERKs 1 and 2. Expression of ERKs 1 and 2, GRB-2 and PCNA (a marker of cell proliferation) was consequently examined during early explant culture of aortic medial VSMC.

In addition, changes in the expression of other components implicated in the regulation of VSMC migration were also examined during early explant culture of VSMC. These included the urokinase-type plasminogen activator receptor (UPAR), which was reported to mediate VSMC migration *in vivo* and *in vitro*, and the small GTP-binding protein Rho, which has also been shown to play a role in migration of VSMC.

4.1 Expression of ERKs 1 and 2 in native aortic tissue.

Expression of ERKs 1 and 2 (p44 and p42 MAP kinases) was first examined in uncultured aortic tissue using samples paralleling those used for analysis of focal adhesion component expression. As shown in Fig. 4.1A, ERKs 1 and 2 were expressed

in extracts of whole aorta in an activated state. The pattern of expression of immunoreactive ERKs 1 and 2 did not alter after endothelial denudation and was modestly decreased in medial extracts. Total ERK2 (p42 MAP Kinase) expression in extracts of whole aorta, deendothelialised aorta and media was not significantly different. Expression of ERKs 1 and 2 was also compared in extracts of fresh media and extracts prepared from medial explants which had been in serum-fed culture for 11 days (Fig. 4.1B). Although activated and total ERKs 1 and 2 expression was readily detectable in fresh media, neither was present in cultured explants.

4.2 Expression of ERKs 1 and 2 during explant culture.

Next the expression of ERKs 1 and 2 was examined during early explant culture in 20% serum over a similar time-course to that used to study expression of focal adhesion components. Evaluation of ERKs 1 and 2 expression showed that expression of activated ERKs 1 and 2 was high in fresh explants and declined significantly during explant culture (Fig. 4.2 above). Expression of total (active and inactive) ERKs 1 and 2 was also higher in fresh explants and declined rapidly during explant culture (Fig 4.2 below).

4.3 Expression of ERKs 1 and 2 during explant culture in the presence of growth factors.

Explant culture in the presence of the defined growth factors PDGF-BB, IGF-1 and bFGF was associated with an even more rapid decline in levels of activated ERKs 1 and 2 (Fig. 4.3). Activated ERKs 1 and 2 were not detectable after two days of culture, but a modest increase in expression occurred after 5 to 7 days.

4.4 Expression of GRB-2 in early explant culture.

The expression of GRB-2, which links receptor tyrosine kinases to ras-dependent activation of the ERK cascade, was examined in early explant culture. As with ERK expression, GRB-2 expression was high in uncultured medial extracts and

declined to almost undetectable levels during the subsequent period of culture from 0 to 7 days (Fig. 4.4).

4.5 Expression of PCNA during explant culture.

As shown in Fig 4.5A (top panel), expression of PCNA was barely detectable in extracts prepared from deendothelialised aorta and fresh medial tissue although an immunoreactive band was observed in extracts from whole aorta. A positive control lysate from an epithelial carcinoma cell line containing equal amounts of protein was noted to stain very strongly for PCNA in the same experiment. A comparison of fresh medial extracts with extracts from 11 day old explants cultured in 20% FCS revealed markedly increased expression of PCNA in the latter samples (Fig. 4.5A bottom panel).

Explants were then cultured in 20% FBS over a 10-day time course and extracts were prepared and immunoblotted as shown in Fig 4.5B. On days 0 and 1 of explant culture PCNA expression was not detectable but an immunoreactive band was visible by day two. Expression thereafter increased markedly, reaching a maximum after day 5 and was sustained thereafter. The increase in expression of PCNA was noted to precede the onset of any VSMC outgrowth from explants, which was not visible until day 5-6 in any of the time courses.

4.6 Expression of UPAR during explant culture.

Protein expression of the urokinase receptor (UPAR) was also examined (Fig. 4.6). UPAR has been previously shown to mediate VSMC migration *in vitro* and *in vivo*. The expression of UPAR was seen to increase over a 7 day period, concomitant with increased expression of focal adhesion components. A detectable increase in UPAR expression was observed as early as after 24 hours in explant culture, several days prior to the onset of VSMC outgrowth from explants.

4.7 Expression of Rho protein in explant culture.

The small GTP-binding protein Rho is implicated in regulation of actin cytoskeletal organisation and is thus thought to be important for cell migration. In extracts prepared from fresh aortic tissue and deendothelialised aorta, Rho expression was high and not significantly different (Fig.4.7A). Rho expression was markedly reduced in extracts prepared from media although still easily detectable (Fig 4.7B).

Protein expression of Rho was examined during a 10 day time course. Although Rho expression was readily detectable at day 0 in uncultured medial extracts, by day 1 this expression was considerably diminished but thereafter increased throughout the remainder of the time course.

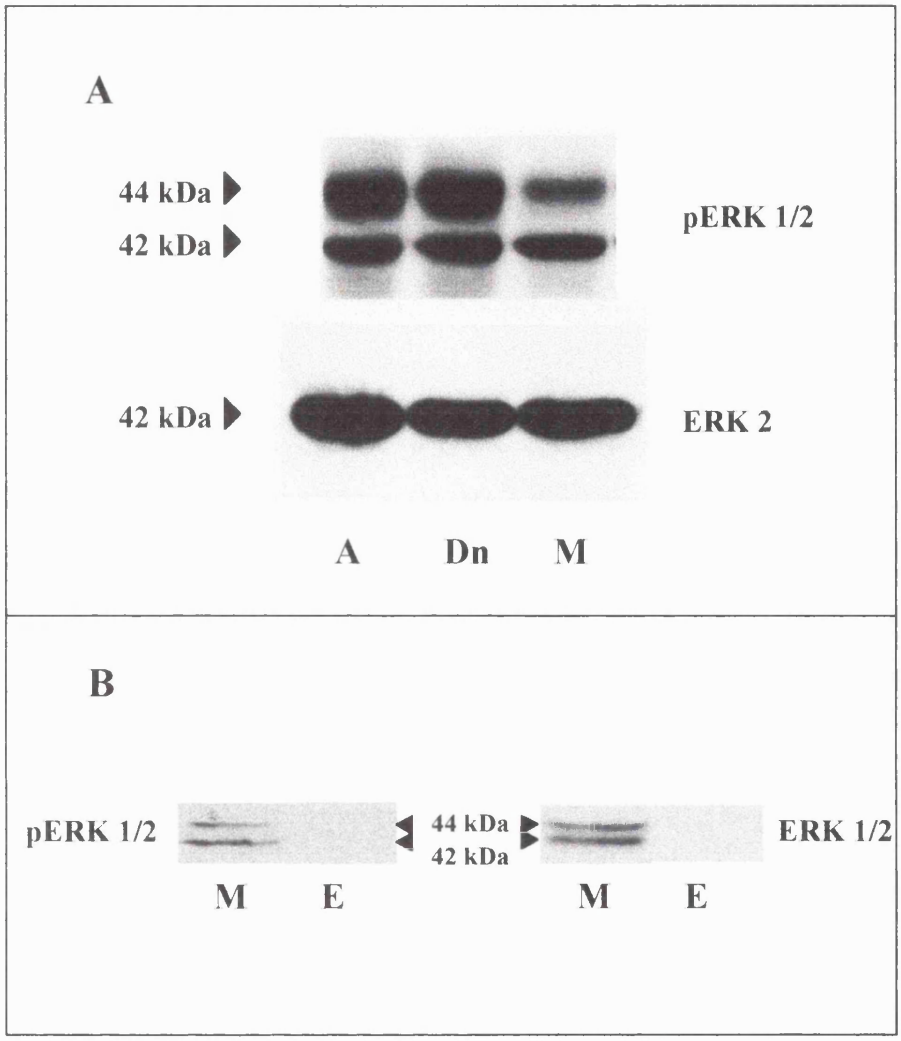


Fig. 4.1 ERKs 1 and 2 are differentially expressed in aortic tissue and cultured medial explants.

A. Protein extracts were prepared from whole aorta (A), deendothelialised aorta (Dn), and media, and equal amounts of protein were immunoblotted with antibodies to either activated ERKs 1 and 2 (pERK 1/2) or total ERK2.

B. Protein extracts were prepared from fresh media (M) and medial explants which had been in culture for 11 days (E) and equal amounts of protein were immunoblotted with antibodies to either activated ERKs 1 and 2 (left) or total ERKs 1 and 2 (right).

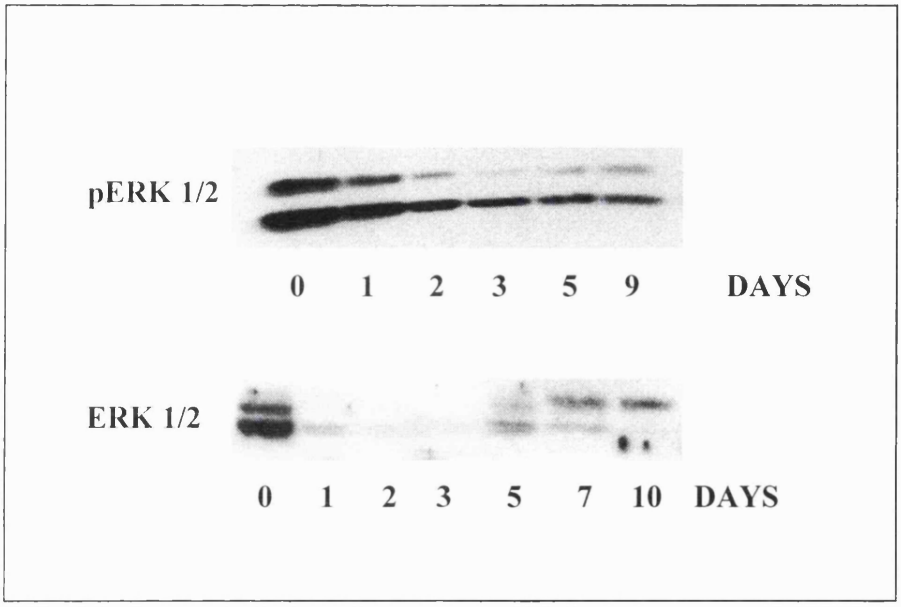


Fig. 4.2 Decreased expression of ERKs 1 and 2 during medial explant culture
Protein extracts were prepared from medial explants cultured in 20% FBS for the times indicated and equal amounts of protein were immunoblotted with antibodies to either activated ERKs 1 and 2 (pERK 1/2) or total ERKs 1 and 2.

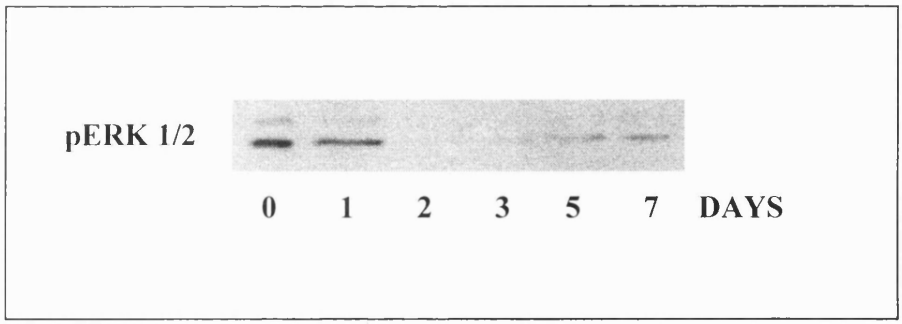


Fig. 4.3 Growth factors do not increase expression of activated ERKs 1 and 2 during medial explant culture.
Extracts were prepared from explants cultured with 25 ng/ml PDGF-BB, 25 ng/ml IGF-1 and 10 ng/ml bFGF for the times indicated in days and equal amounts of protein were directly immunoblotted with antibody to activated ERKs 1 and 2.

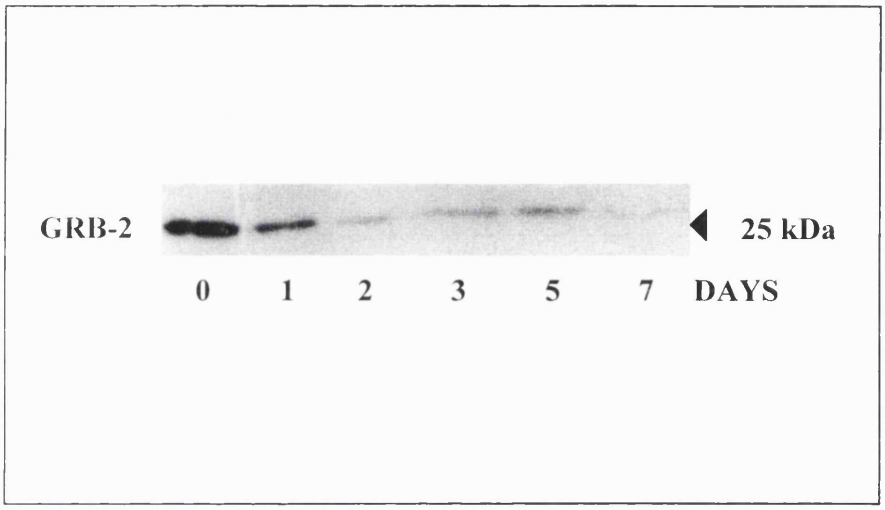


Fig 4.4 Expression of GRB-2 during early explant culture.
Extracts were prepared from explants cultured for the time indicated in days and equal amounts of protein were directly immunoblotted with antibody to GRB-2.

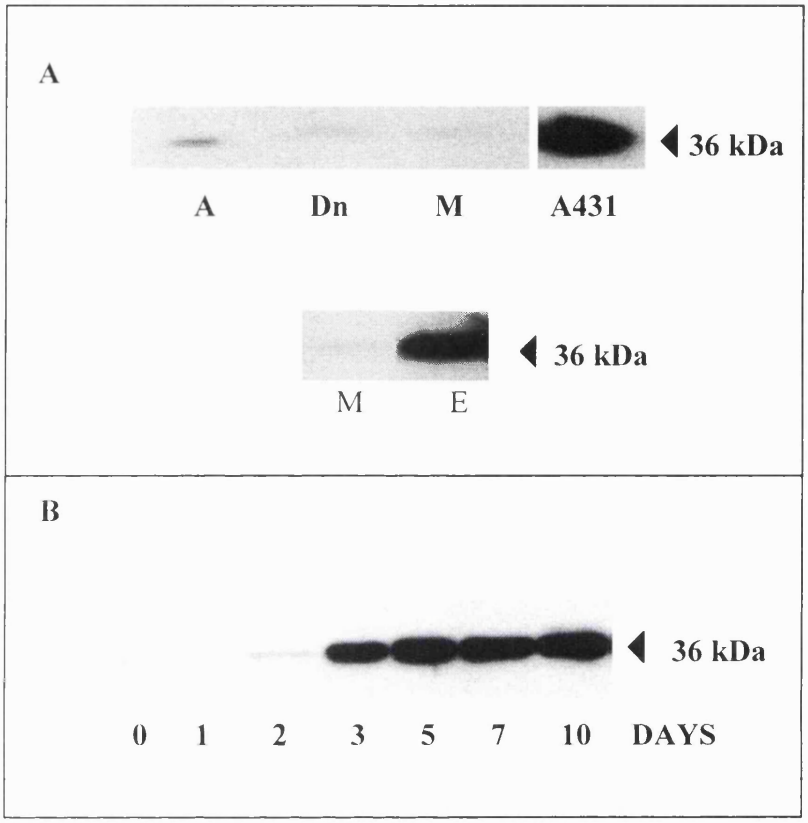


Fig. 4.5 Expression of PCNA in aortic tissue and during medial explant culture.

A: PCNA is differentially expressed in uncultured aortic tissue and cultured explants.

TOP. Protein extracts were prepared from whole aorta (A), deendothelialised aorta (Dn), and media, and equal amounts of protein were immunoblotted with antibody to PCNA. A431 is a positive control epithelial carcinoma cell lysate.

BELOW. Protein extracts were prepared from fresh media (M) and medial explants which had been in culture for 11 days (E) and equal amounts of protein were immunoblotted with antibody to PCNA.

B: Increased expression of PCNA during medial explant culture.

Protein extracts were prepared from medial explants cultured in 20% FBS for the times indicated and equal amounts of protein were immunoblotted with antibody to PCNA.

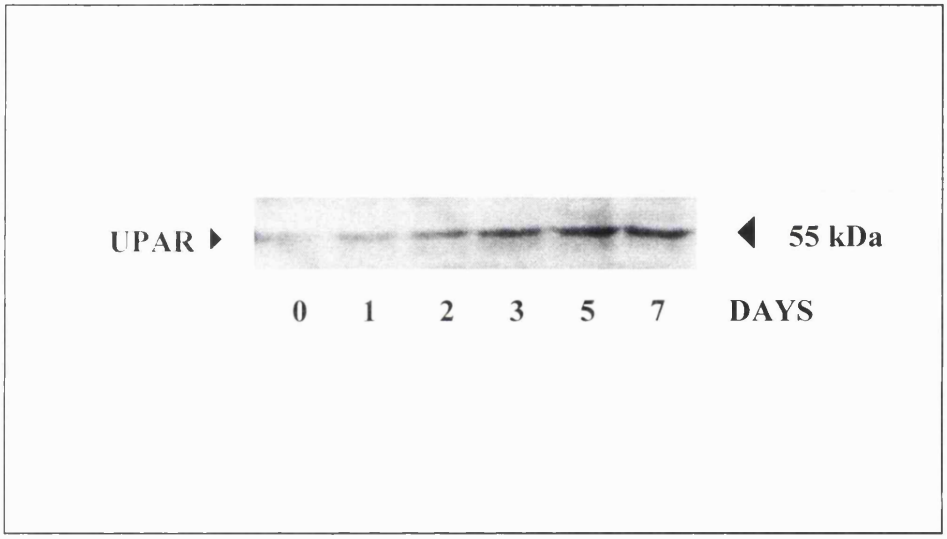


Fig 4.6 Expression of UPAR during early explant culture.
Extracts were prepared from explants cultured for the time indicated in days and equal amounts of protein were directly immunoblotted with antibody to UPAR.

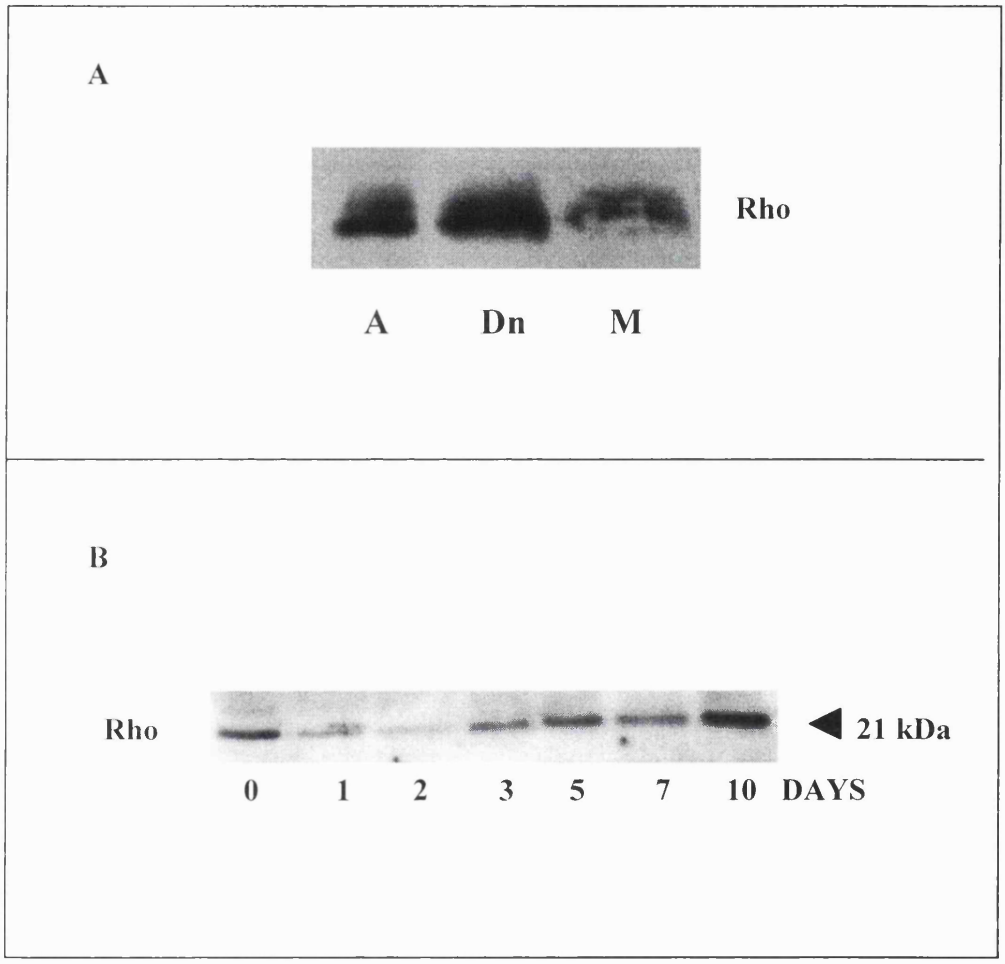


Fig. 4.7 Expression of Rho protein in aortic tissue and during medial explant culture.

A. Protein extracts were prepared from whole aorta (A), deendothelialised aorta (Dn), and media,, and equal amounts of protein were immunoblotted with antibody to Rho.

B. Protein extracts were prepared from medial explants cultured in 20% FBS for the times indicated and equal amounts of protein were immunoblotted with antibody to Rho.

SUMMARY

The results shown in this chapter have demonstrated the expression of other signalling molecules implicated in mitogenic and chemotactic signalling pathways during early explant culture. The rapid increase in expression of PCNA, a marker of cell proliferation, was noted several days prior to the onset of visible outgrowth of VSMC from explants. Surprisingly, however, expression of activated and total ERKs 1 and 2 and GRB-2 diminished during explant culture, in marked contrast to the high expression of activated and total ERKs 1 and 2 in native aortic media.

These results also showed that expression of UPAR, which has been implicated in VSMC migration, increased during early explant culture of medial VSMC. Expression of the small GTP-binding protein Rho appeared to decline initially but subsequently increased during explant culture.

CHAPTER 5

Subcellular Localisation of FAK in VSMC.

Introduction

The expression of FAK in cultured explants and in VSMC was described in chapter three. Although FAK might be expected to localise uniquely to focal adhesions, no prior studies addressed the localisation of FAK in true primary cultures of VSMC. In this chapter the subcellular localisation of FAK in VSMC was addressed in detail using immunofluorescence staining and analysis of subcellular fractions.

5.1 Immunofluorescent localisation of FAK in VSMC

Primary cultures of VSMC were grown to confluence on coverslips and prepared for immunostaining as described in experimental procedures. As shown in Fig. 5.1.1 polyclonal antibodies to the FAK COOH- and NH_2-terminal domains and a monoclonal antibody to the central catalytic domain of FAK (residues 354-533) produced relatively weak staining of focal adhesions although there was prominent staining of nuclei. The specificity of nuclear staining of the antibodies to the FAK COOH- and NH_2-terminal domains was tested by immunofluorescent staining with these antibodies after pre-incubation of VSMC with their respective peptide immunogens. As can be seen from Fig. 5.1.1, all nuclear and focal adhesion staining was abolished by peptide competition of the primary antibody suggesting that the nuclear staining was indeed specific for the immunogen.

Scanning laser confocal microscopy of VSMC with the 354-533 antibody was next performed to assess the intracellular localisation of FAK immunofluorescent staining in greater detail (Fig. 5.1.2). It can be seen that in the plane of the cell substrate, focal adhesion staining is relatively prominent whilst nuclear staining appears weak (Fig. 5.1.2A). Diffuse cytosolic staining of SMC was also noted which was not consistent with discrete focal adhesion staining. At increasing distance from the

basolateral cell surface focal adhesion staining diminishes whilst nuclear staining increased (Fig. 5.1.2B,C). In the apical portion of the cell only nuclear staining was faintly visible and no focal adhesions were detected (Fig. 5.1.2D).

Further confocal microscopic imaging confirmed that, in VSMC co-stained with antibody to the nuclear marker PCNA, there was co-localisation of FAK and PCNA to the nucleus (Fig. 5.1.3). PCNA antibody produced no detectable staining of focal adhesions. Confocal microscopy of cells co-immunostained with FAK and either vinculin or paxillin showed prominent staining paxillin and vinculin staining of focal adhesions but no co-localisation with FAK to the nucleus (Fig. 5.1.3). Antibody to vinculin produced the most prominent staining of focal adhesions which coincided with the tips of actin filaments (Fig 5.1.4), and no detectable nuclear staining. Once again, diffuse cytosolic staining of SMC with FAK was noted. Co-localisation of FAK with either vinculin or paxillin to focal adhesions was relatively weak, reflecting strong paxillin and vinculin focal adhesion staining compared to that of FAK (Fig. 5.1.3). The weakness of focal adhesion staining seen with antibodies to FAK was not due to inability of these antibodies to recognise FAK in focal adhesions as the same reagents produced strong immunofluorescent staining in human endothelial cells (see chapter 6).

5.2 Expression of FAK in nuclear and cytosolic subcellular fractions.

The results shown in Figs. 5.1.1-5.1.4 suggested that FAK is localised to the nucleus as well as focal adhesions in primary cultures of VSMC. To further investigate the distribution of FAK between nuclear and cytosolic compartments, FAK expression was evaluated in nuclear and cytosolic fractions of VSMC. In the first instance, crude subcellular fractions were prepared using the non-ionic detergents Triton X100 and Nonidet-P40 simply by exposing SMC to either detergent and then using low centrifugal forces to remove non-solubilised material which contains nuclear plus insoluble cytoskeletal material. Western blot of FAK in triton-soluble and insoluble fractions detected a significant level of 125 kDa FAK in the triton-insoluble compartment. It was noted however that a 55 kDa COOH-terminal fragment of FAK was detected only in the triton-soluble fraction (Fig. 5.2.1). In addition, western blot

analysis of a Nonidet-P40-insoluble VSMC fraction also readily detected significant expression of 125 kDa FAK.

Subcellular fractions of VSMC were next prepared using either the method of selective plasma membrane permeabilisation or a method based upon hypotonic cell lysis. Western blot analysis of nuclear and cytosolic fractions of VSMC prepared by the digitonin permeabilisation method detected significant amounts of 125 kDa FAK in both nuclear and cytosolic compartments (Fig. 5.2.2 left panel), using an antibody specific for the NH_2-terminal FAK domain. A striking feature of these results was that a major 50 kDa band was detected by the NH_2-terminal domain FAK antibody, predominantly in the nuclear compartment.

The efficacy of the fractionation technique was verified by blotting parallel nuclear and cytosolic extracts to either nuclear markers (nucleoporin), or to focal adhesion components. Immunoblotting with nucleoporin, a component of the nuclear pore complex, showed it to be highly expressed in the nucleus and not expressed in the cytosol using digitonin fractionation (Fig. 5.2.2 left panel). This suggested a minimal degree of cross-contamination of cytosolic fractions with nuclear contents. Expression of vinculin was confined to the cytosolic compartment with barely detectable expression in the nucleus indicating minimal contamination of the nuclear fraction with contents of the cytosol.

An alternative method of subcellular fractionation made use of hypotonic cell lysis followed by isopycnic centrifugation through dense sucrose to separate nuclei from other cellular constituents. The results obtained with this method also showed significant 125 kDa FAK expression in both nuclear and cytosolic fractions (Fig. 5.2.2 right panel). Again, striking segregation of a 50 kDa NH_2-terminal FAK fragment was noted with expression confined largely to the nuclear fraction and barely detected in the cytosol. Although there was no cross-contamination of nucleoporin into the cytosolic fraction using this method of fractionation, heavy contamination of the nuclear compartment with vinculin was observed (Fig. 5.2.2 right panel).

5.3 FAK NH_2-terminal and COOH-terminal fragments are segregated between nuclear and cytosolic fractions in VSMC.

Immunoblots of nuclear and cytosolic VSMC fractions using antibodies specific for the NH2- or COOH-terminal domains of FAK showed a striking segregation of a 55 kDa C00H-terminal FAK fragment exclusively localised to the cytosol from a 50kDa NH_2-terminal FAK fragment which only appeared in the nucleus (Fig. 5.3 above). Using the antibody to the central catalytic domain of FAK, both nuclear and cytosolic p125FAK forms were identifiable and an additional p120 kDa variant in the nuclear compartment only (Fig. 5.3 below).

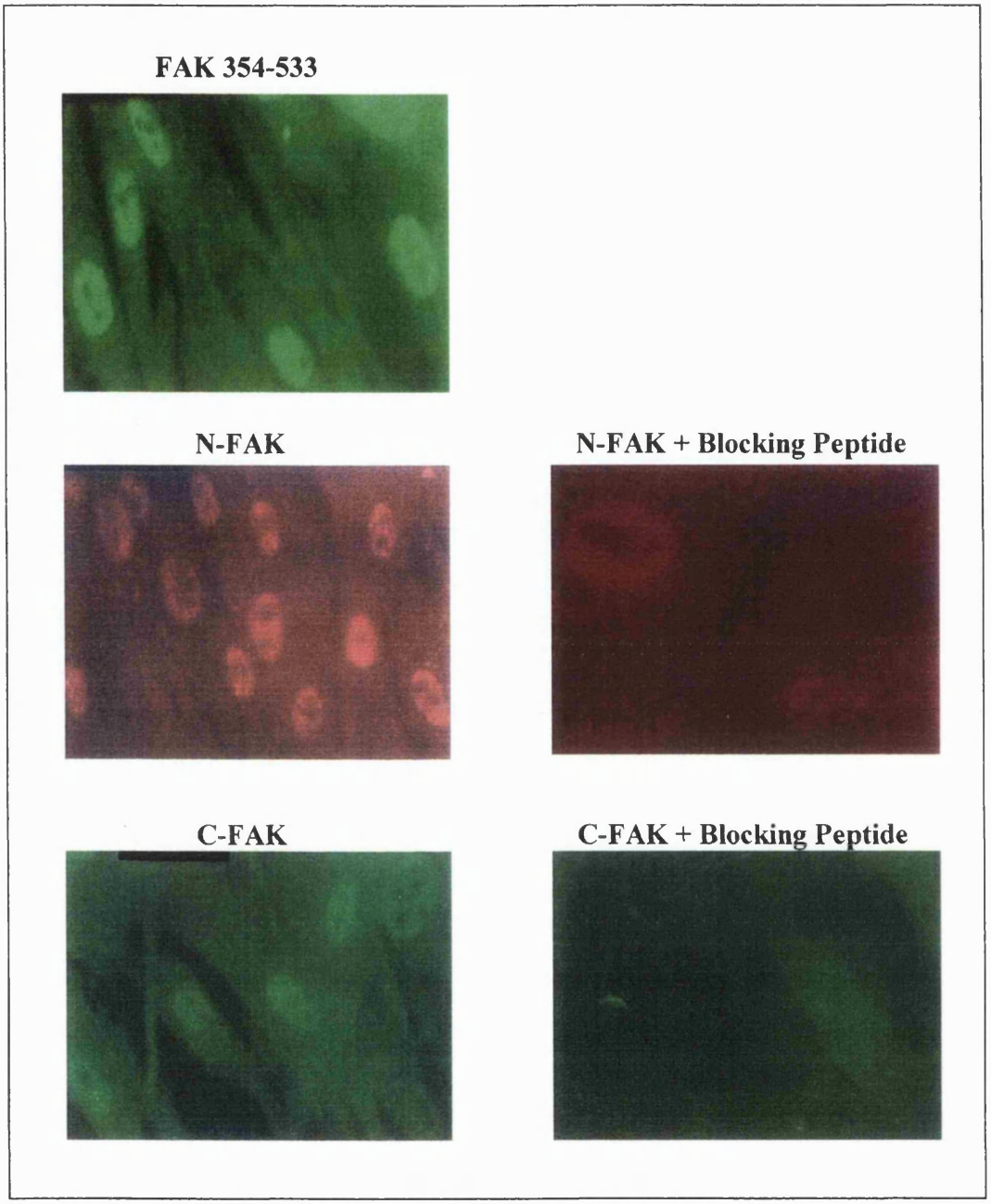


Fig. 5.1.1 Immunofluorescent staining of FAK in primary cultures of VSMC.

Fixed and permeabilised cultures of VSMC were incubated with antibodies to FAK residues 354-533, 1033-1052 (COOH-terminal), or 2-18 (NH2-terminal). Parallel cultures were incubated with antibodies to FAK residues 1033-1052 or 2-18 after pre-incubation with blocking peptides corresponding to the respective immunogenic peptides. Immunofluorescent staining was then performed as described in experimental procedures and photographs were taken using a Nikon Optiphot camera.

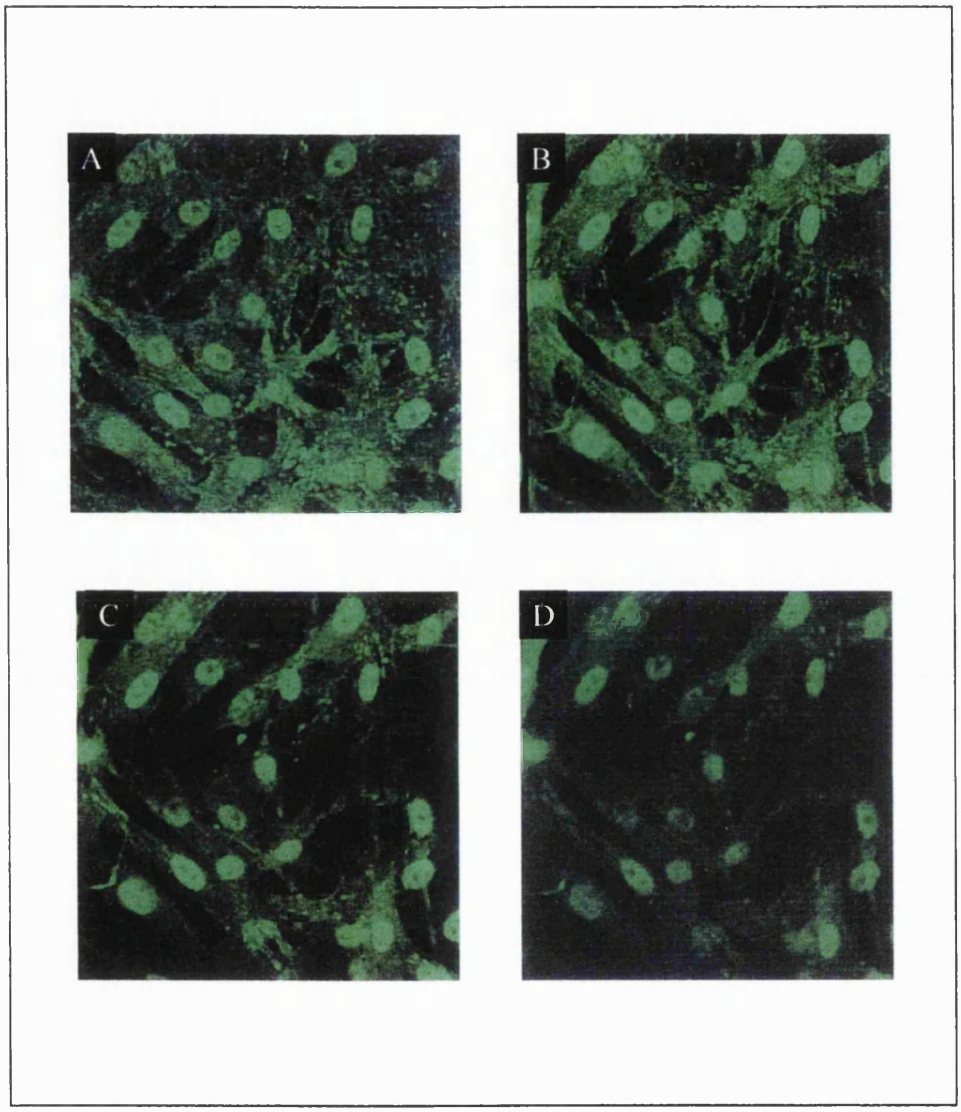


Fig. 5.1.2 Focal adhesion staining diminishes and FAK nuclear staining is more prominent with increasing distance from the basolateral cell surface.

Fixed and permeabilised cultures of VSMC were incubated with antibody to FAK residues 354-533 and immunofluorescent staining was performed followed by confocal laser scanning microscopy at sequential 1.85μm steps through VSMC. Pseudocoloured images were generated of FAK staining (green) starting at the basolateral cell surface (A) and moving up through the cell layer (B and C) to the top of the cell layer (D).

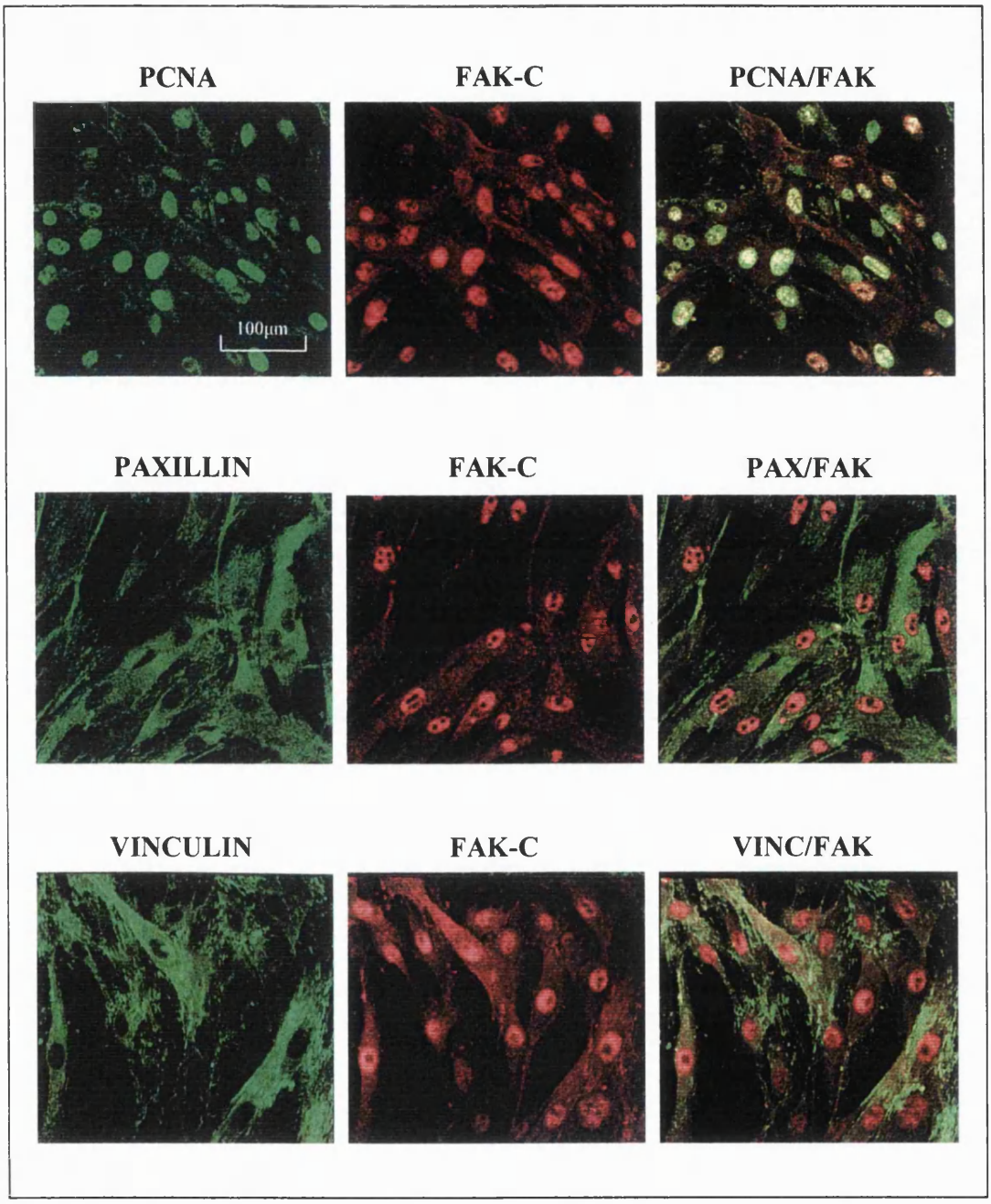


Fig. 5.1.3 Co-immunofluorescent staining of FAK and markers of focal adhesions and the nucleus in primary cultures of VSMC.
Fixed and permeabilised cultures of VSMC were incubated with antibodies to FAK (1033-1052) together with antibody to either paxillin, vinculin or PCNA. FAK immunostaining was visualised using TRITC-conjugated secondary antibody (red) and paxillin, vinculin and PCNA was visualised using FITC-conjugated secondary antibody (green). Confocal pseudocoloured images were generated and are shown above: co-localisation is indicated by yellow staining.

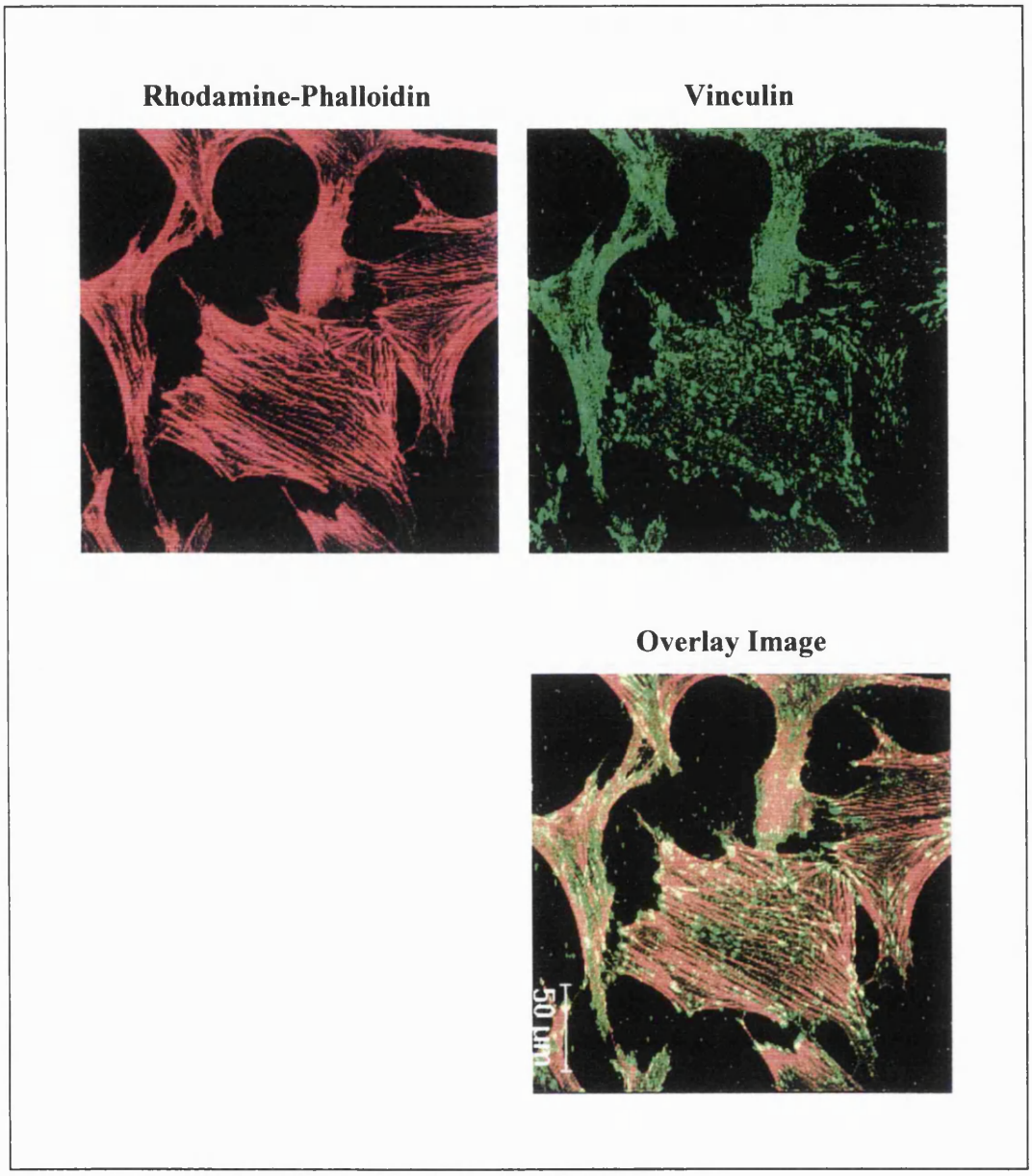


Fig 5.1.4 Focal adhesions are localised at the tips of actin filaments.
Fixed and permeabilised VSMC cultures were incubated with antibody to Rhodamine-Phalloidin (TRITC-conjugated) and vinculin (FITC-conjugated). Immunofluorescent staining was performed and images were collected by scanning laser confocal microscopy. Confocal pseudocoloured images are shown with an overlay image indicating co-localisation of vinculin with actin at the tips of actin filaments (yellow staining).

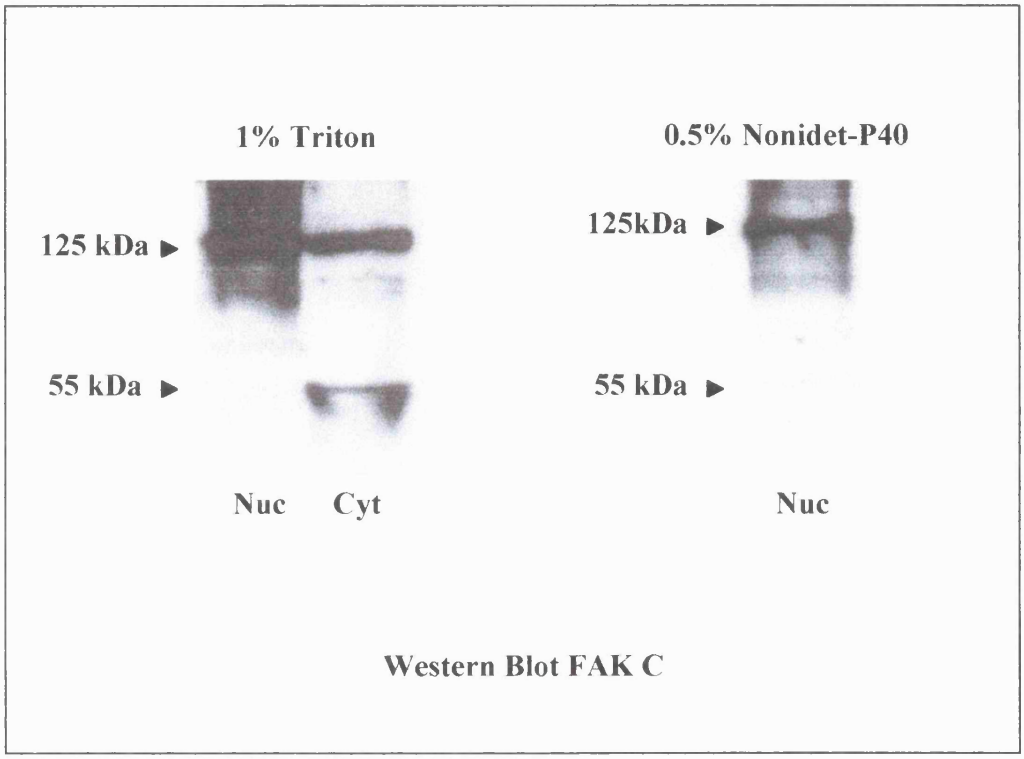


Fig. 5.2.1 FAK is expressed in nuclear and cytosolic cell compartments.
Primary cultures of VSMC were fractionated into nuclear and cytosolic compartments (designated Nuc and Cyt respectively) using the non-ionic detergents Triton X-100 or Nonidet-P40. Equal quantities of protein were then immunoblotted with antibody to the FAK COOH-terminus (FAK-C). The positions of 55 and 125 kDa FAK-immunoreactive species are indicated.

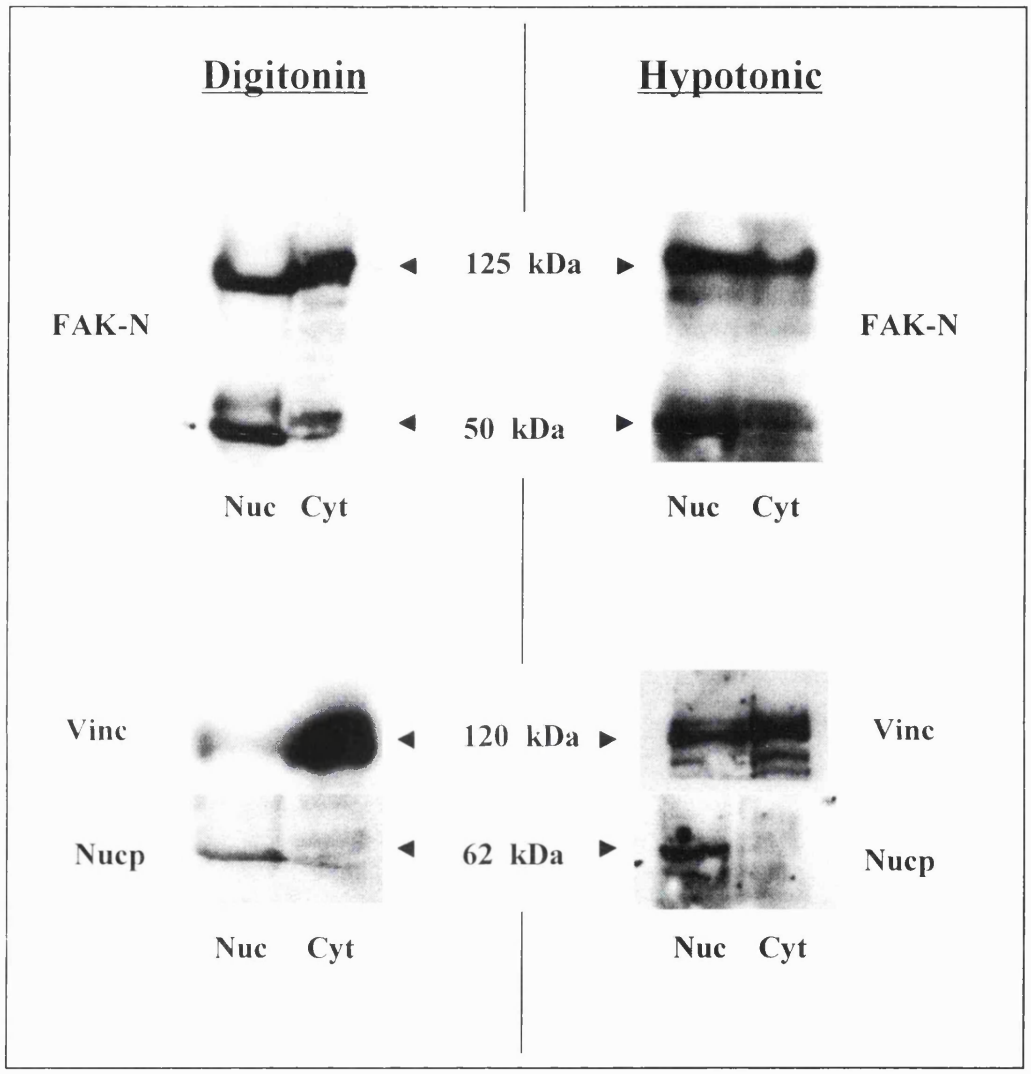


Fig. 5.2.2 Significant expression of FAK immunoreactive species in nuclear fractions of VSMC.

Nuclear (Nuc) and cytosolic (Cyt) fractions were prepared from confluent cultured VSMC either by digitonin permeabilisation (left panels) or hypotonic lysis (right panels). Equal amounts of protein were immunoblotted with antibodies to FAK NH2-terminal residues 2-18 (FAK-N), vinculin (Vinc) and nucleoporin (Nucp). The positions of 50 and 125 kDa FAK-immunoreactive species are indicated. The results are representative of 4 similar experiments.

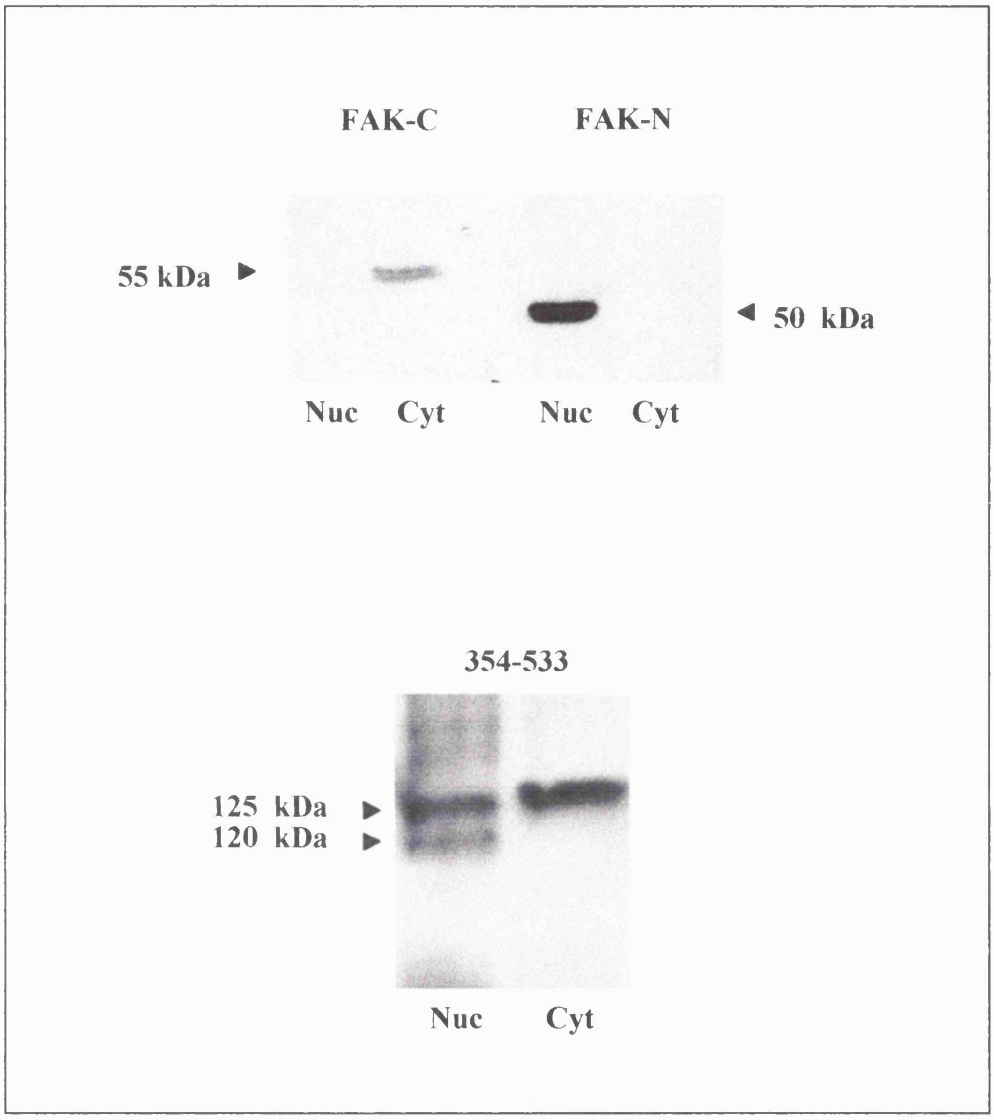


Fig. 5.3 Compartmentalisation of FAK NH2-terminal and COOH-terminal domain fragments in VSMC.
Nuclear (Nuc) and cytosolic (Cyt) fractions were prepared from confluent cultures of VSMC by digitonin permeabilisation. Equal amounts of protein were immunoblotted with antibodies to the FAK COOH-terminus (FAK-C), FAK NH2-terminus (FAK-A) or an antibody to the central catalytic domain of FAK residues 354-533. Note the differential compartmentalisation of 55 kDa FAK COOH-terminal and 50 kDa NH2-terminal domain fragments.

Summary

The results in this chapter have shown that FAK is expressed in the nucleus of VSMC in addition to being localised to focal adhesions. The extent to which FAK was localised to focal adhesions appeared relatively low whilst the nuclear localisation was consistent, reproducible and prominent. The fact that focal adhesions exist in these cells was proven by co-immunostaining with two markers of focal adhesions, vinculin and paxillin, which did not localise to the nucleus. The specificity of FAK nuclear immunofluorescent staining was corroborated by using the blocking immunogenic peptides which abolished all focal adhesion and nuclear staining.

Fractionation of VSMC using several different techniques provided further evidence of nuclear expression of $p125^{FAK}$. A striking finding was that a 55 kDa NH_2-terminal FAK fragment was exclusively localised to the nucleus and a 55 kDa COOH-terminal FAK fragment was only found in the cytosol.

CHAPTER 6

Subcellular localisation of FAK in HUVECs

Introduction

Having defined the subcellular localisation of FAK in primary cultures of VSMC, a similar range of techniques was used to identify its localisation in HUVECs. Previous workers have commented on a nuclear localisation of FAK in HUVECs (Abedi and Zachary, 1997; Levkau et al, 1998) although no detailed studies have been documented which characterise nuclear localisation of FAK in endothelial cells in detail.

6.1 Characterisation of HUVECs in primary culture.

Vascular endothelial cells were derived from collagenase digestion of full term umbilical cords and primary culture in endothelial basal medium (Clonetics) supplemented with 10% FBS as described in experimental procedures. The cells grew in a confluent monolayer and exhibited the typical endothelial cell "cobblestone" morphology (Fig. 6.1A). Immunostaining of these cells with an antibody specific for vascular endothelial cells –Vascular Endothelial Cadherin (VE-Cadherin) – revealed cadherin positive staining at the intercellular border (Fig. 6.1B).

6.2 Immunofluorescent localisation of FAK in HUVECs

Primary cultures of HUVECs were grown on coverslips and immunostained with three separate antibodies to FAK (Fig 6.2.1). The antibodies to the COOH-terminus and the central catalytic domain (354-533) both exhibited strong focal adhesion staining which was more marked than that noted in VSMC using the same antibodies. These antibodies stained the nucleus strongly and, in the case of the COOH-terminus antibody, marked perinuclear immunostaining was also evident. In contrast the

NH_2-terminus antibody appeared to stain only the nuclei of HUVECs and never gave noticeably strong focal adhesion staining. The nuclear localisation was confirmed by DAPI counterstaining. Antibodies to paxillin and vinculin produced strong immunofluorescent staining of focal adhesions with little detectable specific nuclear staining (Fig 6.2.2).

6.3 Subcellular compartmentalisation of FAK in Triton X-100 lysates.

Primary cultures of HUVECs were grown to full confluence and subjected to detergent extraction using 1% Triton X-100 for 15 minutes on ice. In 1% Triton lysates of HUVECs, the major immunoreactive FAK species recognised by an antibody specific for the NH_2-terminus (amino acid residues 2-18) were a 125 kDa band and a less prominent 50 kDa band (termed p50N-FAK) both present in a Triton-soluble fraction (Fig. 6.3.1). In addition, this antibody recognised a 120 kDa band only in the Triton-insoluble fraction. An antibody raised against the COOH-terminal FAT domain comprising amino acids 1033 to 1052, recognised a major 125 kDa band and a minor component of 55 kDa, presumably corresponding to FRNK or a FRNK-related species and henceforth termed p55C-FAK, both present in the Triton-soluble fraction (Fig. 6.3.1). Both antibodies also detected some 125 kDa FAK in the Triton-insoluble fraction. Immunoblotting of parallel 1% Triton-derived fractions with vinculin and paxillin confirmed that the majority of focal adhesion related components were indeed localised to a Triton-soluble compartment (Fig. 6.3.1).

Since triton solubilisation should preserve protein-protein interactions, the distribution of FAK between the triton-insoluble fraction and protein-protein complexes obtained by centrifugation of the triton-soluble fraction at 100,000 *g* for 60 minutes was determined (Fig. 6.3.2). It was noteworthy that while similar amounts of 125 kDa FAK were found in the triton-insoluble compartment and the post-100,000 *g* pellet, p55C-FAK was only detected in the post-100,000 *g* pellet and was also the most abundant immunoreactive form detected in the post-100,000*g* pellet (compare Fig 6.3.2 and 6.3.1). Similarly, blots with the NH_2-terminal antibody showed that p50N-FAK was the most prominent NH_2-terminal species in the post-100,000 *g* pellet. The comparative immunoblot performed with N-FAK and C-FAK antibodies clearly illustrates the

differential expression of 120 kDa and 125 kDa immunoreactive species in the triton-insoluble fraction. Similarly the presence of distinct p50N-FAK and p55C-FAK forms in the post-100,000 *g* pellet was also demonstrated.

Immunoblots of triton-insoluble and post-100,000 *g* fractions with antibodies to vinculin and talin showed that both of these focal adhesion components were more prominent in the post-100,000 *g* pellet (Fig. 6.3.2). In contrast Lamin B, a component of the nuclear envelope, was only found in a triton-insoluble fraction.

6.4 Distribution of FAK between nuclear and cytosolic compartments.

Nuclear and cytosolic compartments were prepared from primary cultures of HUVECs using selective plasma membrane permeabilisation with 0.1% Digitonin as described in Experimental Procedures. The distribution of FAK between these compartments was investigated by western blotting of equal amounts of nuclear and cytosolic protein fractions. As shown in Fig. 6.4.1 (upper panel), the COOH-terminal antibody detected significant amounts of immunoreactive $p125^{FAK}$ in both nuclear and cytosolic fractions but also detected p55C-FAK only in the cytosolic compartment. In striking contrast, the NH_2-terminal antibody detected p50N-FAK only in the nuclear fraction, and this fragment was absent from the cytosol. In addition the NH_2-terminal antibody detected bands of Mr 125,000 120,000 and 90,000 in the nuclear compartment. Consistent with the results obtained from triton-solubilisation, $p125^{FAK}$ was predominantly in the cytosol, while the p120 band was present only in the nuclear fraction. Immunoblotting of parallel nuclear and cytosolic extracts with antibodies to two components of the nuclear envelope, Lamin B and nucleoporin, showed that they were highly expressed in the nuclear fraction and absent or very weakly expressed in the cytosol (Fig. 6.4.1 lower panel). In contrast to this finding, two markers of focal adhesions, vinculin and paxillin, were strongly expressed in the cytosol but barely detectable in the nucleus.

A monoclonal antibody directed against the NH_2-terminal 423 amino acid residues of FAK detected bands of almost identical apparent molecular weight to those detected by the polyclonal NH_2-terminal antibody (Fig. 6.4.2). These included a major 50 kDa band which was present only in the nuclear fraction and bands of Mr 120,000

and 90,000 in the nuclear fraction and bands of Mr 125,000 and 90,000 in the cytosolic fraction. Both polyclonal and monoclonal NH_2-terminal–specific FAK antibodies detected $p125^{FAK}$ predominantly in the cytosol, while an Mr 120,000 form was only present in the nuclear compartment. Occasionally a ~68 kDa band was also detected by both NH_2-terminal–specific antibodies (Fig. 6.4.2). This species however was not consistently observed and may represent a degradation product.

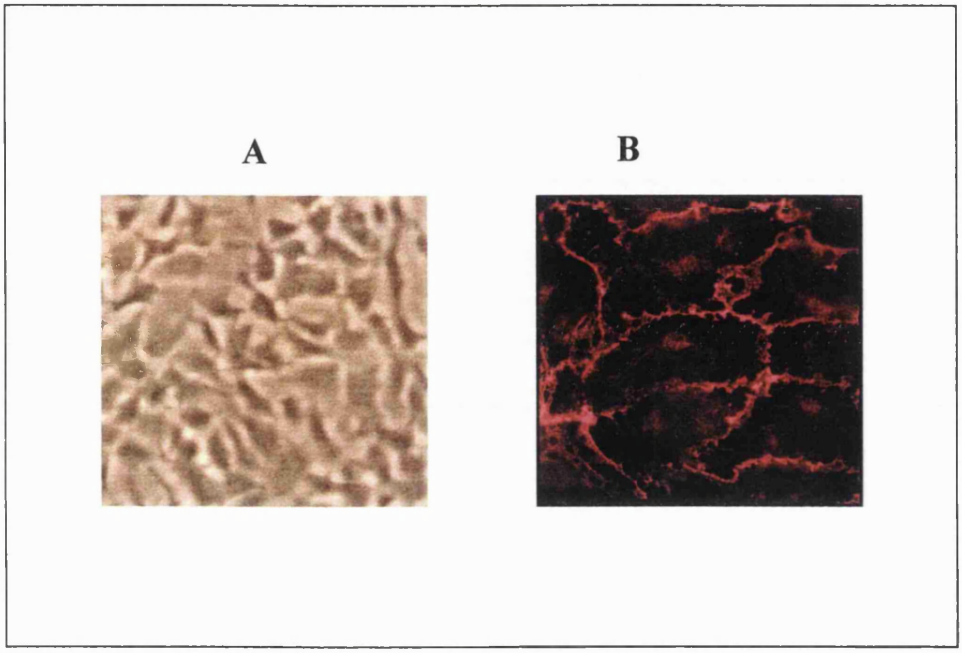


Fig. 6.1 Characterisation of HUVECS in primary culture.
A. Confluent cultures of HUVECs were prepared as described in experimental procedures and photographed using a Nikon Axiophot camera attached to a light microscope.
B. Confluent cultures of HUVECS were immunostained with a mouse monoclonal antibody to VE-Cadherin followed by staining with a goat anti-mouse secondary antibody conjugated to TRITC and photographed using a Nikon Axiophot camera.

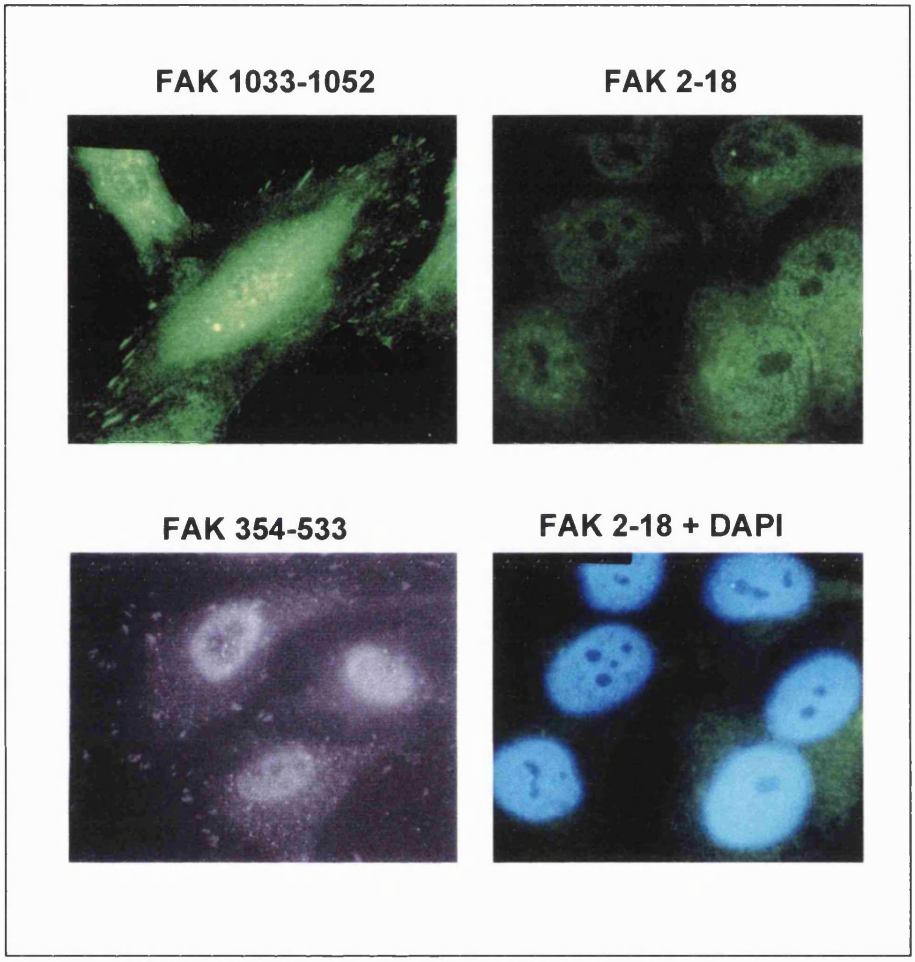


Fig 6.2.1 Nuclear and focal adhesion staining with 3 distinct FAK antibodies.
Confluent cultures of HUVECs were immunostained with an antibody to the COOH-terminus of FAK (1033-1052), to the central catalytic domain of FAK (354-533), and to the NH2-terminus of FAK (2-18). Note focal adhesion and nuclear staining with the first two antibodies. The NH2-terminus antibodies does not appear to stain focal adhesions but co-localises with nuclei as counterstained with DAPI (blue).

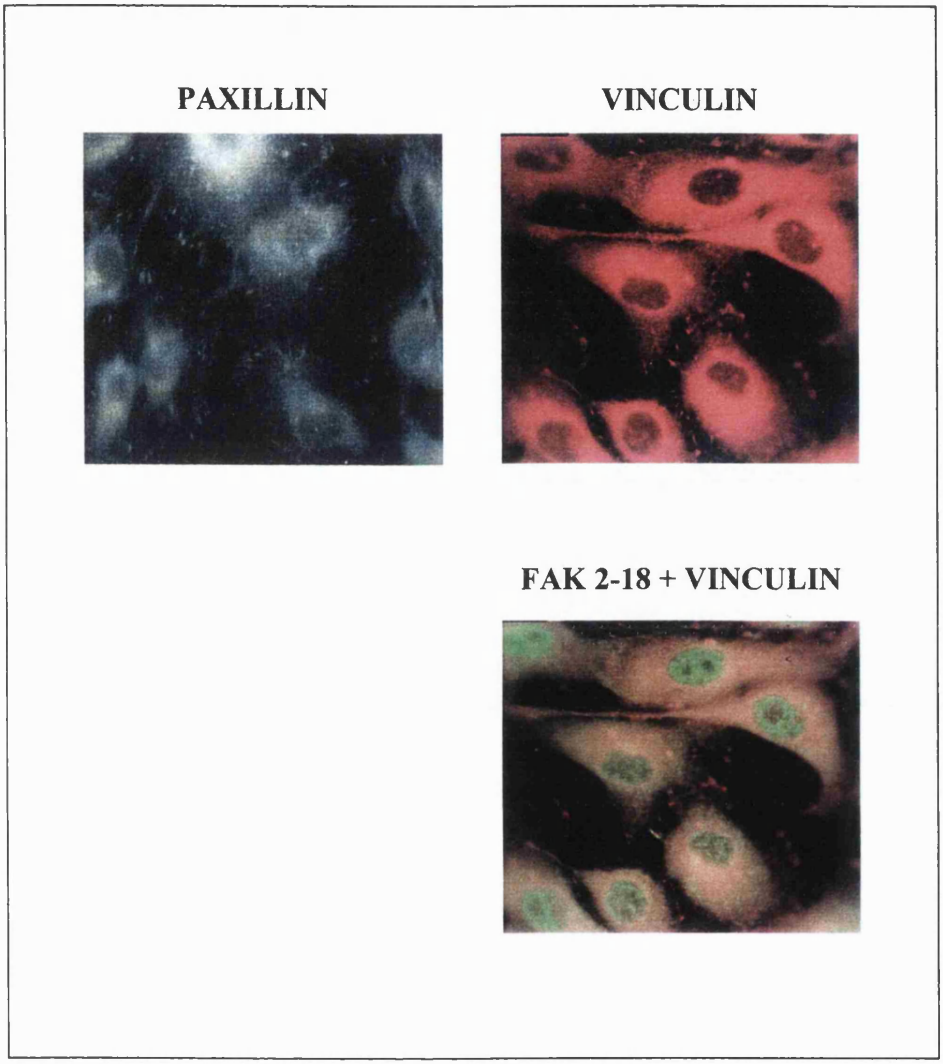


Fig. 6.2.2 Other markers of focal adhesions do not localise to the nucleus.
Confluent cultures of HUVECS were immunostained with antibodies to the focal adhesion components paxillin and vinculin (TRITC). Although focal adhesion staining is clearly defined, nuclei are distinguished in these cells by absence of staining. Vinculin-stained cells were also co-immunostained with antibody to the NH2-terminus of FAK (FITC). Yellow staining represents colocalisation of FAK and vinculin seen in the cytosol and in some focal adhesions. FAK nuclear immunostaining, in contrast, is very prominent.

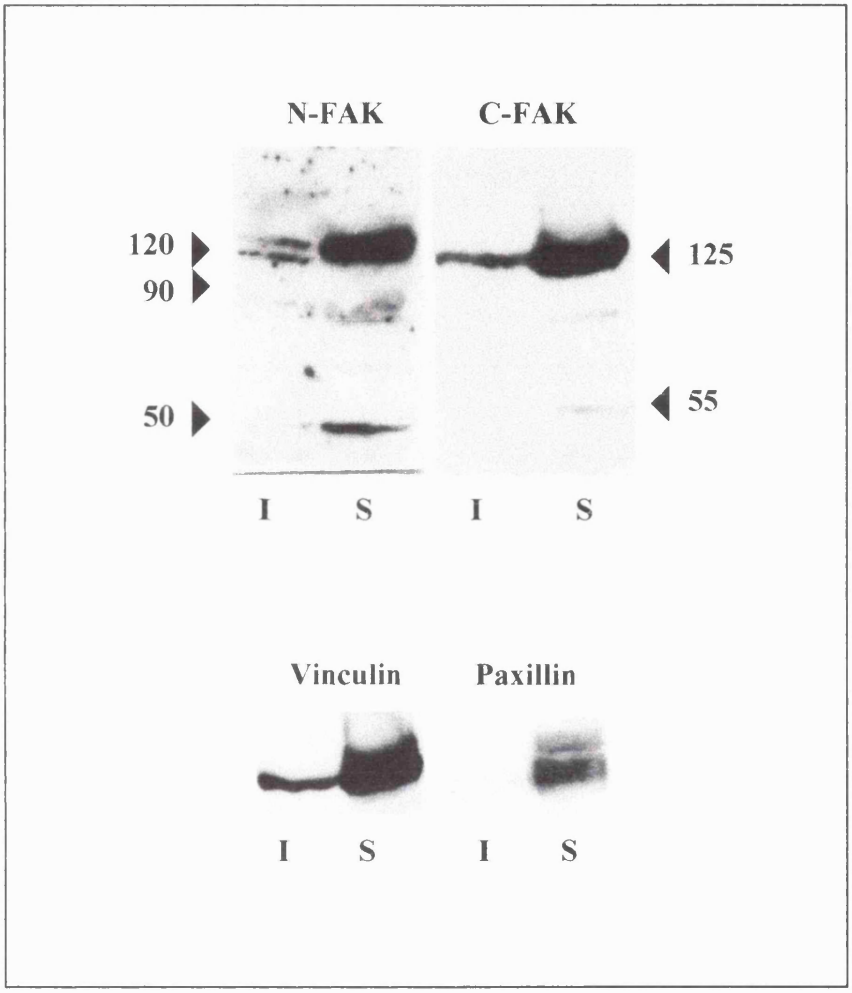


Fig 6.3.1 Differential distribution of p125FAK and a 55 kDa COOH-terminal fragment between Triton-insoluble and -soluble fractions.

Confluent cultures of HUVECS were lysed with 1% Triton X-100 and equal amounts of protein from the triton-soluble compartment (S) and insoluble pellet (I) were adjusted to SDS-PAGE sample buffer and immunoblotted with antibodies to the NH2-terminus of FAK (N-FAK) and the COOH-terminus of FAK (C-FAK). Immunoblots of parallel fractions with vinculin and paxillin are shown for comparison.

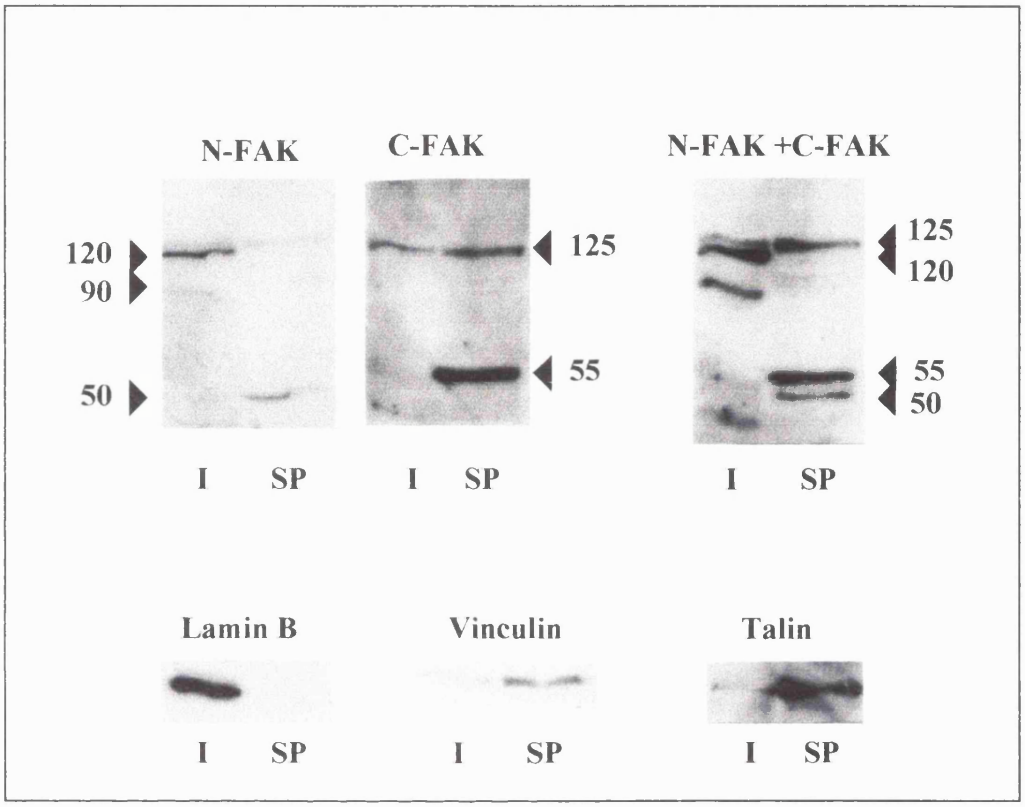


Fig. 6.3.2 Differential distribution of p125FAK and FAK-related fragments in triton-solubilised HUVECS.
Triton-insoluble fractions were prepared and the lysate was centrifuged at 100,000 *g* for 60 minutes. The pellet (SP) was solubilised and immunoblotted in parallel with the Triton-insoluble fractions (I). Fractions were immunoblotted with antibodies to the FAK NH2-terminus (N-FAK), the FAK COOH-terminus (C-FAK), and both antibodies simultaneously (N-FAK + C-FAK). Immunoblots of parallel fractions with Lamin B, vinculin and talin are shown for comparison.

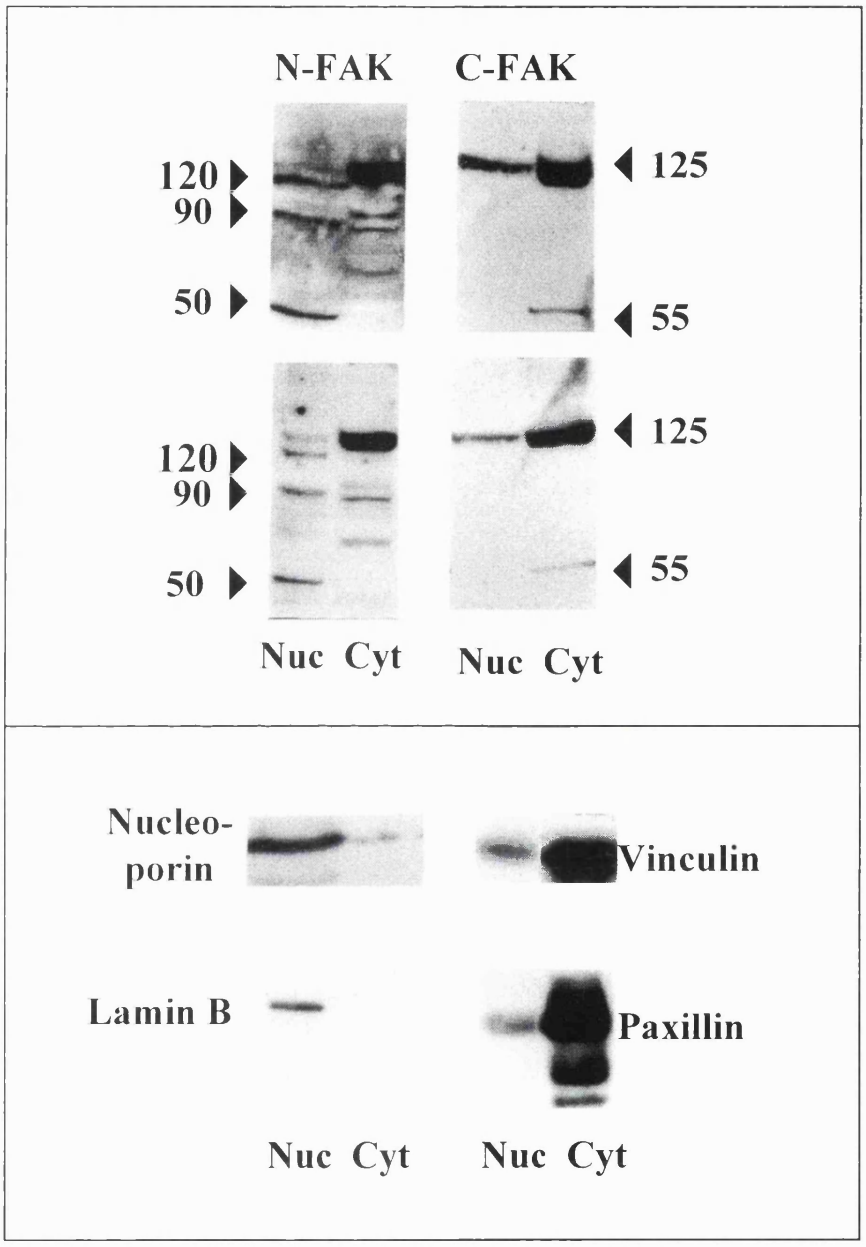


Fig 6.4.1 Differential compartmentalisation of FAK 50 kDa NH2-terminal and 55 kDa COOH-terminal fragments between nuclear and cytosolic fractions.

Nuclear (Nuc) and cytosolic (Cyt) fractions were prepared from confluent cultures of HUVECS and immunoblotted with antibodies to the FAK NH2-terminus (N-FAK), the FAK COOH-terminus (C-FAK), nucleoporin, Lamin B, vinculin and paxillin. The positions of 50, 55, 90, 120 and 125 kDa FAK-immunoreactive species are indicated. Two representative immunoblots with NH2-terminal and COOH-terminal antibodies are shown. The results are representative of 15 similar experiments.

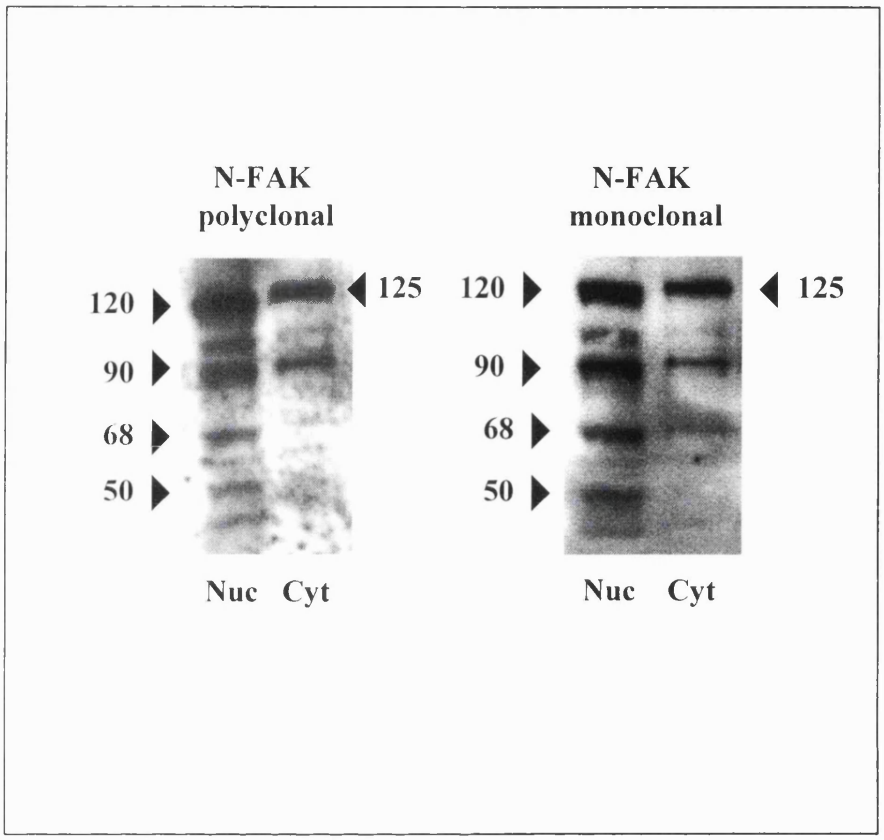


Fig 6.4.2 Differential compartmentalisation of FAK immunoreactive species between nuclear and cytosolic fractions.
Nuclear (Nuc) and Cytosolic (Cyt) fractions of HUVECS were prepared and immunoblotted with a monoclonal and polyclonal antibody to the NH2-terminus of FAK. Note the identical pattern of immunoreactive bands as recognised by the separate antibodies. A 50 kDa fragment is exclusively localised to the nucleus as observed before.

Summary

In this chapter the results demonstrated that, similar to the findings in VSMC, FAK is expressed in the nucleus of endothelial cells. HUVECs appeared to express FAK more noticeably in focal adhesions than VSMC as detected by immunofluorescent staining. Marked perinuclear staining was also detected with an antibody to the COOH-terminus of FAK. Following 1% Triton lysis of HUVECs, most $p125^{FAK}$ was recovered from a triton-soluble fraction but an intriguing result was the consistent finding of FAK immunoreactive species in the triton-insoluble compartment which is mostly comprised of nuclear and triton-insoluble cytoskeletal components. This finding was further corroborated by specific fractionation of HUVECs into nuclear and cytosolic compartments: again significant quantities of FAK immunoreactive species were found in the nuclear fraction. In addition, a p50N-FAK fragment and a p120 FAK band were also exclusively localised to the nucleus. Cross-contamination of nuclear and cytosolic compartments was shown to be small by immunoblotting parallel samples with markers of the nucleus and focal adhesions respectively.

CHAPTER 7

Apoptotic regulation of Focal adhesion Kinase

Introduction

A growing body of evidence has indicated a critical role for FAK in transducing survival signals in anchorage-dependent cells. Although FAK proteolytic cleavage during apoptosis has already been described, the tyrosine phosphorylation sites important for FAK-mediated anti-apoptotic signalling are presently unknown. In addition the temporal relationships between FAK phosphorylation status, FAK cleavage and intracellular redistribution of cleavage products have not been clearly defined. In this chapter the regulation and intracellular compartmentalisation of FAK fragments and FAK site-specific tyrosine phosphorylation were investigated during apoptosis in HUVECs. Studies were also performed to identify signalling pathways which might be important in mediating apoptotic regulation of FAK tyrosine phosphorylation.

7.1 Characterisation of Apoptosis in HUVECs

Apoptosis was induced in HUVECs by either serum withdrawal or treatment with 1 μM staurosporine, a broad-spectrum kinase inhibitor and potent apoptogenic agent in a variety of cell types (Secrist *et al.*, 1990; Hsu *et al.*, 1997; Janicke *et al*, 1996; Lazarovici *et al.*, 1996). Treatment of cells with staurosporine led to rapid changes in morphology with striking cell retraction and rounding within 10 minutes (Fig. 7.1.1A,B). Similar changes did not occur in serum-deprived HUVECs until after 6 to 8 hours and were less pronounced (Fig. 7.1.3). Within 4 hours of the addition of staurosporine, cells had begun to detach from the culture dish, and by 8 hours following treatment, large numbers of cells were detached (Fig. 7.1.1C). By this time there were no morphologically normal looking HUVECs on the culture dish and those that remained attached had lost all cell-cell connections and were fully rounded (Fig. 7.1.1D).

As seen in Fig. 7.1.2, staurosporine-induced morphological abnormalities were well advanced two hours following treatment and a marked increase in TUNEL-positive staining was observed after 4 hours treatment. In contrast, TUNEL-positive staining was not evident in serum-deprived HUVECs until after 12 hours of treatment (not shown). In addition, whereas staurosporine had caused marked abnormalities in cell morphology within 10 minutes, no changes were detectable in serum-deprived HUVECs even after 1 hour. Although these cells did begin to detach from the substrate after 4 hours, at 8 hours following serum-withdrawal relatively few cells had detached (Fig. 7.1.3).

7.2 Proteolytic cleavage of $p125^{FAK}$ during endothelial cell apoptosis

FAK immunoreactivity was next determined in HUVECs induced to undergo apoptosis by either staurosporine treatment or serum deprivation. Following treatments, nuclear and cytosolic fractions of cells were prepared and immunoblotted with an antibody to the NH_2-terminus of FAK. Staurosporine increased FAK proteolysis to a cytosolic 85 kDa fragment and increased expression of nuclear p50N-FAK after 4 to 8 hours (Fig. 7.2A). Expression of $p125^{FAK}$ and the p120 FAK variant (see chapter 6) concomitantly decreased in staurosporine-treated cells. After 16 hours with staurosporine, $p125^{FAK}$ and p120 FAK had completely disappeared and p50N-FAK in the nuclear fraction was the only major detectable FAK-immunoreactive species (Fig. 7.3.2). Increased p50N-FAK and the p120 FAK variant were only detected in the nuclear compartment during apoptosis, while the 90 and 85 kDa fragments were predominantly in the cytosolic fraction of the staurosporine-treated HUVECs (Fig. 7.2A and 7.3.2). Western blotting of parallel extracts with antibody to COOH-terminal FAK revealed a decrease in the level of $p125^{FAK}$, and showed that the p55C-FAK species remained in the cytosolic fraction for up to 16 hours after addition of staurosporine (Fig. 7.3.2).

Compared with staurosporine, serum deprivation induced a much less marked increase in $p125^{FAK}$ proteolysis and nuclear accumulation of p50N-FAK (Fig. 7.2B). This was consistent with a slower onset of morphological changes, and subsequent cell detachment in these cells (Fig. 7.1.3).

7.3 FAK proteolysis during apoptosis is caspase-mediated

Previous studies have demonstrated proteolytic cleavage of FAK during apoptosis to be caspase-mediated. Therefore the ability of a widely used cell-permeable caspase inhibitor to inhibit apoptosis in endothelial cells was next examined. The broad-specificity caspase inhibitor ZVAD-fmk was used for this purpose: cells were pre-incubated with 100μM ZVAD-fmk for 45 minutes prior to staurosporine treatment. Pre-incubation of HUVECs with ZVAD-fmk did not cause any detectable changes in cell morphology (Fig. 7.3.1). In addition, ZVAD-fmk pre-treatment did not abrogate staurosporine-induced changes in cell morphology nor the progression to cell detachment in cells treated with staurosporine (Fig. 7.3.1).

ZVAD-fmk markedly inhibited the staurosporine-induced reduction in cytosolic $p125^{FAK}$ as determined by immunoblotting with the COOH-terminal and the NH_2-terminal antibodies (Fig. 7.3.2). Immunoblotting with the NH_2-terminal antibody showed that ZVAD-fmk blocked the increase in p50N-FAK and the decrease in p120 FAK in the nuclear fraction and completely blocked the increase in the cytosolic 85 kDa proteolytic product without decreasing expression of the 90 kDa band.

7.4 A calpain-dependent pathway does not mediate apoptogenic FAK proteolysis

Calpains are another class of proteases whose activity has been implicated in programmed cell death. To investigate whether apoptogenic FAK proteolysis was in part calpain-dependent in addition to the proven caspase-dependent pathway, the effects of the calpain inhibitor PD150606 were examined. Cells were pre-incubated with this compound for up to 1 hour prior to staurosporine treatment or at the time of serum-withdrawal. Pre-treatment of HUVECs with PD150606 had no visible effect upon subsequent apoptotic morphological changes in cells. In addition, there was little effect on the decrease in $p125^{FAK}$ expression and the accumulation of 85 and 90 kDa proteolytic fragments in the cytosol. Calpain inhibition also failed to prevent the nuclear expression of p50N-FAK induced by either staurosporine treatment or serum deprivation (Fig. 7.4).

7.5 Site-specific tyrosine phosphorylation of p125 ^{FAK} in HUVECs

FAK tyrosine phosphorylation in nuclear and cytosolic compartments of HUVECs was next examined using antibodies specific for individual FAK phosphotyrosine residues. Whole cell extracts of confluent, serum-fed HUVECs were prepared by lysing cells directly in 2X SDS-PAGE sample buffer. Thereafter extracts were immunoblotted with antibodies to FAK phosphorylated at residues Y397, Y407, Y576, Y577, Y861 and Y925. As shown in Fig. 7.5, these cells contained significant amounts of immunoreactivity to anti-pY861, anti-pY407 and anti-pY397, although immunoreactivity to other phospho-specific FAK antibodies was undetectable. Antibodies to pY861 and Y397FAK detected major 125 kDa bands in both nuclear and cytosolic fractions. In addition, anti-pY861FAK recognised a lower abundance band of ~ Mr 130,000 in the nuclear but not the cytosolic compartment. It was found that similar levels of pY861FAK immunoreactivity were present in the nuclear and cytosolic compartments, while pY397 and pY407FAK immunoreactivity was mainly in the cytosol.

7.6 Regulation of FAK tyrosine phosphorylation at residues Y397, Y861 and Y407

7.6.1 Regulation by staurosporine treatment.

Treatment of HUVECs with 1 μM staurosporine resulted in a rapid loss of phosphorylation of FAK at Y861 and Y397 in nuclear and cytosolic compartments which was almost maximal after 10 minutes and sustained thereafter (Fig. 7.6.1). The decrease in tyrosine phosphorylation of FAK preceded any cell detachment or apoptosis as judged by TUNEL staining (see Fig 7.1.2), but did occur concomitantly with marked and rapid changes in HUVEC morphology characterised by cell retraction and loss of intercellular adhesions (Fig. 7.1.1). FAK dephosphorylation induced by staurosporine was not associated with any detectable shift in gel mobility of the major 125 kDa immunoreactive FAK band.

7.6.2 Regulation by serum withdrawal.

Next the effects of serum withdrawal upon FAK tyrosine phosphorylation at residues Y397, Y861 and Y407 were investigated in HUVECs. Longer periods of serum deprivation caused a marked decrease in tyrosine phosphorylation at Y397, Y861 and Y407 (Fig. 7.6.2) which was slower than that induced by staurosporine (Fig. 7.6.1) but preceded a detectable decrease in p125FAK and increase in FAK proteolysis (Fig. 7.2B). A salient feature of the effect of serum withdrawal upon HUVECs was that the overall decrease in immunoreactivity to anti-pY861FAK was preceded at earlier times by a marked shift in the gel mobility of the major anti-pY861FAK immunoreactive band in the nuclear compartment from a band of Mr 125,000 to a more slowly migrating form of ~ Mr 130,000 which was present constitutively at a lower level (Fig. 7.6.2 and also Fig. 7.5). The mobility shift in the nuclear pY861 immunoreactive band was detectable as early as 60 minutes, reached a maximum after 4 hours and was sustained up to 8 hours. In contrast, little significant mobility shift occurred in the cytosolic pY861 125 kDa FAK band, and the most striking change in cytosolic Y861 phosphorylation status was a marked decrease, while total phospho-Y861 immunoreactivity persisted at a higher level for up to 8 hours. No detectable mobility shift in FAK was detected by antibodies to pY407FAK and pY397FAK (Fig 7.6.2).

7.7 Mechanism of staurosporine-induced FAK tyrosine dephosphorylation

Although it is well recognised that staurosporine is a broad-spectrum kinase inhibitor, it has been established to exhibit greater potency for protein Kinase C (PKC) than other kinases. Therefore the role of PKC inhibition in mediating staurosporine-induced FAK dephosphorylation and proteolysis was examined using the selective PKC inhibitor, GF109203X (Toullec *et al.*, 1991). This compound strongly inhibited FAK phosphorylation at Y861 with a very similar time dependence to that for staurosporine determined in parallel cell cultures (Fig. 7.7.1). Similar to staurosporine, GF109203X also induced an increase in expression of nuclear p50N-FAK, loss of $p125^{FAK}$ and increased generation of the cytosolic 85 kDa proteolytic cleavage product (Fig 7.7.1, bottom panel). All of these changes were detectable after 4 hours and occurred after

FAK dephosphorylation was complete. Interestingly, despite producing very similar changes in FAK dephosphorylation and proteolysis, GF109203X did not induce marked changes in morphology of HUVECs. Indeed the changes noted were very similar to those described following serum withdrawal with morphological abnormalities not evident until after several hours and reduced cell detachment as compared to staurosporine-treated cells (Fig. 7.7.2).

The role of PKC was further examined using the structurally unrelated and mechanistically distinct PKC inhibitor, calphostin C. A comparison of the effects of staurosporine and the two PKC inhibitors was made using basal tyrosine phosphorylation of Y861 in untreated HUVECs as a control. As shown in Fig. 7.7.3, FAK is constitutively phosphorylated at Y861 in whole cell lysates. Following 1 hour of serum deprivation, Y861 immunoreactivity underwent mobility shift to a doublet comprising 130 and 125 kDa forms. This was consistent with earlier results (Fig. 7.6.2). Calphostin C induced dephosphorylation of Y861 more slowly with inhibition evident after 30 and 60 minutes. Like GF109203X, calphostin C did not cause significant changes in cell morphology over the 1 hour time-course examined in contrast to rapid induction of cell retraction by staurosporine over the same period.

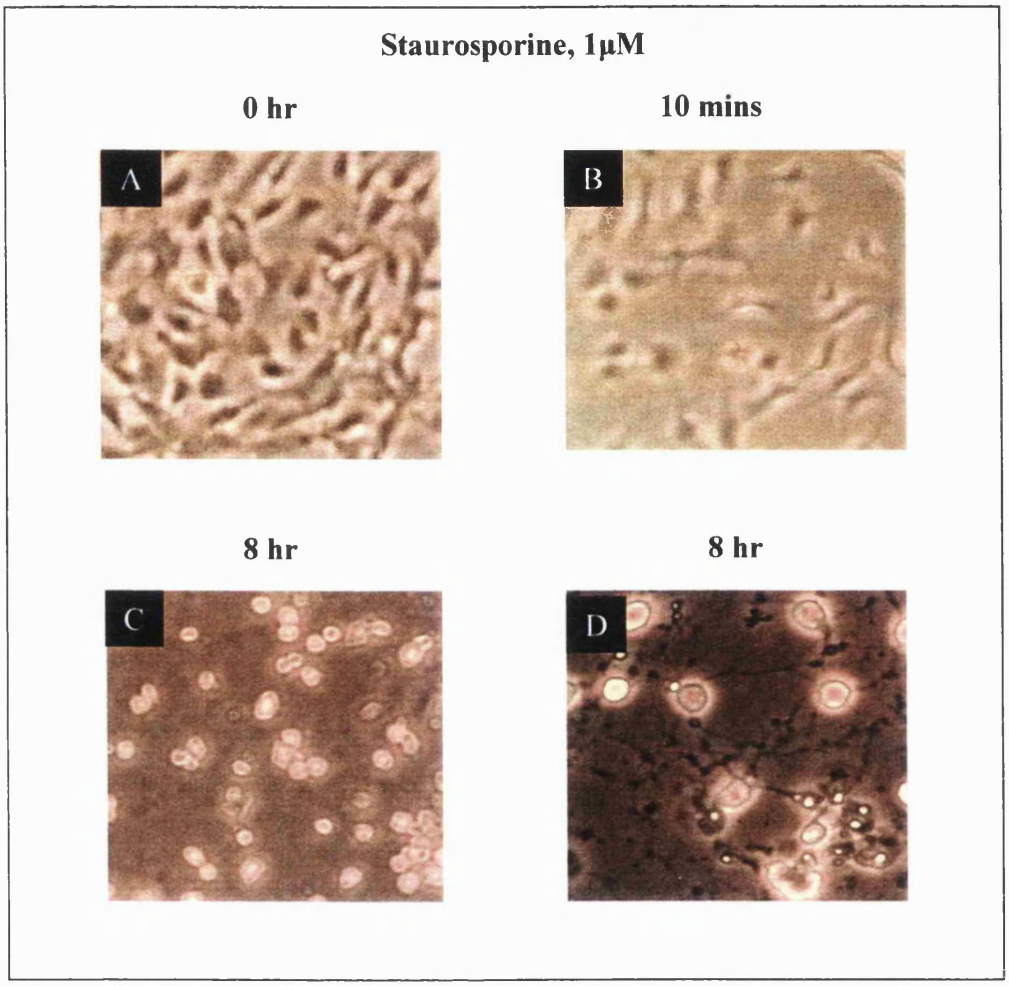


Fig. 7.1.1 Staurosporine induces rapid changes in cell morphology in confluent primary culture of HUVECs.
Confluent cultures of HUVECs (**A**), were treated with 1μM staurosporine After 10 minutes, cell retraction and rounding were already detectable (**B**). Cell detachment was visible within 4 hours of treatment and by 8 hours was marked with >30% of the cell population floating. These cells appeared as bright, rounded bodies on bright-field microscopy (**C**). At higher magnification, no normal cells are visible at 8 hours, and no intercellular connections are apparent (**D**).
Photographs were taken with a Nikon Axiophot camera using phase contrast (A and B) or bright field microscopy (C and D).

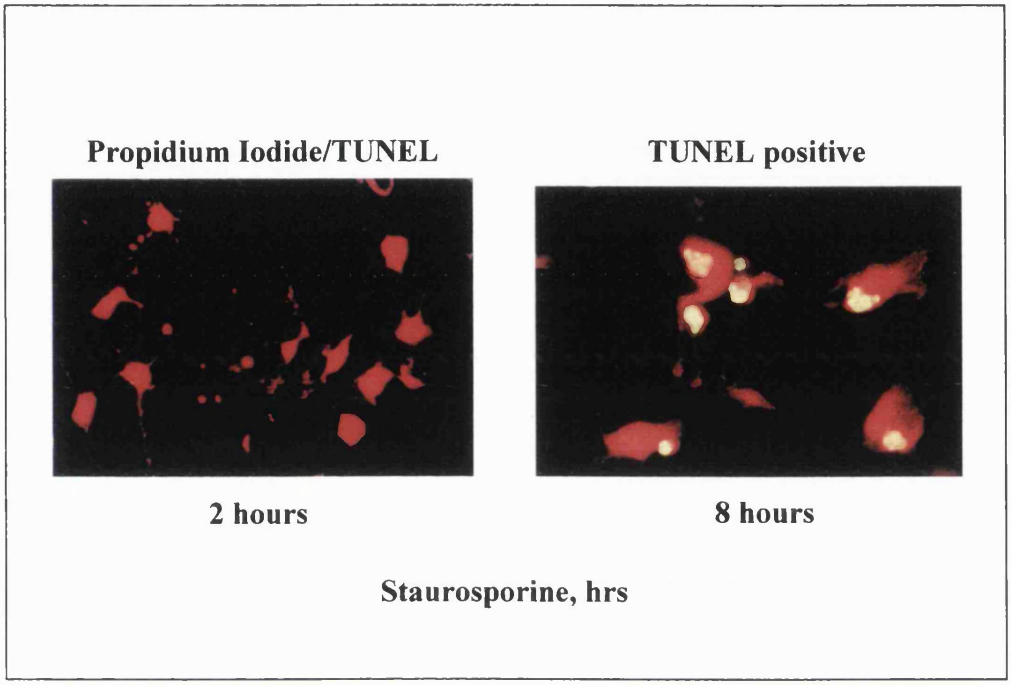


Fig. 7.1.2 Staurosporine-induced TUNEL staining in HUVECs. HUVECS were treated with 1μM staurosporine for the times indicated and then subjected to TUNEL staining and co-immunostaining with the fluorescent marker Propidium Iodide. Dysmorphic changes, including marked cell retraction, are an early finding. Consistent with the gross morphological changes seen in Fig. 7.1.1 at 8 hours following staurosporine treatment, most of these cells are TUNEL positive at this time.

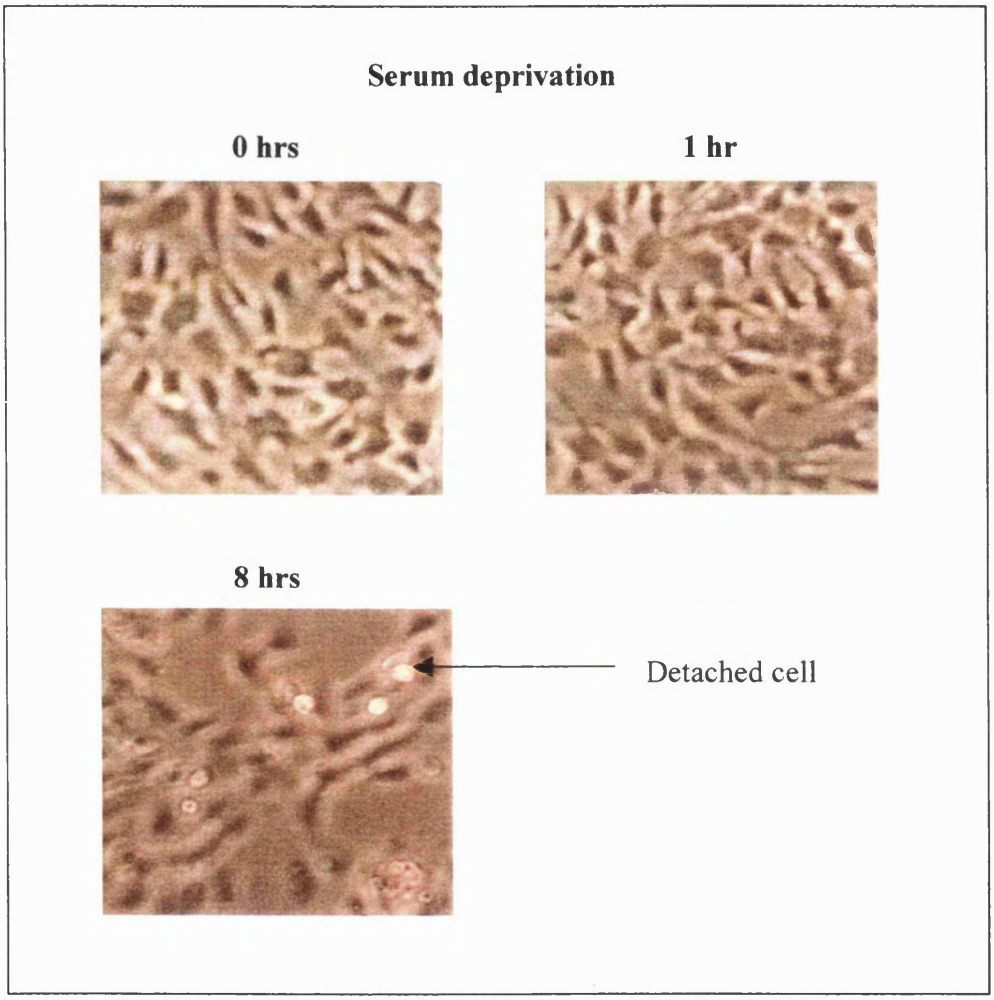


Fig. 7.1.3 Gradual loss of cell-cell contact and delayed cell detachment in serum-deprived HUVECs.
No detectable changes in HUVEC morphology were noted as early as 1 hour following serum deprivation. However, after 8 hours cell retraction and loss of cell-cell contact was noted along with detachment of small numbers of cells (see arrow).
Photographs were taken with a Nikon Axiophot camera using phase contrast (upper panels) or bright field microscopy (lower panel).

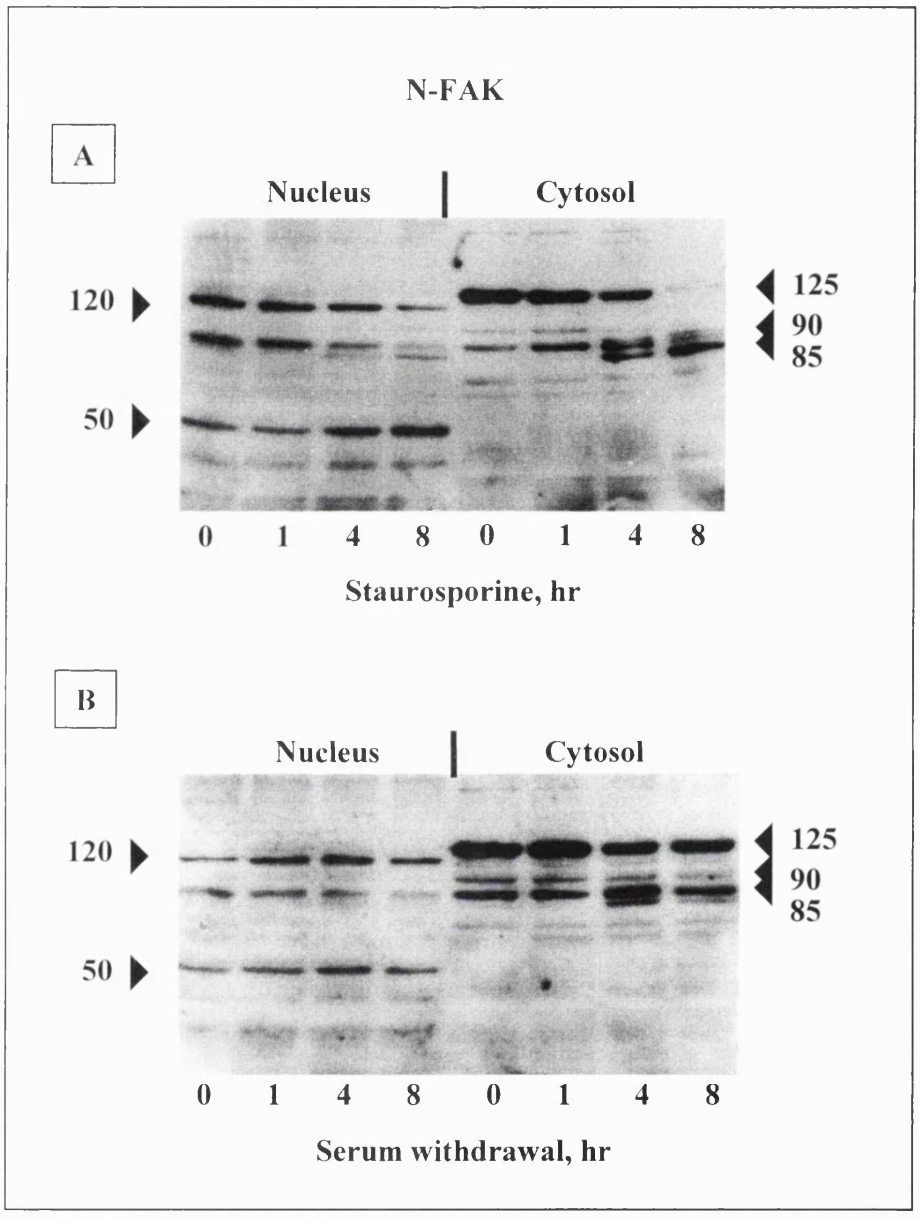


Fig. 7.2 Protein expression of a FAK 50 kDa NH2-terminal fragment increases in the nucleus during endothelial cell apoptosis.
HUVECS were either treated with 1μM staurosporine (A) or incubated in serum-free medium (B) for the times indicated in hours. Nuclear and cytosolic extracts were then prepared and immunoblotted with antibody to the FAK NH2-terminus. The result shown is representative of 4 similar experiments.

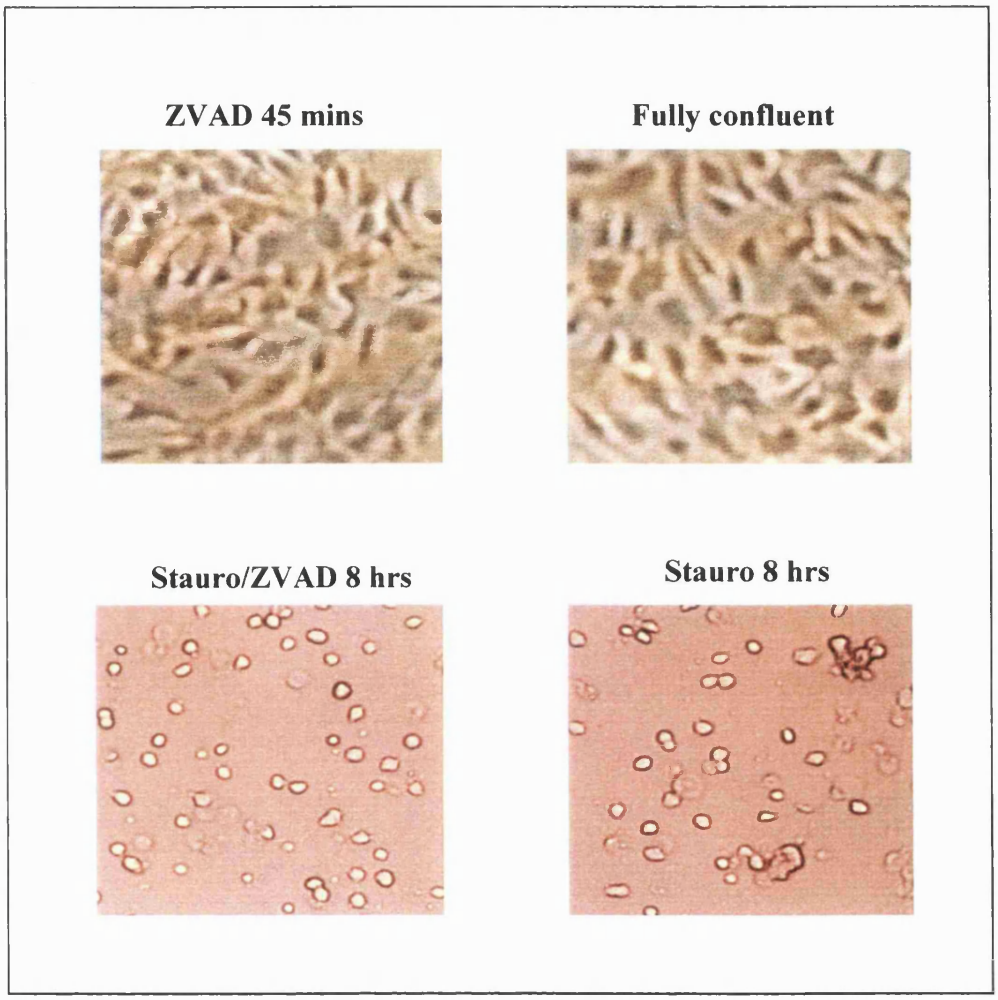


Fig. 7.3.1 Caspase inhibition does not abrogate staurosporine-induced changes in cell morphology and the progression to cell detachment.
Fully confluent HUVECs were pre-incubated with the broad-spectrum caspase inhibitor ZVAD-fmk for 45 minutes (upper panels). HUVECs pre-treated with ZVAD-fmk were given 1μM staurosporine and compared to cells treated with 1μM staurosporine alone (lower panels). Similar proportions of floating cells were visible in HUVEC cultures incubated with ZVAD-fmk and staurosporine together as compared with staurosporine treatment alone.
Photographs were taken with a Nikon Axiophot camera using phase contrast (upper panels) or bright field microscopy (lower panels).

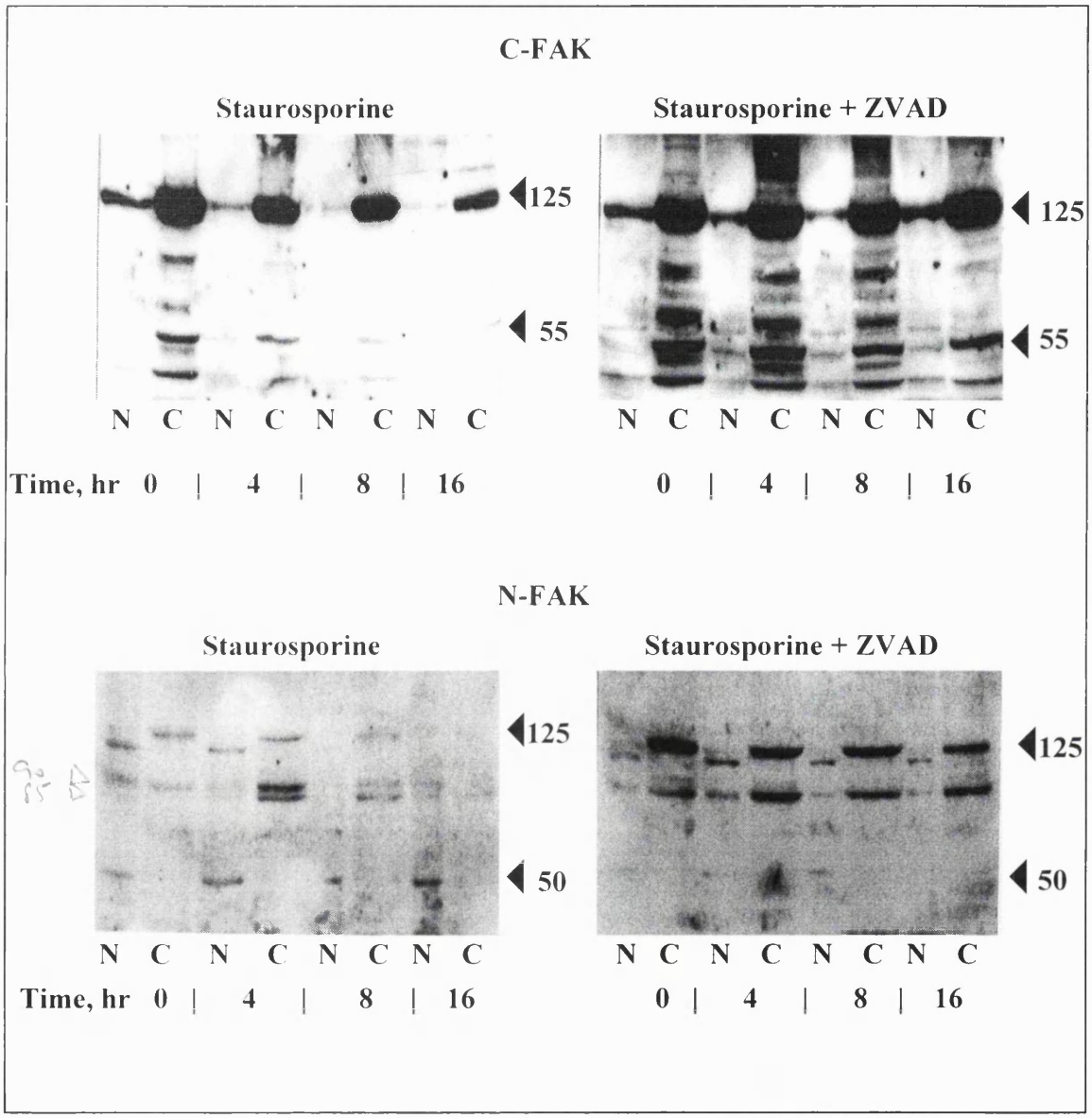


Fig 7.3.2 Nuclear accumulation of a FAK 50 kDa NH2-terminal fragment is reduced by a caspase inhibitor.

Cells were treated with 1 μM staurosporine for the time indicated in hours in the presence or absence of 100 μM ZVAD-fmk. Nuclear (N) and cytosolic (C) extracts were then prepared and immunoblotted with antibodies to the FAK COOH-terminus (C-FAK) or FAK NH2-terminus (N-FAK). The result shown is representative of 3 similar experiments.

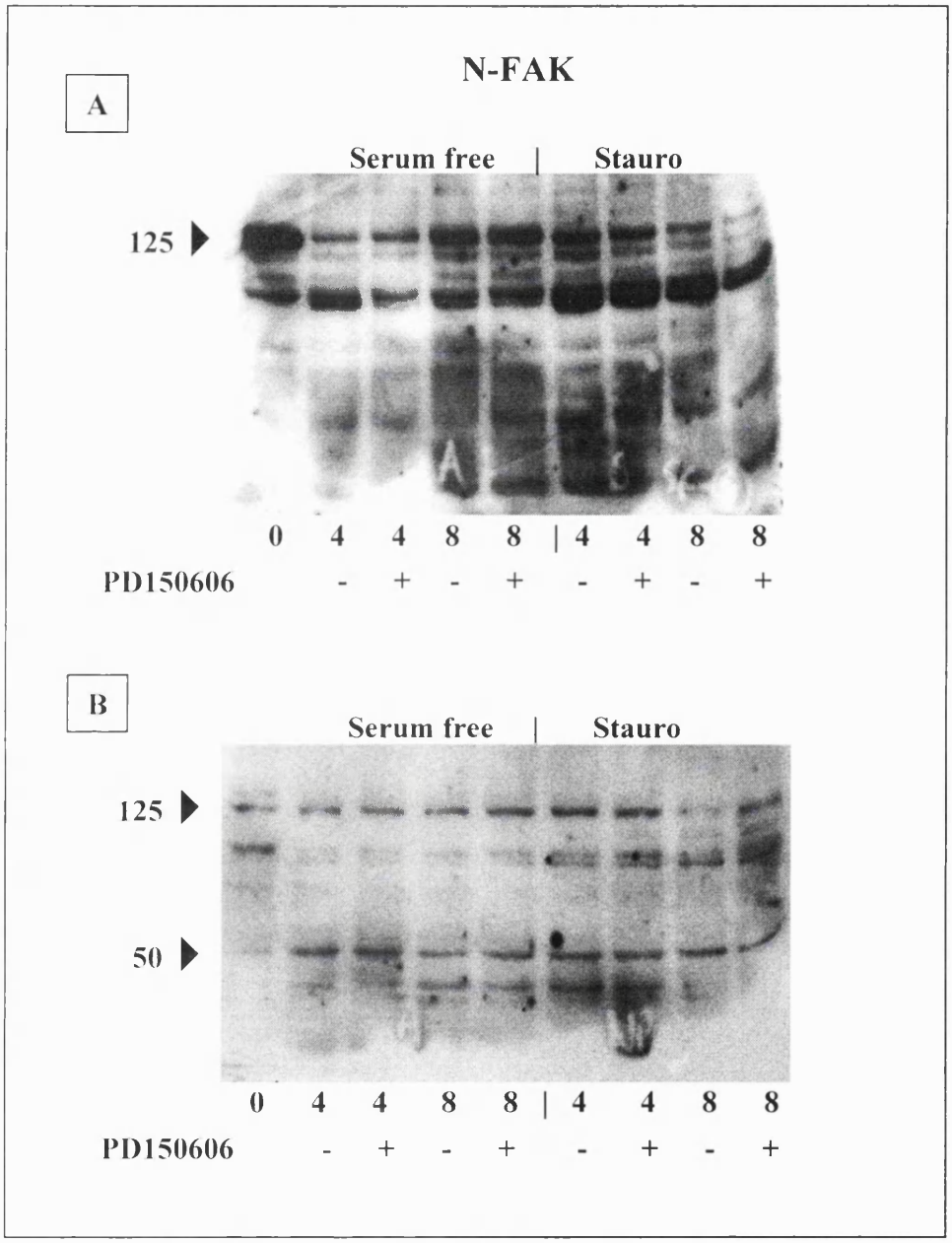


Fig. 7.4 FAK proteolytic cleavage during apoptosis is not calpain mediated.
Cells were incubated in serum-free medium or treated with 1 μM staurosporine in the presence or absence of 50 μM PD150606. Cytosolic (A) and nuclear (B) extracts were then prepared and immunoblotted with an antibody to the NH2-terminus of FAK (N-FAK). The results shown are representative of 3 similar experiments.

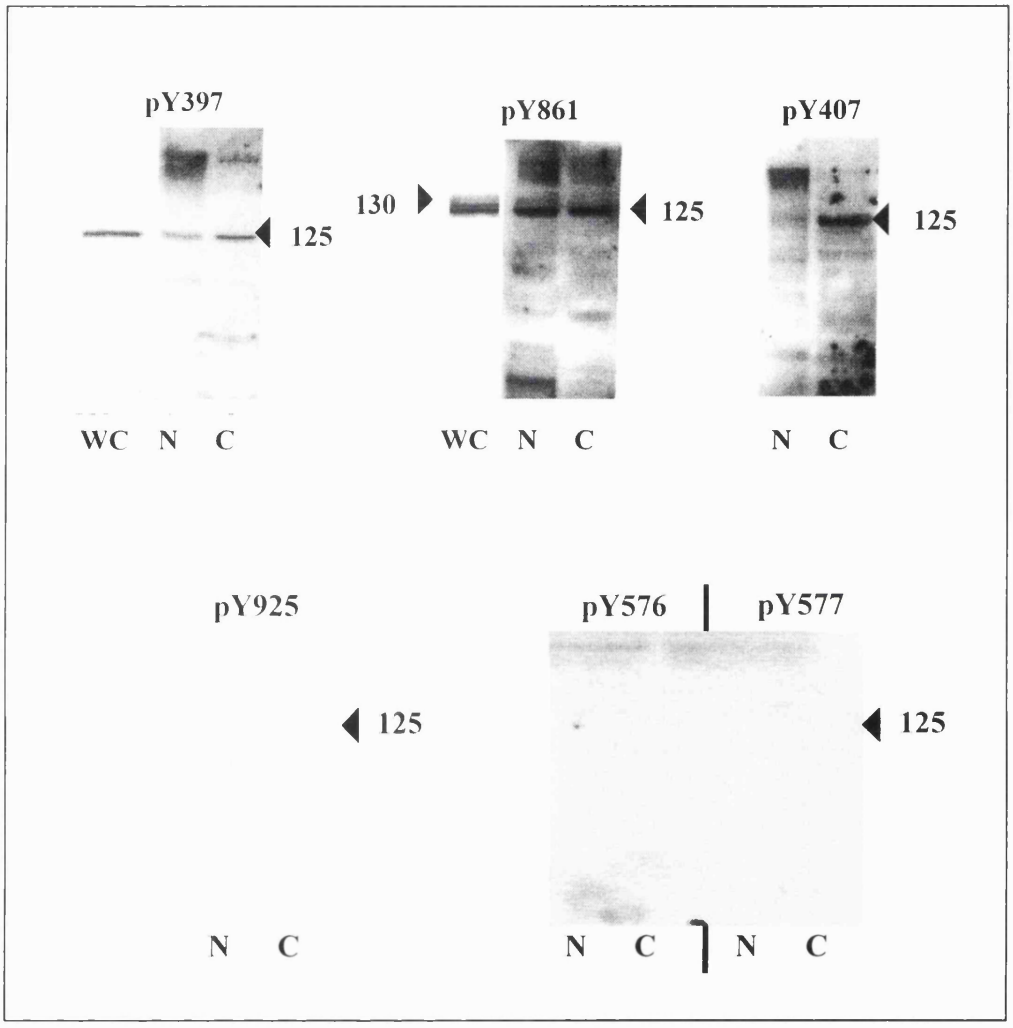


Fig 7.5 Basal FAK tyrosine phosphorylation at Y397, Y861 and Y407 in HUVECs.

Nuclear (N) and cytosolic (C) extracts were prepared from confluent unstimulated HUVECs and immunoblotted with antibodies to FAK phosphorylated at Y397, Y861, Y407, Y576, Y577 and Y925. Whole cell extracts were also prepared and immunoblotted with antibodies to FAK phosphorylated at Y397 and Y861. The positions of 125 and 130 kDa FAK-immunoreactive species are indicated. The results shown are representative of 3 similar experiments. WC refers to whole cell lysates.

Fig. 7.6.1 Staurosporine induces rapid dephosphorylation of FAK at Y861 and Y397.
Cells were treated with 1μM staurosporine for the times indicated in minutes. Nuclear and cytosolic extracts were then prepared and immunoblotted with antibodies to FAK phosphorylated at Y861 (A) and Y397 (B). The results shown are representative of 3 similar experiments.

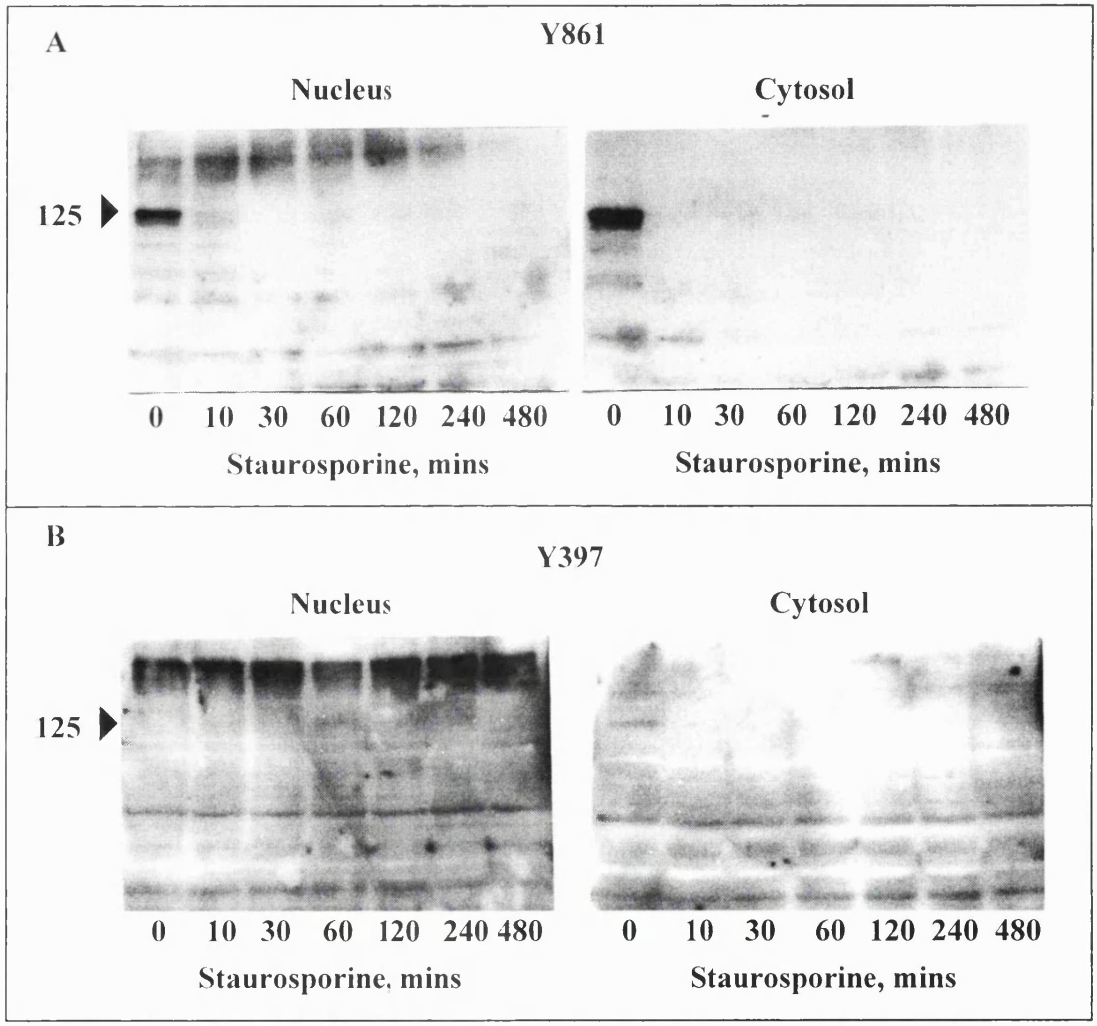


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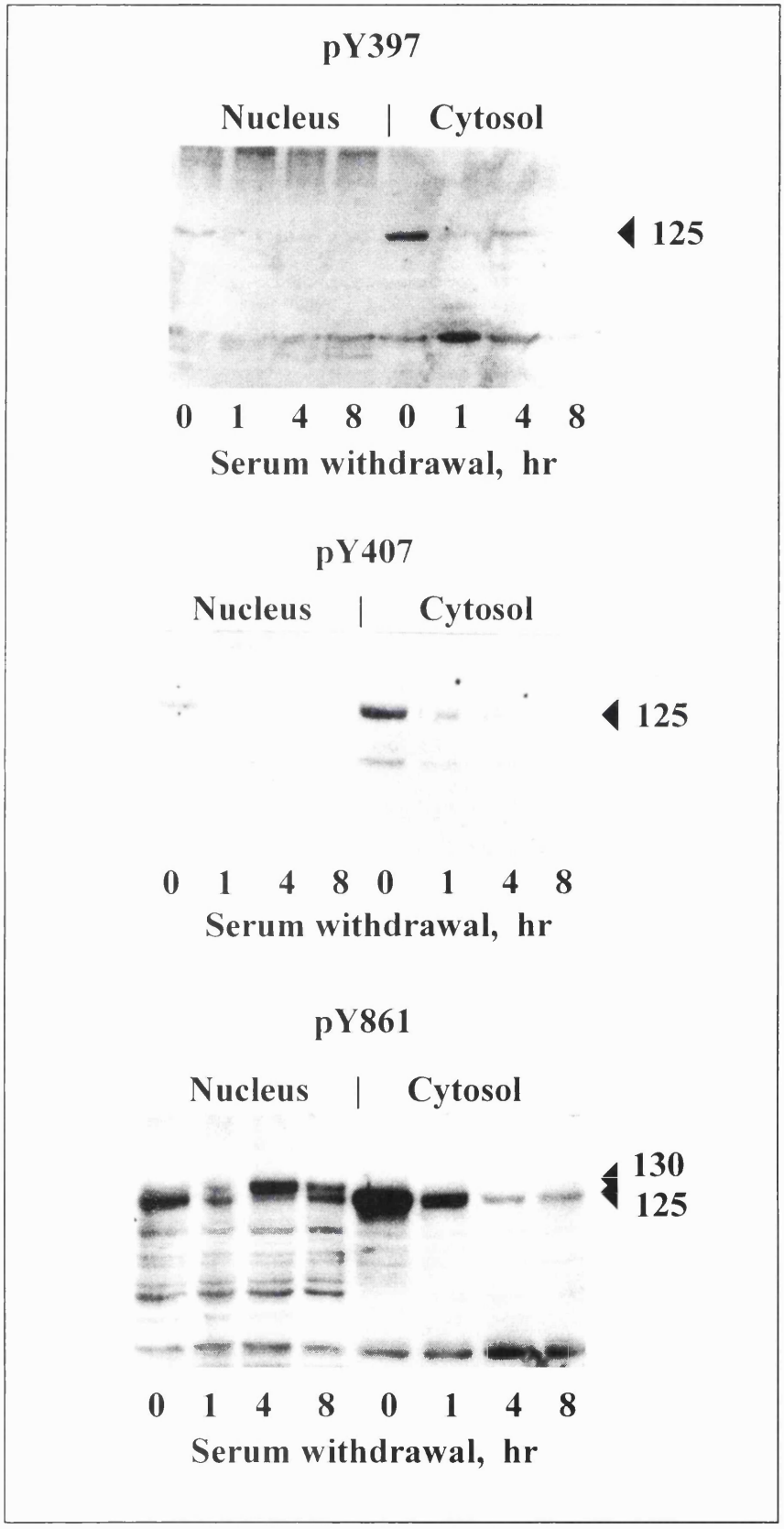
Fig. 7.6.2 serum deprivation induces cytosolic dephosphorylation of FAK at Y861, Y397 and Y407 and a mobility shift in pY861FAK.
HUVECS were incubated with serum-free medium for the time indicated in hours. Nuclear and cytosolic extracts were then prepared and immunoblotted with antibodies to FAK phosphorylated at Y397, Y407 or Y861. The positions of of 125 and 130 kDa FAK-immunoreactive species are indicated. The results shown are representative of three similar experiments.


pY397
Nucleus | Cytosol
125
0 1 4 8 0 1 4 8
Serum withdrawal, hr
pY407
Nucleus | Cytosol
125
0 1 4 8 0 1 4 8
Serum withdrawal, hr
pY861
Nucleus | Cytosol
130
125
0 1 4 8 0 1 4 8
Serum withdrawal, hr

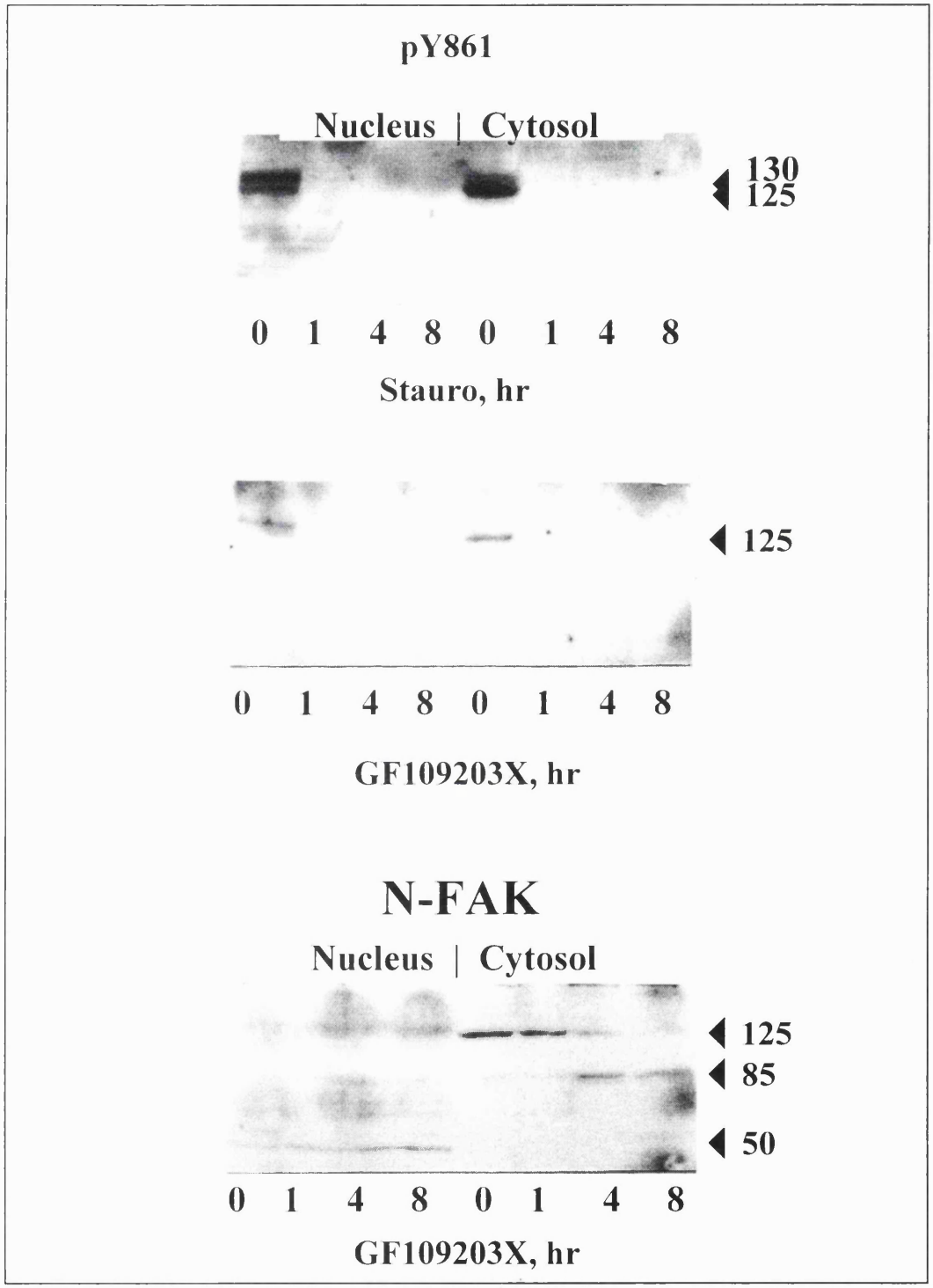


Fig. 7.7.1 The PKC inhibitor GF109203X induces rapid dephosphorylation of FAK at Y861 and nuclear accumulation of a 50 kDa NH2-terminal fragment.

Cells were treated with 1 μM staurosporine or 3 μM GF109203X for the times indicated in hours. Nuclear and cytosolic extracts were then prepared and immunoblotted with antibodies to FAK phosphorylated at Y861 or the FAK NH2-terminus.

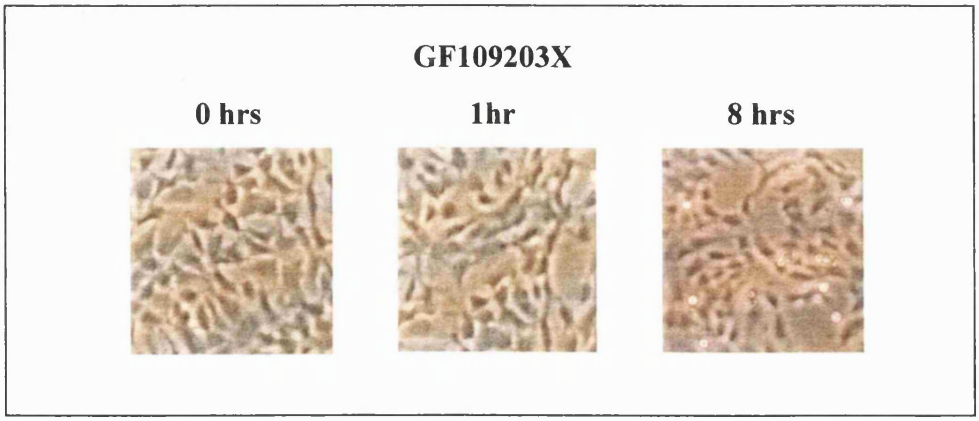


Fig. 7.7.2 The PKC inhibitor GF109203X has similar effects on HUVEC morphology as serum deprivation alone.
Despite marked effects of PKC inhibition on FAK tyrosine dephosphorylation and proteolysis, there are no discernible differences on HUVEC morphology between treatment with GF109203X and treatment with serum deprivation alone.

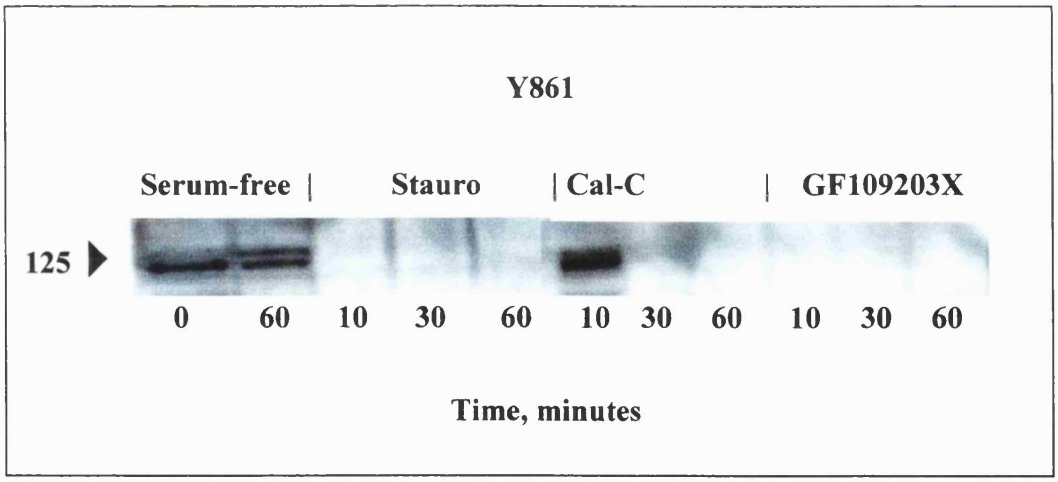


Fig 7.7.3 Staurosporine-induced dephosphorylation of FAK at pY861 is PKC-mediated.
Cells were incubated in serum-free medium or treated with 1μM staurosporine (Stauro) or 3 μM GF109203X or 1μM calphostin-C (Cal-C) for the times indicated in minutes. Cells were then directly extracted into 2XSDS sample buffer and immunoblotted with antibody to FAK phosphorylated at Y861. The position of 125 kDa FAK-immunoreactive species is shown.

Summary

The results in this chapter have demonstrated FAK proteolysis in endothelial cells undergoing apoptosis and compartmentalisation and apoptotic regulation of an amino-terminal FAK fragment, p50N-FAK, previously described in chapter 6. Apoptosis in HUVECs – induced by either chemical means or growth factor withdrawal – is identified by characteristic changes in cell morphology and the development of TUNEL-positive staining. These changes are accompanied by striking changes in expression of $p125^{FAK}$ with evidence of proteolytic cleavage to 90 and 85 kDa fragments taking place within 4 hours of staurosporine treatment and a corresponding decrease in the expression of $p125^{FAK}$. Whilst these changes occurred in the cytosolic compartment, there was a concomitant increase in the expression of p50N-FAK in the nuclear compartment. Serum withdrawal led to a similar pattern of proteolytic events but at a slower rate and with less marked changes in expression levels of p125FAK and related species.

Using a broad-spectrum caspase inhibitor, ZVAD-fmk, it was demonstrated that FAK proteolytic cleavage during staurosporine-induced apoptosis was caspase-dependent. Caspase inhibition effectively inhibited loss of $p125^{FAK}$ expression and prevented the generation of proteolytic fragments as well as abrogating nuclear accumulation of the p50N-FAK fragment. Interestingly, despite the protective effects of caspase inhibition on FAK proteolysis, treatment with ZVAD-fmk did not prevent the morphological changes associated with staurosporine treatment. FAK proteolysis during apoptosis did not appear to be mediated by a calpain-dependent pathway as inhibition of calpains by a broad-spectrum calpain inhibitor failed to prevent apoptotic cleavage of FAK during either serum withdrawal or staurosporine treatment.

In the remainder of this chapter, results characterising apoptotic regulation of FAK tyrosine phosphorylation were presented. The results demonstrated basal tyrosine phosphorylation at residues Y397 and Y407 predominantly in the cytosolic fraction and Y861 phosphorylation in both nuclear and cytosolic fractions of endothelial cells. The

effects on FAK phosphorylation of inducing apoptosis were then examined. Serum withdrawal led to rapid dephosphorylation of FAK at residues Y407 and Y397 with almost complete loss of signal by one hour. Changes in pY861 were more complex with a gel mobility shift from 125 to 130 kDa forms in the nuclear compartment and a decrease in p125 Y861FAK in the cytosol. Staurosporine treatment induced much more rapid loss of tyrosine phosphorylation at residues Y861 and Y397 without any accompanying changes in gel mobility. A striking feature of these data was that, following either staurosporine treatment or serum withdrawal, tyrosine dephosphorylation preceded detectable loss of $p125^{FAK}$ and FAK proteolysis.

Finally data were presented implicating a role for PKC in early regulation of pY861FAK phosphorylation during endothelial cell apoptosis. Inhibition of PKC was not only shown to cause tyrosine dephosphorylation at Y861, but also to cause decreased expression of $p125^{FAK}$ and nuclear accumulation of p50N-FAK.

CHAPTER 8

Discussion

Atherosclerosis and other vasculoproliferative disorders result in part from changes in the two major cellular constituents of the vessel wall, VSMC and the endothelium. It is thought that endothelial dysfunction may be a primary event or process which initiates a chronic inflammatory response in turn leading to phenotypic change in VSMC. Two consequences of this are excessive migration and proliferation of VSMC, which lead to the formation of a neo-intima. This process is subsequently maintained by ongoing endothelial dysfunction and VSMC migration/proliferation with the involvement of other cells, particularly the monocyte/macrophage.

The molecular events underlying VSMC phenotypic change and loss of endothelial function were still largely unknown at the outset of this thesis, although some aspects of these phenomena had previously been documented. Earlier work from this laboratory had established that a non-receptor protein tyrosine kinase called FAK and the FAK-associated proteins paxillin and $p130^{Cas}$, were regulated by PDGF-BB in primary cultures of rabbit VSMC and implicated activation of FAK in the VSMC chemotactic response to PDGF-BB (Abedi *et al.*, 1995). It had also been shown that VEGF, a specific mitogen and survival factor for ECs, stimulates FAK and paxillin tyrosine phosphorylation and increases FAK recruitment to focal adhesions in HUVECs (Abedi and Zachary, 1997). Focal adhesions are thought to be important for mediating signal transduction in cell migration and are closely linked to cell survival signalling, particularly in anchorage dependent cells such as VSMC and HUVECs.

FAK has also emerged as a key player in a number of biological pathways including cell migration and adhesion, cell survival and proliferation and in development. The overall goal of this thesis was twofold. Firstly, to investigate the regulation of FAK with respect to phenotypic modulation of VSMC using primary rabbit aortic VSMC as a model system. Secondly, given that focal adhesion integrity is thought to be a critical determinant of cell survival and signalling in ECs, it was examined whether FAK played a role in the apoptotic response of HUVECs.

The results presented in chapter three showed the expression of focal adhesion components in early explant culture. The explant model of rabbit aortic medial VSMC outgrowth provides a useful *in vitro* model in which the phenotypic modulation of VSMC from a contractile to a synthetic phenotype (as seen in the lesions of neointimal hyperplasia) is consistently reproduced. It has been established that VSMC derived from this method are able to proliferate with a doubling time of ~ 36 hours and undergo loss of expression of contractile proteins in culture (Fritz *et al.*, 1970). This is in contrast to the enzyme dispersal technique wherein VSMC proliferate much more slowly (doubling time ~68 hours) and retain expression of contractile proteins through several passages in culture (Kirschenlohr *et al.*, 1995). The explant model therefore presents an opportunity to examine the molecular mechanisms underlying the phenotypic transition of VSMC in primary culture. The initiation of outgrowth of VSMC from rabbit aortic explants occurs after several days in culture and outgrowth involving the majority of explants takes from 10 to 14 days. These findings are consistent with the results of other workers using explant models (Campbell and Campbell, 1993). The lag interval of several days preceding the appearance of VSMC outside explants presumably reflects the inability of the differentiated VSMC to migrate.

The results presented have shown for the first time that increased expression of FAK and other signalling components of focal adhesions occurs during the early stages of medial VSMC culture, preceding and overlapping with cell migration and outgrowth from explants. Thus FAK, paxillin and $p130^{Cas}$ proteins were upregulated in primary VSMC cultures established by explant outgrowth as compared to fresh uncultured medial tissue. The expression of these proteins increased after culture of fresh medial explants in either serum or a combination of the defined growth factors PDGF-BB, bFGF and IGF-1. Although these growth factors are well established mitogenic and/or chemotactic agents in vascular cells, other than a study correlating PDGF-BB-induced chemotaxis with FAK and paxillin tyrosine phosphorylation (Abedi *et al.*, 1995), there have been no reports of the effects of these molecules on regulation of focal adhesion components in vascular cell migration.

It was noted on several occasions that expression of $p125^{FAK}$ initially declined from a higher basal level in the early stages of explant culture prior to increasing again. An initial upregulation may have arisen during the process of harvesting the explants:

rabbit aortic dissection involves subjecting the aorta to mechanical stretch, which is known to stimulate FAK tyrosine phosphorylation in cardiomyocytes and ECs (Seko *et al.*, 1999; Naruse *et al.*, 1998) and may also induce FAK expression. This is a possibility which warrants further experimentation. The subsequent decrease in expression may in turn reflect a return to an unstimulated basal level.

Increased protein expression of FAK and $p130^{Cas}$ during early explant culture in serum or in growth factor-supplemented medium was accompanied by a marked increase in association of these components. Western blots of proteins derived from either direct cell extracts or co-immunoprecipitation experiments showed immunoreactive $p130^{Cas}$ bands migrating at approximately 130 and 125 kDa with the former predominant. Similar $p130^{Cas}$ bands have been reported by other workers (Sakai *et al*, 1994a; Polte and Hanks, 1995) and may reflect modifications to $p130^{Cas}$ such as different levels of tyrosine phosphorylation or serine/threonine phosphorylation.

Increased expression of the focal adhesion components FAK, paxillin and $p130^{Cas}$ in early explant culture was accompanied by a striking concomitant decrease in expression of VSMC-specific MHC and later by a decrease in expression of VSMC-specific α-actin. Hence, upregulation of FAK, paxillin and $p130^{Cas}$ appears to be associated with an early stage in the transition of VSMC from a contractile state to a phenotype characterised by loss of components of the contractile machinery and increased capacity for cell migration and proliferation. In the context of these findings it is of interest to note that modulation of rat VSMC phenotype by fibronectin was reported to be associated with increased FAK tyrosine phosphorylation and phosphotyrosine staining of focal adhesions (Hedin *et al.*, 1997).

It is tempting to speculate that the increased expression of FAK, paxillin and $p130^{Cas}$ are part of the molecular sequence necessary for the outgrowth of VSMC during explant culture. Greater expression of FAK and other focal adhesion components could play a role in mediating the phenotypic modulation of VSMC during explant culture from a contractile form to one that has the capacity to migrate/proliferate. Focal adhesions play a key role in cell adhesion and locomotion and several lines of evidence support a role for FAK in regulating the rate of cell migration. FAK-deficient mice develop gross abnormalities in the development of mesenchymal tissue organisation, and cells from the embryos of these mice have reduced rates of migration and a more disorganised actin cytoskeleton (Ilic *et al.*, 1996). Overexpression of wild-type FAK

enhances cell migration (Cary *et al.*, 1996, 1998) and overexpression of FRNK inhibits FAK tyrosine phosphorylation and delays focal adhesion formation in fibroblasts (Gilmore and Romer, 1996; Richardson and Parsons, 1996). FAK has been previously reported to associate with $p130^{Cas}$ in other cell types and co-expression of $p130^{Cas}$ with FAK enhanced the migratory response of cells compared with that induced by expression of FAK alone (Cary *et al.*, 1998). An alternative explanation for the upregulation of FAK in VSMC during early explant culture may be linked to its cell survival functions. Although the role of FAK in survival signalling in VSMC was not addressed in this thesis, a detailed consideration of FAK survival signalling in ECs follows in the discussion of chapter 7 results.

An important caveat to the results in chapter 3 is that regulation of mRNA levels was not examined and therefore it is unclear whether FAK is regulated at the level of protein expression or mRNA expression. Further investigation to elucidate the expression of FAK in explant culture should entail examination of mRNA expression of FAK.

Given the results obtained for expression of focal adhesion components, it was of further interest to examine changes in expression of other signalling molecules implicated in signalling linked to cell migration. An increase in the expression of UPAR was noted in early explant culture prior to the onset of detectable VSMC outgrowth. The role for UPAR in such outgrowth is yet to be determined although evidence is accumulating which suggests a role for UPAR in VSMC migration (Okada *et al.*, 1996; Kenagy *et al.*, 1996; Dumler *et al.*, 1998). It is possible therefore that the phenotypic modulation of VSMC also involves changes in expression of UPAR which may serve to mediate cell migration. This could be facilitated either through enhanced ECM degrading capacity or by non-proteolysis-dependent signalling mechanisms. In the context of the latter possibility, a recent report has implicated UPAR-associated activation of the Jak/Stat signalling cascade in the regulation of migration of human VSMC (Dumler *et al.*, 1998).

Expression of the small GTP-binding protein Rho was greatly reduced in extracts prepared from medial tissue as compared to extracts from fresh, whole aortic tissue and deendothelialised aorta. In a 10 day explant culture time course Rho expression was increased in uncultured medial tissue, declined to almost undetectable

levels and thereafter increased markedly prior to the onset of VSMC outgrowth. Considering Rho is thought to play a role in cell migration, which does not occur in native aortic tissue *in vivo*, it is not clear why Rho expression should be detectable in uncultured aortic tissue. However integrin signalling, which is implicated in the transmission of mechanical stress (reviewed in Lehoux and Tedgui, 1998), is thought to be regulated at least in part by Rho proteins. Thus given that the aorta is subject to a variety of mechanical forces in the form of stretch (tensile stress), blood pressure, and shear stress, one might expect high basal levels of expression of proteins involved in the propagation of signals responsible for and arising from mechanical forces. Direct evidence of a role for Rho proteins in mediating smooth muscle cell contractility came from studies in permeabilised smooth muscle where introduction of activated Rho greatly enhanced contractility at given calcium concentrations and lowered the calcium requirement for contractility (Hirata *et al.*, 1992). Rho is thought to stimulate muscle contractility through inhibition of myosin light chain kinase (MLCK) phosphatase resulting in enhanced phosphorylation of myosin light chain kinase (Noda *et al.*, 1995) MLCK regulates the interaction of myosin with actin (reviewed in Burridge and Chrzanowska-Wodnicka, 1996).

Alternatively handling of the aorta during the dissection procedure may also contribute to increased basal Rho expression. It might be postulated that the subsequent decline in Rho expression simply reflects the absence of mechanical stress once the explants are harvested and that the subsequent rise in Rho expression, enabling stress fibre formation and focal adhesion formation, may be a prerequisite for VSMC outgrowth.

The expression of signalling molecules involved in mitogenic signalling in early explant culture was also examined in chapter 4. The results showed that PCNA expression increased dramatically after three days in culture, prior to the onset of visible explant outgrowth. This finding is consistent with re-entry of explant VSMC into the G1 phase of the cell cycle and although it does not prove that those cells are proliferating, studies from other workers have demonstrated proliferation of cells (using [^{3}H]thymidine incorporation into DNA) in explant models of VSMC culture (Fritz *et al.*, 1970; Rossi *et al.*, 1973; Wight *et al.*, 1977). It was therefore of interest to compare and contrast the time-course of FAK expression with that of ERKs 1 and 2, which are

thought to be centrally involved in mitogenic signalling pathways in VSMC and other cell types.

In striking contrast to FAK, ERKs 1 and 2 showed a marked decrease in expression during early explant culture either in serum or in the presence of PDGF-BB, IGF-1 and bFGF, as judged by western blotting with antibodies to either activated or total ERKs 1 and 2. The decrease in ERK expression was paralleled by a decrease in expression of GRB-2, which links RTKs to ras-dependent activation of the ERK cascade. These results are intriguing given the large body of evidence which demonstrates the involvement of the ERK cascade in the proliferation of many cell types. This finding suggests that in explant VSMCs, mitogenic pathways other than those utilising ERKs may be involved in cell proliferation. In support of this, it was reported that in human arterial VSMC that express the inducible form of cyclooxygenase-2 (COX-2), activation of ERKs serves as a negative regulator of proliferation (Bornfeldt *et al.*, 1997). In addition, COX-2 expression is increased in rat aortic VSMC stimulated to proliferate by serum or growth factors (Serhan *et al.*, 1996; Pritchard *et al.*, 1994; Rimarachin *et al.*, 1994). It therefore seems plausible that explant VSMC are also able to upregulate COX-2 and that negative regulation of COX-2 through inhibition of expression and activation of ERKs may be permissive for phenotypic modulation and subsequent mitogenic signalling in these cells. Furthermore, it is well established that activation of ERKs by distinct ligands in the same cell type leads to different biological responses. In PC12 cells, for example, epidermal growth factor-stimulated ERK activation may mediate mitogenesis whereas nerve growth factor-stimulated ERK activation has been implicated in growth arrest and differentiation (Traverse *et al.*, 1992).

Although focal adhesions are thought to be the key sites of action of FAK, the subcellular localisation of FAK in VSMC had not previously been addressed in detail. In chapter 5, immunofluorescence studies of FAK in primary cultures of VSMC led to the unexpected finding that FAK is at least partly localised to the nucleus in VSMC. FAK immunostaining showed surprisingly weak FAK staining of focal adhesions, while paxillin and vinculin focal adhesion immunostaining was abundant and vivid. In contrast, nuclear FAK immunostaining was relatively strong, reproducible and was obtained using several distinct antibodies. Furthermore FAK immunostaining of both

the nucleus and focal adhesions was abolished by peptide competition of the primary antibody. Western blotting of crude subcellular fractions prepared from Triton X-100 or Nonidet-P40 lysates showed significant levels of $p125^{FAK}$ in detergent-insoluble compartments and a 55 kDa COOH-terminal FAK fragment (p55C-FAK) solely in the Triton-soluble compartment. Further evaluation of FAK intracellular distribution by blotting purified nuclear and cytosolic extracts of VSMC also showed a significant fraction of $p125^{FAK}$ and most of a 50 kDa NH_2-terminal domain FAK fragment were present in the nuclear fraction, while the COOH-terminal fragment was found only in the cytosol. Because the 50 kDa band (p50N-FAK) was recognised only by antibodies directed to NH_2-terminal residues of FAK, it is highly likely that this fragment corresponds to the NH_2-terminal non-catalytic domain of FAK. On the basis of its apparent molecular weight it is predicted to contain amino acid residues 1 to 407 which would make it identical or very similar to a previously reported NH_2-terminal apoptotic cleavage product in ECs (Levkau *et al.*, 1998). P50N-FAK is also constitutively expressed in the nuclear fraction of HUVECs (see chapter 6).

The less prominent immunostaining of focal adhesions in VSMC with antibodies to FAK compared with antibodies to vinculin and paxillin was a surprising feature and could possibly reflect masking of FAK antibody recognition epitopes in focal adhesions. It is, however, somewhat implausible that epitopes at the COOH- and NH_2-termini, and in the middle portion of FAK, would be concealed from 3 separate FAK antibodies. Alternatively, FAK may simply not be recruited to focal adhesions in VSMC to the same extent as other focal adhesion components nor to the same extent as noted in other cell types. Interestingly, diffuse cytosolic immunostaining of VSMC which was not related to focal adhesions was noted with antibodies to FAK. When FAK was first cloned in mouse, it was reported to immunolocalise to focal adhesions and in a perinuclear distribution (Hanks *et al.*, 1992). Other studies have reported FAK localisation to focal adhesions in rodent VSMCs , but used cells after multiple passages (Wilson *et al.*, 1995; Zheng *et al.*, 1998). A recent study of FAK proteolysis and focal adhesion disassembly in human VSMC did not examine FAK immunolocalisation (Carragher *et al.*, 1999). It is possible that FAK association with focal adhesions may increase with time and/or passage number in culture. Hence the cytosolic localisation of FAK noted in primary cultures of explant VSMC may represent a cytoplasmic pool from which FAK can be recruited to focal adhesions or perhaps other intracellular

destinations. One line of evidence to support this came from data showing translocation of FAK from a perinuclear distribution in attached, unstimulated cardiomyocytes to focal adhesions, following VEGF stimulation (Takahashi *et al.*, 1999).

Nuclear localisation of FAK is a novel finding in VSMC although it has been noted in ECs previously (Abedi and Zachary, 1997; Levkau *et al.*, 1998). It is of interest that in a study detailing the cloning and characterisation of a novel FAK homologue (Dfak56) in *Drosophila*, Dfak56 nuclear as well as focal adhesion immunostaining was reported in a *Drosophila* neuronal cell line (Fujimoto *et al.*, 1999). Other focal adhesion and cytoskeletal-associated components, including the $p130^{Cas}$-related protein HEF1, and Zyxin, have also been reported to be localised in part to the nucleus (Law *et al.*, 1996; Nix and Beckerle, 1997). Relatively small molecules (less than 60 kDa) may be able to diffuse through the nuclear pore; this is true of actin (Wada *et al.*, 1998) and may be the case for p50N-FAK. Larger molecules however have been shown to gain entry to the nucleus either by virtue of nuclear localisation sequences (NLS) or by interactions with NLS-containing partners (reviewed in Nigg, 1997). Although FAK does not contain a classical nuclear localisation signal, nuclear targetting of several proteins can also involve atypical localisation motifs (Nigg, 1997; Sachdev *et al.*, 1998; Schmalz *et al.*, 1998).

Efficient mechanisms also exist in cells for translocating proteins from the nucleus to the cytoplasm. Such mechanisms appear to be dependent upon nuclear export signals (NESs) prototypical examples of which are found in HIV-1 Rev protein and in the polypeptide inhibitor of cAMP-dependent protein kinase, PKI-α (Fisher *et al.*, 1995; Wen *et al.*, 1995)(see Fig. 8.1). A typical NES consists of a short hydrophobic sequence (up to 12 amino acids), with a high leucine content (Nix and Beckerle, 1997). Inspection of the FAK amino acid sequence revealed a previously unreported consensus nuclear export signal between residues 166-176, IALKLGCLEI, which is conserved between human, murine and chicken FAK and shares homology with other canonical export signals (see Fig 8.1).

The role of this motif is unknown and its possible function warrants further investigation. This may involve mutating the sequence to see if it can cause mislocalisation of FAK which, if this is a functional NES, might be predicted to cause excessive nuclear accumulation relative to the cytoplasm and focal adhesions. Alternatively the motif could be added to a protein which is too large to exit the nucleus

by diffusion, such as bovine serum albumin, and observing if nuclear export is facilitated.

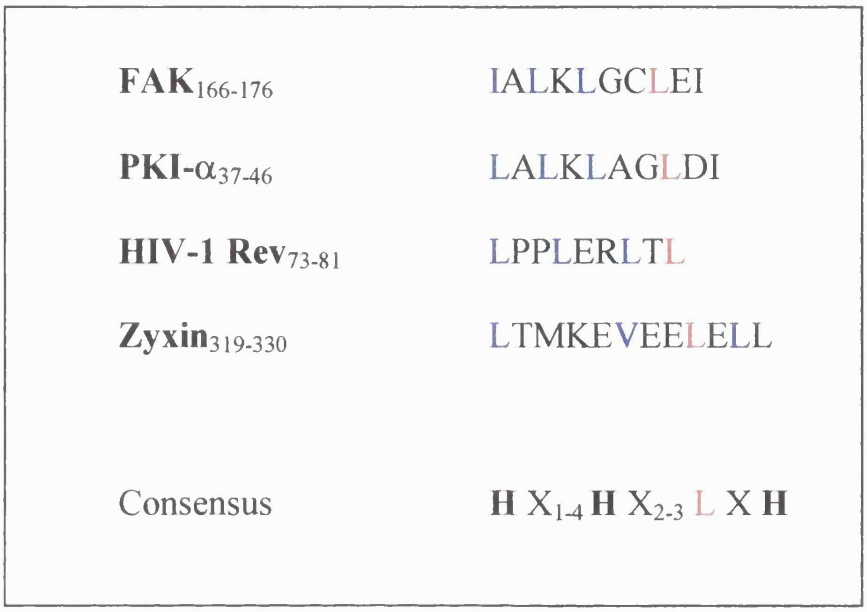


Fig. 8.1 A canonical nuclear export signal is localised in the NH_2-terminal domain of FAK.
H refers to the hydrophobic amino acid residues (leucine, isoleucine or valine). The L residue is always conserved.

The p55C-FAK fragment was only recognised by the antibody to the COOH-terminus of FAK (immunogenic epitope residues 1033-1052) and therefore is most likely to be similar to p41/43 FRNK (Schaller *et al.*, 1993). A COOH-terminal FAK fragment of this size has not been previously reported although a smaller (35 kDa) fragment was detected in apoptotic Jurkat cell extracts (Gervais *et al.*, 1998). Given that p55C-FAK is constitutively expressed in VSMC, it may well arise from an alternative mRNA transcript, in much the same way as FRNK, and function in the negative regulation of FAK in VSMC. Although it may be small enough to enter the nucleus by diffusion, the presence of the FAT domain is perhaps sufficient to ensure its constitutive focal adhesion localisation.

The finding that FAK fragments corresponding to the NH_2-terminal and COOH-terminal domains are differentially compartmentalised is of particular note. The NH_2-

terminal non-catalytic domain has been implicated in binding to the cytoplasmic domains of β_1 and β_3 integrin subunits (Schaller *et al.*, 1995). However the motifs in this domain which are involved in integrin associations have not been defined and its functions are otherwise unknown. Furthermore, recent findings indicate that the NH_2-terminal domain is dispensable for adhesion-dependent regulation of FAK and paxillin tyrosine phosphorylation (Shen and Schaller, 1999). One possible role for p50N-FAK is the regulation of $p125^{FAK}$ functions. Lack of COOH-terminal FAT domain residues prevent FAK from localising to focal adhesions (Hildebrand *et al.*, 1993). In this context it is of interest that a FAK mutant construct lacking the COOH-terminal 14 amino acid residues interferes with FAK function and inhibits cell cycle progression by competing with endogenous FAK binding to Src and Fyn (Zhao *et al.*, 1998). Hence it is plausible that segregation of NH_2-terminal fragments lacking a functional COOH-terminal domain may be necessary to prevent interference with focal adhesion associated functions of full-length $p125^{FAK}$. Alternatively NH_2-terminal variants may perform other, as yet unknown, functions. Regardless of the precise role of nuclear FAK in VSMC, these findings indicate that cellular functions of FAK may not be determined solely by its association with focal adhesions and suggest novel regulatory roles for FAK and FAK fragments in these cells.

Given the unexpected nature of the findings presented in chapter 5, it was of interest to examine whether nuclear localisation of FAK and the segregation of NH_2- and COOH-terminal domain fragments was a specific feature of VSMC or was also observed in ECs. Accordingly, in chapter 6, the subcellular localisation of FAK in HUVECs was investigated. Nuclear localisation of FAK was demonstrated by immunostaining with different antibodies and immunoblotting of nuclear and cytosolic cell fractions. The results showed that 3 different FAK antibodies produced immunofluorescent staining of the nucleus as well as focal adhesions. Focal adhesion staining with these antibodies was much more prominent in HUVECs than observed in VSMC. The antibody to the COOH-terminal domain of FAK also produced very pronounced perinuclear and cytoplasmic staining which was also seen with the antibody to the catalytic region although less prominently. In addition NH_2-terminal and COOH-terminal antibodies detected some $p125^{FAK}$ in the nuclear fraction of digitonin-permeabilised HUVECs although most immunoreactive $p125^{FAK}$ was in the cytosolic

fraction. As noted in VSMC, there was a striking segregation of p50N-FAK, found only in the nuclear compartment, and p55C-FAK which was present only in the cytosolic fraction of these cells. Furthermore a p120 band recognised solely by the NH_2-terminal-specific antibody was detected only in the nuclear fraction. Since this band was not recognised by the COOH-terminal-specific antibody, it seems most likely to represent an endogenous FAK species truncated at the COOH terminus. However the possibility that this is a protein immunologically related to FAK cannot be excluded. Although p55C-FAK and p50N-FAK comprised only a small fraction of total FAK immunoreactivity relative to $p125^{FAK}$, they appeared to be present at similar levels in HUVECs.

Differential compartmentalisation of $p125^{FAK}$ relative to both p55C-FAK and p50N-FAK was suggested by Triton solubilisation. The findings that similar amounts of $p125^{FAK}$ were associated with the Triton-insoluble compartment and with protein-protein complexes resistant to Triton solubilisation, while p55C-FAK and p50N-FAK were highly enriched in the 100,000 *g* pellet relative to the Triton-insoluble fraction, suggest that the FAK COOH- and NH_2-terminal domains are more abundant than the 125 kDa form in multi-component aggregates resistant to Triton solubilisation. It may also be tentatively inferred from these studies that a large proportion of $p125^{FAK}$ is Triton-soluble and likely to constitute a free cytosolic pool. Immunofluorescence data from HUVECs showing copious perinuclear and cytoplasmic FAK immunostaining support this interpretation.

The studies presented in chapter 7 examined the apoptotic regulation of FAK in HUVECs. Staurosporine was shown to induce apoptosis rapidly compared to serum deprivation of HUVECs, as determined both by TUNEL staining and cell detachment. Proteolytic cleavage of FAK to 90 and 85 kDa fragments localised to the cytosol and concomitant loss of $p125^{FAK}$ and the 120 kDa FAK variant were also demonstrated. The p55C-FAK species remained localised to the cytosolic compartment and its expression was much reduced after 16 hours of staurosporine treatment. In contrast, expression of the p50N-FAK fragment in the nuclear fraction increased with time in staurosporine-treated cells. Serum-deprivation caused similar but less marked changes in protein expression of FAK and FAK-related species to those noted with staurosporine. Consistent with these findings, serum-deprived cells were slower to exhibit TUNEL-

positive staining and did not detach as rapidly as staurosporine-treated HUVECs. Although caspase inhibition with ZVAD-fmk was effective in limiting FAK proteolysis, it did not prevent changes in cell morphology or the progression to cell detachment in staurosporine-treated cells. Pre-treatment of cells with a calpain inhibitor failed to prevent FAK proteolysis to 85 and 90 kDa fragments or the increase in nuclear expression of p50N-FAK induced by either staurosporine or serum-deprivation.

The finding that p50N-FAK expression increased only in the nuclear fraction during HUVEC apoptosis and could not be detected at significant levels in the cytosolic fraction in non-apoptotic cells, suggests that this fragment is either generated in the nuclear fraction or is rapidly sequestered to this compartment after proteolysis. It has been previously suggested that caspase-dependent FAK cleavage products produced during apoptosis may undergo translocation from the cytosol to the nucleus (Levkau *et al.*, 1998). Since p50N-FAK could not be detected in the cytosolic fraction of HUVECs, and 90 and 85 kDa proteolytic cleavage products occurred predominantly in the cytosol, the results presented here do not provide evidence in support of nuclear translocation during apoptosis. The ability of the broad-specificity caspase inhibitor ZVAD-fmk to reduce the staurosporine-induced increase in p50N-FAK while inhibiting cleavage of $p125^{FAK}$ and the accumulation of the 90 kDa product, suggests that generation of p50N-FAK is mediated in part by a caspase proteolytic pathway, as previously reported (Levkau *et al.*, 1998). The decrease in p55C-FAK in staurosporine-treated cells, was also prevented by ZVAD-fmk pre-treatment, indicating that this protein is subject to caspase-dependent proteolysis. Further supporting evidence for these findings is provided by the identification of two consensus sites for caspase cleavage in the amino acid sequence of FAK (Gervais *et al.*, 1998). One of these sites, $DQTD^{772}$ is preferentially cleaved by caspase-3 and is conserved in human and chicken FAK. The other site, $VSWD^{704}$ is conserved in all animal FAK species so far cloned and cleaved by caspase-6. The action of caspases at these sites was shown to generate FAK fragments of 90 and 85 kDa (Gervais *et al.*, 1998), similar to the fragments produced during staurosporine-induced apoptosis in chapter 7 (Fig. 8.2). These fragments were recognised by the antibody to the NH_2-terminal domain of FAK. However, the 35 and 40 kDa FAK COOH-terminal cleavage products predicted to be generated by cleavage at these sites were not detected. Cleavage of $p125^{FAK}$ at the sites described would additionally result in generation of COOH-terminal fragments which would be

hypothesised to inhibit FAK phosphorylation (Gervais *et al.*, 1998). FAK proteolytic cleavage is one mechanism underlying disruption of FAK function and concomitant perturbation of focal adhesion structure and function. FAK cleavage would act in two ways. Firstly, by decreasing the level of full-length $p125^{FAK}$. Secondly by increasing the level of small FAT-domain fragments which, similar to results obtained for FRNK overexpression, might act in a dominant-negative fashion to inhibit $p125^{FAK}$ function.

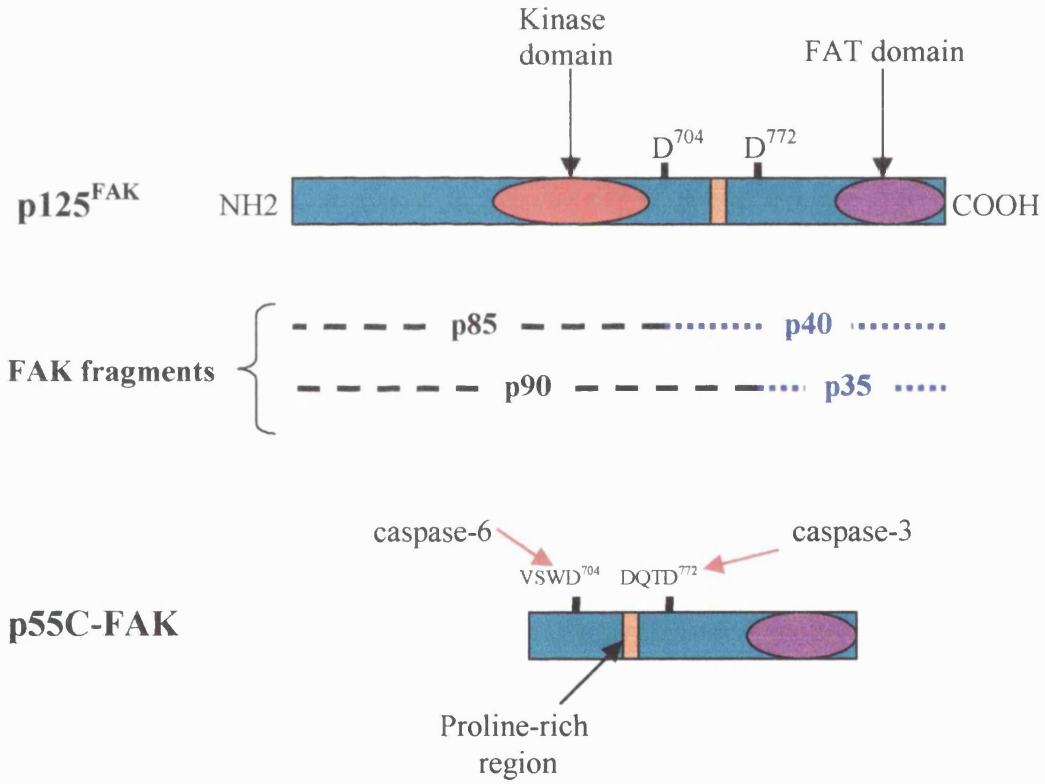


Fig. 8.2 FAK fragments generated by caspase cleavage.
Both $p125^{FAK}$ and p55C-FAK can be cleaved at consensus cleavage sites for caspases-3 and –6 generating extreme COOH-terminus FAK fragments containing the focal adhesion targetting (FAT) domain.

However, as discussed below, the time-course of staurosporine- and serum deprivation-induced FAK cleavage did not correlate with the rapid loss of FAK phosphorylation found in subsequent experiments. Furthermore, staurosporine-induced morphological changes and loss of focal adhesions occurred prior to, and were temporally distinct from, FAK proteolysis. These results indicate that FAK cleavage is likely to be a consequence of, rather than an early initiating event, in apoptosis.

The differences noted between staurosporine treatment and serum withdrawal may reflect engagement of different apoptotic pathways or simply more efficient harnessing of a single, common pathway by staurosporine. The effects of staurosporine treatment upon cells are, however, unclear and this is well illustrated by the findings with ZVAD-fmk pretreatment. Despite blocking staurosporine-induced caspase-mediated cleavage of FAK efficiently, cell retraction and rounding followed by detachment still occurred as rapidly as in cells treated with staurosporine alone. Although the effects of staurosporine on focal adhesions and cytoskeletal integrity were not examined, it seems likely that the early rounding of cells and detachment from the substrate occur as a result of focal adhesion disassembly and loss of non-caspase-mediated signal transduction pathways. In another study ZVAD-fmk was shown to prevent apoptosis induced by the nephrotoxicant dichlorovinylcysteine (DCVC), but was unable to prevent focal disassembly and dephosphorylation of FAK (van de Water *et al.*, 1999).

The results in chapter 7 did not provide any evidence to support a role for calpain-mediated FAK proteolysis in HUVECs undergoing apoptosis. The findings do not preclude a role for calpains which have already been demonstrated to participate in apoptosis in HUVECs (Meredith *et al.*, 1998). In addition, calpain-mediated proteolysis of FAK has been demonstrated in platelets indicating that the relevant cleavage sites exist in the FAK sequence (Cooray *et al.*, 1996). It will be important to address the role of calpains in EC apoptosis further.

The FAK tyrosine phosphorylation sites identified so far are residues Y397, Y407, Y576, Y577, Y861, and Y925. In resting ECs FAK was phosphorylated at Y397, Y407 and Y861 based on immunoreactivity to phospho-specific antibodies. Phospho-Y861FAK appeared to be the major phosphorylated form in both the nuclear and

cytosolic compartments in resting ECs, and little phospho-Y397 or –Y407FAK were detected in the nuclear fraction. Dephosphorylation at Y861, Y407 and Y397 was rapidly induced by staurosporine and serum deprivation, and preceded the decrease in $p125^{FAK}$, the onset of FAK proteolysis and detectable apoptosis. FAK tyrosine dephosphorylation has also been shown to precede cleavage during chemically induced apoptosis in renal epithelial cells (van de Water *et al.*, 1999), though neither the mechanism nor the phosphorylation sites involved were investigated. These findings indicate that FAK dephosphorylation at specific tyrosine residues may be a primary event in the initiation of EC apoptosis, and suggest that regulation of FAK tyrosine phosphorylation status rather than proteolysis plays a critical role in maintaining focal adhesion integrity and survival signalling in these cells.

Two separate and mechanistically distinct inhibitors of protein kinase C were shown to induce dephosphorylation of FAK at pY861, although neither caused cell retraction nor increased the rate of cell detachment above that caused by serum deprivation alone. The ability of the selective PKC inhibitor GF109203X to induce rapid pY861FAK dephosphorylation followed by proteolysis suggests that the effects of staurosporine are mediated at least in part via inhibition of PKC. Since Y861 and Y407 are phosphorylation sites for Src *in vivo* and *in vitro* (Calalb *et al.*, 1995, 1996), and staurosporine is known to inhibit protein tyrosine kinases (Secrist *et al.*, 1990), it is possible that this agent may inhibit FAK itself or another upstream kinase, such as Src, required for maintaining FAK phosphorylation. PKC, a serine/threonine kinase, has not been previously thought to play a major role in the regulation of FAK activity. However FAK tyrosine phosphorylation is stimulated by phorbol esters and diacylglycerol (Sinnett-Smith *et al.*, 1993) and GF109203X inhibited VEGF-induced FAK tyrosine phosphorylation in HUVECs (Abedi and Zachary, 1997). Inhibition of PKC with calphostin C resulted in slower dephosphorylation of pY861FAK as compared to GF109203X. Calphostin C was also reported to inhibit FAK serine phosphorylation and induce loss of $p125^{FAK}$ in BALBc/3T3 cells (Mogi *et al.*, 1995). Early cell retraction and detachment were not a feature of PKC inhibition despite causing rapid FAK dephosphorylation and proteolysis. This might indicate that effects of staurosporine on apoptosis and cell morphology are also due to its ability to inhibit other kinases important for maintaining survival functions in HUVECs.

These findings point towards an important role for PKC in the maintenance of basal FAK tyrosine phosphorylation in ECs which may be critical for the propagation of survival signals. Given that PKC is implicated in integrin signalling pathways and, in some cell types, PKC inhibitors impair cell adhesion and spreading (Disatnik and Rando, 1999; Clark and Brugge, 1995; Woods and Couchman, 1992), it is plausible that integrin-dependent survival signalling is relayed to FAK via PKC. Furthermore, since VEGF is an important survival factor for ECs and induces both PKC activation (Xia *et al.*, 1996) and GF109203X-sensitive FAK tyrosine phosphorylation (Abedi and Zachary, 1997), these results suggest a role for PKC-mediated FAK tyrosine phosphorylation in anti-apoptotic signalling by VEGF.

While only the 125 kDa form was detected by antibody to phospho-Y861FAK in the cytosol, phospho-Y861FAK was present in the nuclear compartment in two forms corresponding to $p125^{FAK}$ and a more slowly migrating band of ~Mr 130,000. A noteworthy feature of the results was the marked shift in phospho-Y861FAK mobility to the more slowly migrating form specifically in the nuclear fraction during apoptosis induced by serum withdrawal. The shift was detectable only in the nuclear fraction, was evident after 60 minutes, had reached a maximum by 4 hours and was sustained for up to 8 hours. Shifts in SDS-PAGE mobility of protein bands to more slowly migrating forms are indicative of increased phosphorylation. In the case of phospho-Y861FAK, gel retardation is likely to result from increased phosphorylation at additional sites. Whether this is due to tyrosine or serine/threonine phosphorylation is unclear, but since other phospho-specific antibodies could not detect any mobility shift in $p125^{FAK}$, it is perhaps most likely that the mobility shift is primarily due to serine/threonine phosphorylation. The mobility shift in nuclear phospho-Y861FAK mobility was not readily detected by the NH_2- and COOH-terminal FAK antibodies indicating that only a small fraction of total cellular FAK is affected by these changes. Increased serine phosphorylation has been proposed to disrupt FAK complexes with $p130^{Cas}$ and $pp60^{c\text{-}src}$ and maintain FAK in an inactive state until mitosis is complete (Yamakita *et al.*, 1999). Since mitosis and EC apoptosis involve similar marked changes in cell shape and adhesiveness - involving cell retraction from ECM and loss of attachments - it is intriguing to speculate that apoptosis-induced changes in phospho-Y861FAK mobility may be analogous to the increased serine phosphorylation reported in mitotic cells.

Conclusions

The data presented in this thesis provide new insights into the regulation and localisation of FAK in vascular cells. The finding that expression of FAK, paxillin and $p130^{Cas}$ are upregulated during explant culture of medial VSMC suggests that these signalling molecules may play an important role in mediating phenotypic transition of VSMC from a contractile to a synthetic phenotype. Evidence demonstrating nuclear expression of FAK and FAK-related species in both VSMC and HUVECs also indicates that FAK may play other novel regulatory roles in vascular cells and that its cellular functions are not necessarily contingent upon its association with focal adhesions. The finding of constitutively expressed NH_2-terminal and COOH-terminal FAK fragments which are targetted to different subcellular compartments and regulated during EC apoptosis is intriguing. Presumably the COOH-terminal fragment (p55C-FAK) is akin to p41/43 FRNK and functions in a similar manner. However the role of the NH_2-terminal fragment (p50N-FAK) is unclear and its highly restricted nuclear localisation suggests that it may perform a novel role in FAK-mediated regulation of EC function. Finally the identification of PKC-dependent FAK tyrosine phosphorylation sites which are subject to rapid regulation during EC apoptosis provides a potentially important insight into the signalling mechanisms underlying FAK-dependent survival.

The fate of a cell, with particular regard to commitment to survival or programmed cell death, appears to depend upon a complex interplay between apoptotic signalling pathways and multiple cell survival signals which act to suppress apoptosis. Anchorage-dependent cells, such as HUVECs, are exquisitely sensitive to loss of survival signals generated by cell-substrate contact (resulting in anoikis) and in such cells FAK appears to be critically important in anti-apoptotic signal transduction. The data presented in this thesis forms the basis for proposing the following model for the regulation of FAK tyrosine phosphorylation in HUVEC survival and apoptosis (Fig. 8.3). Survival signals arise from diverse sources: cell-ECM interactions, fluid shear stress or from ligand engagement of growth factor receptors (e.g. VEGF binding to KDR). In some cases, such as VEGF ligation of KDR, signal is then propagated to FAK via PKC-dependent mechanisms. FAK activation, and in particular phosphorylation at Y861, Y407 and Y397 in HUVECs, appears to be important for survival signalling and

is able to suppress a p53-mediated apoptotic pathway (Ilic *et al.*, 1998). FAK tyrosine dephosphorylation, secondary to inhibition of PKC-mediated pathways or serum deprivation, is followed by focal adhesion disassembly and cell retraction (early apoptosis). These events are followed subsequently by caspase activation leading to FAK proteolysis and cell detachment (late apoptosis). Such a model may also be relevant for VSMC apoptosis, although this remains to be determined.

The challenges ahead will be to elucidate the role of PKC in regulation of FAK phosphorylation and to determine the importance and functions of individual sites of FAK tyrosine phosphorylation. Whether FAK serine/threonine phosphorylation has a role to play in apoptosis should also be examined. Finally, much remains to be discovered concerning the functions of the NH_2-terminal FAK fragment although its exclusive nuclear localisation suggests further exciting and novel functions for FAK-related species.

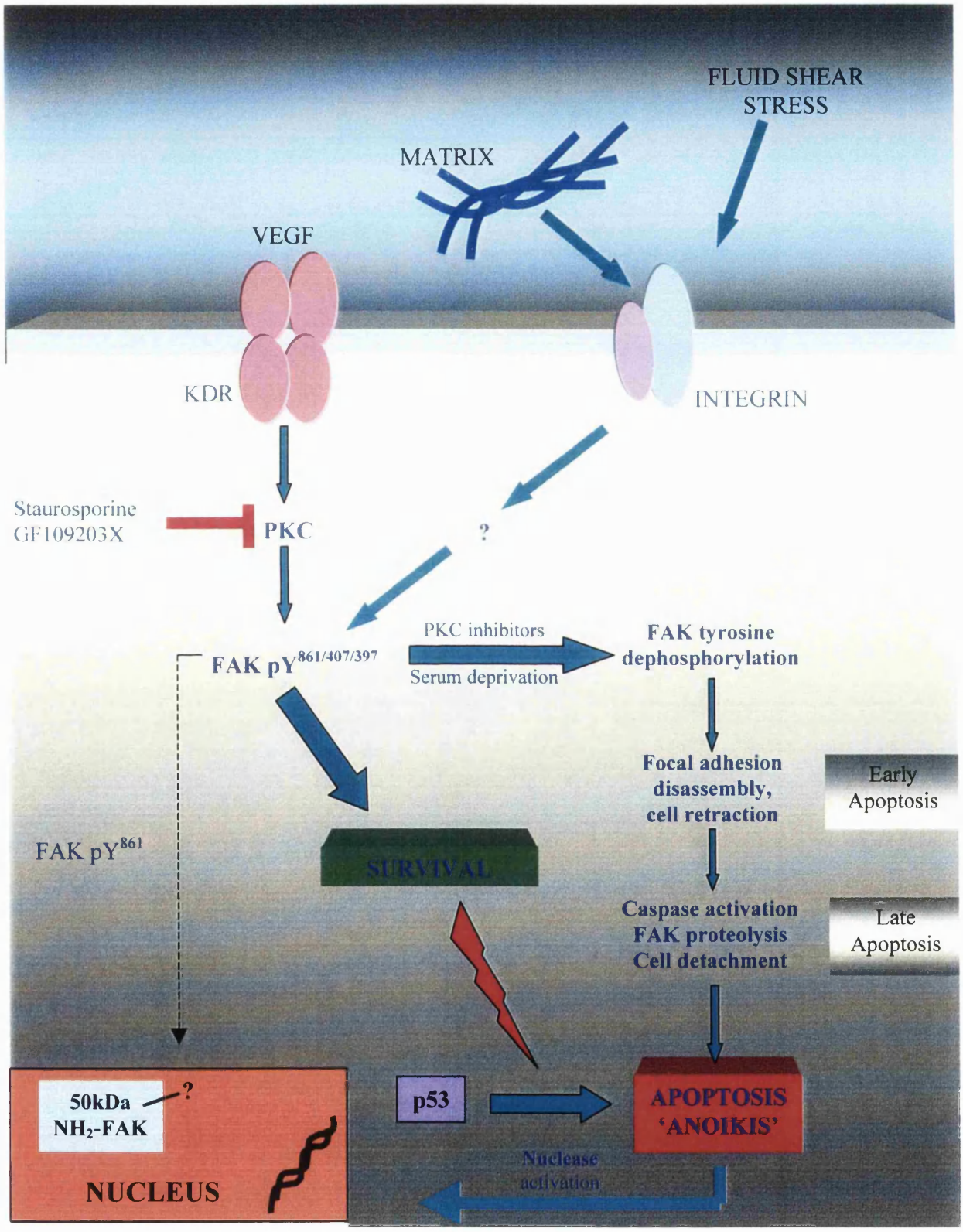


Fig. 8.3 Model for Regulation of FAK Tyrosine Phosphorylation in HUVEC Survival and Apoptosis

Bibliography

ABE, J., TAKAHASHI, M., ISHIDA, M., LEE, J.D. & BERK, B.C. (1997). c-Src is required for oxidative stress-mediated activation of big mitogen-activated protein kinase 1. *Journal of Biological Chemistry* **272**, 20389-20394.

ABEDI, H., DAWES, K.E. & ZACHARY, I. (1995). Differential effects of platelet-derived growth factor BB on p125 focal adhesion kinase and paxillin tyrosine phosphorylation and on cell migration in rabbit aortic vascular smooth muscle cells and Swiss 3T3 fibroblasts. *Journal of Biological Chemistry* **270**, 11367-11376.

ABEDI, H. & ZACHARY, I. (1997). Vascular endothelial growth factor stimulates tyrosine phosphorylation and recruitment to new focal adhesions of focal adhesion kinase and paxillin in endothelial cells. *Journal of Biological Chemistry* **272**, 15442-15451.

ADAM, L.P., FRANKLIN, M.T., RAFF, G.J. & HATHAWAY, D.R. (1995). Activation of mitogen-activated protein kinase in porcine carotid arteries. *Circulation Research* **76**, 183-190.

ADAM, S.A., MARR, R.S. & GERACE, L. (1990). Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. *Journal of Cell Biology* **111**, 807-816.

ALBLAS, J., VAN CORVEN, E.J., HORDIJK, P.L., MILLIGAN, G. & MOOLENAAR, W.H. (1993). Gi-mediated activation of the p21ras-mitogen-activated protein kinase pathway by alpha 2-adrenergic receptors expressed in fibroblasts. *Journal of Biological Chemistry* **268** , 22235-22238.

ALON, T., HEMO, I., ITIN, A., PE'ER, J., STONE, J. & KESHET, E. (1995). Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. *Nature Medicine* **1**, 1024-1028.

ALVAREZ, R.J., GIPS, S.J., MOLDOVAN, N., WILHIDE, C.C., MILLIKEN, EE, HOANG, A.T., HRUBAN, R.H., SILVERMAN, H.S., DANG, C.V. & GOLDSCHMIDT-CLERMONT, P.J. (1997). 17beta-estradiol inhibits apoptosis of endothelial cells. *Biochemical & Biophysical Research Communications* **237**, 372-381.

AMEISAN, J.C. (1994). Programmed cell death (apoptosis) and cell survival regulation: relevance to AIDS and cancer. *AIDS* **8**, 1197-1213.

ANDO, J. & KAMIYA, A. (1996). Flow-dependent regulation of gene expression in vascular endothelial cells. [Review] [40 refs]. *Japanese Heart Journal* **37**, 19-32.

ANDRE, E. & BECKER-ANDRE, M. (1993). Expression of an N-terminally truncated form of human focal adhesion kinase in brain. *Biochemical & Biophysical Research Communications* **190**, 140-147.

ARAKI, S., SHIMADA, Y., KAJI, K. & HAYASHI, H. (1990). Apoptosis of vascular endothelial cells by fibroblast growth factor deprivation [published erratum appears in Biochem Biophys Res Commun 1990 Jun 29;169(3):1248]. *Biochemical & Biophysical Research Communications* **168**, 1194-1200.

ASAHARA, T., BAUTERS, C., PASTORE, C., KEARNEY, M., ROSSOW, S., BUNTING, S., FERRARA, N., SYMES, J.F. & ISNER, J.M. (1995). Local delivery of vascular endothelial growth factor accelerates reendothelialization and attenuates intimal hyperplasia in balloon-injured rat carotid artery [see comments]. *Circulation* **91**, 2793-2801.

ASAOKA, Y., NAKAMURA, S., YOSHIDA, K. & NISHIZUKA, Y. (1992). Protein kinase C, calcium and phospholipid degradation. [Review] [25 refs]. *Trends in Biochemical Sciences* **17**, 414-417.

AVIEZER, D., LEVY, E., SAFRAN, M., SVAHN, C., BUDDECKE, E., SCHMIDT, A., DAVID, G., VLODAVSKY, I. & YAYON, A. (1994). Differential structural requirements of heparin and heparan sulfate proteoglycans that promote binding of basic fibroblast growth factor to its receptor. *J.Biol.Chem.* **269**, 114-121.

AVRAHAM, S., LONDON, R., FU, Y., OTA, S., HIREGOWDARA, D., LI, J., JIANG, S., PASZTOR, L.M., WHITE, R.A. & GROOPMAN, J.E. (1995). Identification and characterization of a novel related adhesion focal tyrosine kinase (RAFTK) from megakaryocytes and brain. *Journal of Biological Chemistry* **270**, 27742-27751.

AVRAHAM, S. & AVRAHAM, H. (1997). Characterization of the novel focal adhesion kinase RAFTK in hematopoietic cells. [Review] [79 refs]. *Leukemia & Lymphoma* **27**, 247-256.

AZMI, T.I. & O'SHEA, J.D. (1984). Mechanism of deletion of endothelial cells during regression of the corpus luteum. *Laboratory Investigation* **51**, 206-217.

BACHMAIER, K., PUMMERER, C., KOZIERADZKI.I., PFEFFER, K., MAK, T.W., NEU, N. & PENNINGER, J.M. (1997). Low-molecular-weight tumour necrosis factor receptor p55 controls induction of autoimmune heart disease. *Circulation* **95**, 655-661.

BALL, R.Y., STOWERS, E.C., BURTON, J.H., CARY, N.R.B., SKEPPER, J.N. & MITCHISON, M.J. (1995). Evidence that death of macrophage foam cells contributes to the lipid core of atheroma. *Atherosclerosis* **114**, 45-54.

BANAI, S., SHWEIKI, D., PINSON, A., CHANDRA, M., LAZAROVICI, G. & KESHET, E. (1994). Upregulation of vascular endothelial growth factor expression induced by myocardial ischaemia: implications for coronary angiogenesis. *Cardiovascular Research* **28**, 1176-1179.

BANSKOTA, N.K., TAUB, R., ZELLNER, K. & KING, G.L. (1989). Insulin, insulin-like growth factor and platelet-derived growth factor interact additively in the induction of the proto-oncogene *c-myc* and cellular proliferation in cultured bovine aortic smooth muscle cells. *Mol.Endocrinol.* **3**, 1183-1190.

BASERGA, R., RESNICOFF, M. & DEWS, M. (1997). The IGF-1 receptor and cancer. *Endocrine* **7**, 99-102.

BAUTERS, C., ASAHARA, T., ZHENG, L.P., TAKESHITA, S., BUNTING, S., FERRARA, N., SYMES, J.F. & ISNER, J.M. (1995). Recovery of disturbed endothelium-dependent flow in the collateral-perfused rabbit ischemic hindlimb after administration of vascular endothelial growth factor [see comments]. *Circulation* **91**, 2802-2809.

BECKERLE, M.C., BURRIDGE, K., DEMARTINO, G.N. & CROALL, D.E. (1987). Colocalisation of calcium-dependent protease II and one of its substrates at sites of cell adhesion. *Cell* **51** , 569-577.

BELLIS, S.L., MILLER, J.T. & TURNER, C.E. (1995). Characterization of tyrosine phosphorylation of paxillin in vitro by focal adhesion kinase. *Journal of Biological Chemistry* **270** , 17437-17441.

BENJAMIN, L.E. & KESHET, E. (1997). Conditional switching of vascular endothelial growth factor (VEGF) expression in tumors: induction of endothelial cell shedding and regression of hemangioblastoma-like vessels by VEGF withdrawal. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 8761-8766.

BENNETT, M.R., ANGLIN, S., MCEWAN, J.R., JAGOE, R., NEWBY, A.C. & EVAN, G.I. (1994). Inhibition of vascular smooth muscle cell proliferation in vitro and in vivo by c-myc antisense oligodeoxynucleotides. *Journal of Clinical Investigation* **93**, 820-828.

BENNETT, M.R., GIBSON, D.F., SCHWARTZ, S.M. & TAIT, J.F. (1995a). Binding and phagocytosis of apoptotic vascular smooth muscle cells is mediated in part by exposure of phosphatidylserine. *Circ.Res.* **77**, 1136-1142.

BENNETT, M.R., EVAN, G.I. & SCHWARTZ, S.M. (1995b). Apoptosis of human vascular smooth cells derived from normal vessels and coronary atherosclerotic plaques. *J.Clin.Invest.* **95**, 2266-2274.

BERG, N.N. & OSTERGAARD, H.L. (1997). T cell receptor engagement induces tyrosine phosphorylation of FAK and Pyk2 and their association with Lck. *Journal of Immunology* **159**, 1753-1757.

BERROU, E., FONTENAY-ROUPIE, M., QUARCK, R., MCKENZIE, F.R., LEVY-TOLEDANO, S., TOBELEM, G. & BRYCKAERT, M. (1996). Transforming growth factor β-1 inhibits mitogen-activated protein kinase induced by basic fibroblast growth factor in smooth muscle cells. *Biochem.J.* **316**, 167-173.

BERRY, V., RATHOD, H., PULMAN, L.B. & DATTA, H.K. (1994). Immunofluorescent evidence for the abundance of focal adhesion kinase in the human and avian osteoclasts and its down regulation by calcitonin. *Journal of Endocrinology* **141**, R11-R15

BIALIK, S., GEENE, D.L., SASSON, I.E., CHENG, R., HORNER, J.W., EVANS, S.M., LORD, E.M., KOCH, C.J. & KITSIS, R.N. (1997). Myocyte apoptosis during acute myocardial infarction in the mouse localises to hypoxic regions but occurs independently of p53. *J.Clin.Invest.* **100**, 1363-1372.

BILATO, C., CURTO, K.A., MONTICONE, R.E., PAULY, R.R., WHITE, A.J. & CROW, M.T. (1997). The inhibition of vascular smooth muscle cell migration by peptide and antibody antagonists of the alphavbeta3 integrin complex is reversed by activated calcium/calmodulin- dependent protein kinase II. *Journal of Clinical Investigation* **100**, 693-704.

BILLINGHAM, M.E. (1987). Cardiac transplant atherosclerosis. *Transplantation Proceedings* **19**, 19-25.

BIRNBAUM, M.J., CLEM, R.J. & MILLER, L.K. (1994). An apoptosis-inhibiting gene from a nuclear polyhedrosis virus encoding a polypeptide with Cys/His sequence motifs. *J.Virol.* **68** , 2521-2528.

BJORKERUD, S. & BJORKERUD, B. (1996). Apoptosis is abundant in human atherosclerotic lesions, especially in inflammatory cells (macrophages and T cells), and may contribute to the accumulation of gruel and plaque instability. *Am.J.Pathol.* **149**, 367-380.

BLENIS, J. (1993). Signal transduction via the MAP kinases: proceed at your own RSK. [Review] [72 refs]. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 5889-5892.

BLOBEL, G. & POTTER, V.R. (1966). Nuclei from rat liver: isolation method that combines purity with high yield. *Science* **154**, 1662-1665.

BOISE, L.H., GONZALEZ-GARCIA, M., POSTEMA, C.E., DING, L., LINDSTEN, T., TURKA, L.A., MAO, X., NUNEZ, G. & THOMPSON, C.B. (1993). BCL-$_x$, a bcl-2 related gene that functions as a dominant receptor of apoptotic cell death. *Cell* **74**, 597-608.

BOLDIN, M.P., GONCHAROV, T.M., GOLTSEV, Y.V. & WALLACH, D. (1996). Involvement of MACH, a novel Mort1/Fadd-interacting protease, on Fas/APO-1-and TNF receptor-induced cell death. *Cell* **85**, 803-815.

BORNFELDT, K.E., GIDLÖF, R.A., WASTESON, A., LAKE, M., SKOTTNER, A. & ARNQVIST, H.J. (1991). Binding and biological effects of insulin, insulin homologues and insulin-like

growth factors in rat aortic smooth muscle cells: comparison of maximal growth-promoting activites. *Diabetologia* **34**, 307-313.

BORNFELDT, K.E., RAINES, E.W., NAKANO, T., GRAVES, L.M., KREBS, E.G. & ROSS, R. (1994a). Insulin-like growth factor-1 and platelet-derived growth factor-BB induce directed migration of human arterial smooth muscle cells via signalling pathways that are distinct from those of proliferation. *J.Clin.Invest.* **93**, 1266-1274.

BORNFELDT, K.E., RAINES, E.W., BURSTEN, S.L., GRAVES, L.M. & ROSS, R. (1994b). Signalling pathways leading to chemotaxis and proliferation of human arterial smooth muscle cells - are they distinct or common? *J.Cell.Biochem.* **265**, 637

BORNFELDT, K.E., CAMPBELL, J.S., KOYAMA, H., ARGAST, G.M., LESLIE, CC, RAINES, E.W., KREBS, E.G. & ROSS, R. (1997). The mitogen-activated protein kinase pathway can mediate growth inhibition and proliferation in smooth muscle cells. Dependence on the availability of downstream targets. *Journal of Clinical Investigation* **100**, 875-885.

BOULTON, T.G., YANCOPOULOS, G.D., GREGORY, J.S., SLAUGHTER, C., MOOMAW, C., HSU, J. & COBB, M.H. (1990). An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. *Science* **249**, 64-67.

BOULTON, T.G., NYE, S.H., ROBBINS, D.J., IP, N.Y., RADZIEJEWSKA, E., MORGENBESSER, S.D., DEPINHO, R.A., PANAYOTATOS, N., COBB, M.H. & YANCOPOULOS, G.D. (1991). ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell* **65**, 663-675.

BOWEN-POPE, D.F., HART, C.E. & SEIFERT, R.A. (1989). Sera and conditioned media contain different isoforms of platelet-derived growth factor (PDGF) which binds to different classes of PDGF receptor. *J.Biol.Chem.* **264**, 2502-2508.

BOYLE, W.J., LAMPERT, M.A., LIPSICK, J.S. & BALUDA, M.A. (1984). Avian myeloblastosis virus and E26 virus oncogene products are nuclear proteins. *Proceedings of the National Academy of Sciences of the United States of America* **81**, 4265-4269.

BRANCOLINI, C., BENEDETTI, M. & SCHNEIDER, C. (1995). Microfilament organisation during apoptosis: the role of Gas2, a possible substrate for ICE-like proteases. *EMBO J.* **14**, 5179-5190.

BROCKDORFF, J., KANNER, S.B., NIELSEN, M., BORREGAARD, N., GEISLER, C., SVEJGAARD & ODUM, N. (1998). Interleukin-2 induces beta2-integrin-dependent signal transduction involving the focal adhesion kinase-related protein B (fakB). *Proceedings of the National Academy of Sciences of the United States of America* **95**, 6959-6964.

BROWN, M.C., PERROTTA, J.A. & TURNER, C.E. (1996). Identification of LIM3 as the principal determinant of paxillin focal adhesion localization and characterization of a novel motif on paxillin directing vinculin and focal adhesion kinase binding. *Journal of Cell Biology* **135**, 1109-1123.

BROWN, T.L., PATIL, S., CIANCI, C.D., MORROW, J.S. & HOWE, P.H. (1999). Transforming growth factor beta induces caspase 3-independent cleavage of alpha II-spectrin (alpha-fodrin) coincident with apoptosis. *J.Biol.Chem.* **274**, 23256-23262.

BURGAYA, F., MENEGON, A., MENEGOZ, M., VALTORTA, F. & GIRAULT, J.A. (1995). Focal adhesion kinase in rat central nervous system. *European Journal of Neuroscience* **7**, 1810-1821.

BURGAYA, F. & GIRAULT, J.A. (1996). Cloning of focal adhesion kinase, pp125FAK, from rat brain reveals multiple transcripts with different patterns of expression. *Brain Research* **Molecular Brain Research. 37**, 63-73.

BURGAYA, F., TOUTANT, M., STUDLER, J.M., COSTA, A., LE BERT, M., GELMAN, M., GIRAULT & JA (1997). Alternatively spliced focal adhesion kinase in rat brain with increased autophosphorylation activity. *Journal of Biological Chemistry* **272**, 28720-28725.

BURGERING, B.M. & COFFER, P.J. (1995). Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction [see comments]. *Nature* **376**, 599-602.

BURGESS, W.H. & MACIAG, T. (1989). The heparin-binding (fibroblast) growth factor family of proteins. *Ann.Rev.Biochem.* **58**, 575-606.

BURNHAM, M.R., HARTE, M.T., RICHARDSON, A., PARSONS, J.T., BOUTON & AH (1996). The identification of p130cas-binding proteins and their role in cellular transformation. *Oncogene* **12**, 2467-2472.

BURRIDGE, K. & CHZRANOWSKA-WODNICKA, M. (1996). Focal adhesions, contractility and signalling. *Annu.Rev.Cell Dev.Biol.* **12**, 463-519.

BUSSO, N., MASUR, S.K., LAZEGA, D., WAXMAN, S. & OSSOWSKI, L. (1994). Induction of cell migration by pro-urokinase binding to its receptor: possible mechanism for signal transduction in human epithelial cells. *Journal of Cell Biology* **126**, 259-270.

BUTTERY, L.D., CHESTER, A.H., SPRINGALL, D.R., BORLAND, J.A., MICHEL, YACOUB, M.H. & POLAK, J.M. (1996). Explanted vein grafts with an intact endothelium demonstrate reduced focal expression of endothelial nitric oxide synthase specific to atherosclerotic sites. *Journal of Pathology* **179**, 197-203.

CAI, W., DEVAUX, B., SCHAPER, W. & SCHAPER, J. (1997). The role of Fas/APO 1 and apoptosis in the development of human atherosclerotic lesions. *Atherosclerosis* **131**, 177-186.

CAI, Z.Z., BETTALIEB, A., EL-MADHANI, N., LEGRES, L.G., STANCOU, R., MASLIAH, J. & CHOUAIB, S. (1997). Alteration of the sphingomyelin/ceramide pathway is assocaited with resistance of human breast carcinoma MCF7 cells to tumour necrosis factor-α-mediated cytotoxicity. *J.Biol.Chem.* **272**, 6918-6926.

CALALB, M.B., POLTE, T.R. & HANKS, S.K. (1995). Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases. *Molecular & Cellular Biology* **15**, 954-963.

CALALB, M.B., ZHANG, X., POLTE, T.R. & HANKS, S.K. (1996). Focal adhesion kinase tyrosine-861 is a major site of phosphorylation by Src. *Biochemical & Biophysical Research Communications* **228**, 662-668.

CAMPBELL, G.R., CAMPBELL, J.H., MANDERSON, J.A., HORRIGAN, S. & RENNICK, R.E. (1988). Arterial smooth muscle. A multifunctional mesenchymal cell. [Review] [93 refs]. *Archives of Pathology & Laboratory Medicine* **112**, 977-986.

CAMPBELL, J.H. & CAMPBELL, G.R. (1993). Culture techniques and their applications to studies of vascular smooth muscle. [Review] [69 refs]. *Clinical Science* **85**, 501-513.

CANTLEY, L.C., AUGER, K.R., CARPENTER, C., DUCKWORTH, B., GRAZIANI, A., CAPELLER, R. & SOLTOFF, S. (1991). Oncogenes and signal transduction. *Cell* **64**, 281-302.

CANTLEY, L.C. & SONGYANG, Z. (1994). Specificity in recognition of phosphopeptides by src-homology 2 domains. *Journal of Cell Science - Supplement* **18**, 121-126.

CARDONE, M.H., SALVESEN, G.S., WIDMANN, C., JOHNSON, G. & FRISCH, S.M. (1997). The regulation of anoikis: MEKK-1 activation requires cleavage by caspases. *Cell* **90**, 315-323.

CARMELIET, P., FERREIRA, V., BREIER, G., POLLEFEYT, S., KIECKENS, GERTSENSTEIN, M., FAHRIG, M., VANDENHOECK, A., HARPAL, K., EBERHARDT, C., DECLERCQ, C., PAWLING, J., MOONS, L., COLLEN, D., RISAU, W. & NAGY, A. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* **380**, 435-439.

CARMELIET, P., NG, Y.S., NUYENS, D., THEILMEIER, G., BRUSSELMANS, CORNELISSEN, I., EHLER, E., KAKKAR, V.V., STALMANS, I., MATTOT, V., PERRIARD, JC, DEWERCHIN, M., FLAMENG, W., NAGY, A., LUPU, F., MOONS, L., COLLEN, D., D'A, PA & SHIMA, D.T. (1999a). Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188 [see comments]. *Nature Medicine* **5**, 495-502.

CARMELIET, P., LAMPUGNANI, M.G., MOONS, L., BREVIARIO, F., COMPERNOLLE, V., BONO, F., BALCONI, G., SPAGNUOLO, R., OOSTUYSE, B., DEWERCHIN, M., ZANETTI, A., ANGELLILO, A., MATTOT, V., NUYENS, D., LUTGENS, E., CLOTMAN, F., DE, RUITER, M.C., GITTENBERGER-DE, G.A., POELMANN, R., LUPU, F., HERBERT, J.M., COLLEN, D. & DEJANA, E. (1999b). Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell* **98**, 147-157.

CARO, C.G., FITZ-GERALD, J.M. & SCHROTER, R.C. (1971). Atheroma and arterial wall shear. Observation, correlation and proposal of a shear dependent mass transfer mechanism for atherogenesis. *Proceedings of the Royal Society of London - Series B: Biological Sciences* **177**, 109-159.

CARRAGHER, N.O., LEVKAU, B., ROSS, R. & RAINES, E.W. (1999). Degraded collagen fragments promote rapid disassembly of smooth muscle focal adhesions that correlates with cleavage of pp125(FAK), paxillin, and talin. *Journal of Cell Biology* **147**, 619-630.

CARY, L.A., CHANG, J.F. & GUAN, J.L. (1996). Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn. *Journal of Cell Science* **109**, 1787-1794.

CARY, L.A., HAN, D.C., POLTE, T.R., HANKS, S.K. & GUAN, J.L. (1998). Identification of p130Cas as a mediator of focal adhesion kinase-promoted cell migration. *Journal of Cell Biology* **140**, 211-221.

CASAMASSIMA, A. & ROZENGURT, E. (1997). Tyrosine phosphorylation of p130(cas) by bombesin, lysophosphatidic acid, phorbol esters, and platelet-derived growth factor. Signaling pathways and formation of a p130(cas)-Crk complex. *Journal of Biological Chemistry* **272**, 9363-9370.

CELERMAJER, D.S., SORENSEN, K.E., GEORGAKOPOULOS, D., BULL, C., THOMAS, O., ROBINSON, J. & DEANFIELD, J.E. (1993). Cigarette smoking is associated with dose-related and potentially reversible impairment of endothelium-dependent dilation in healthy young adults. *Circulation* **88**, 2149-2155.

CHAMLEY-CAMPBELL, J.H. & CAMPBELL, G.R. (1981). What controls smooth muscle phenotype? *Atherosclerosis* **40**, 347-357.

CHAMLEY-CAMPBELL, J.H., CAMPBELL, G.R. & ROSS, R. (1981). Phenotype-dependent response of cultured aortic smooth muscle to serum mitogens. *Journal of Cell Biology* **89**, 379-383.

CHANG, M.W., BARR, E., LU, M.M., BARTON, K. & LEIDEN, J.M. (1995a). Adenovirus-mediated over-expression of the cyclin/cyclin-dependent kinase inhibitor, p21 inhibits vascular smooth muscle cell proliferation and neointima formation in the rat carotid artery model of balloon angioplasty. *Journal of Clinical Investigation* **96**, 2260-2268.

CHANG, M.W., BARR, E., SELTZER, J., JIANG, Y.Q., NABEL, G.J., NABEL, EG, PARMACEK, M.S. & LEIDEN, J.M. (1995b). Cytostatic gene therapy for vascular proliferative disorders with a constitutively active form of the retinoblastoma gene product. *Science* **267**, 518-522.

CHATTERJEE & , S. (1998). Sphingolipids in Atherosclerosis and Vascular Biology. *Arterioscler.Thromb.Vasc.Biol.* **18**, 1523-1533.

CHEN, C.S., MRKSICH, M., HUANG, S., WHITESIDES, G.M. & INGBER, D.E. (1997). Geometric control of cell life and death. *Science* **276**, 1425-1428.

CHEN, H.C., APPEDDU, P.A., ISODA, H. & GUAN, J.L. (1996). Phosphorylation of tyrosine 397 in focal adhesion kinase is required for binding phosphatidylinositol 3-kinase. *Journal of Biological Chemistry* **271**, 26329-26334.

CHEN, Q., KINCH, M.S., LIN, T.H., BURRIDGE, K. & JULIANO, R.L. (1994). Integrin-mediated cell adhesion activates mitogen-activated protein kinases. *Journal of Biological Chemistry* **269**, 26602-26605.

CHENG, W., KAJSTURA, J., LI, P., WOLIN, M.S., SONNENBLICK, E.H., HINTZE, T.H., OLIVETTI, G. & ANVERSA, P. (1995). Stretch-induced programmed myocyte cell death. *J.Clin.Invest.* **96**, 2247-2259.

CHINNAIYAN, A.M., O'ROURKE, K., TEWARI, M. & DIXIT, V.M. (1995). FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell* **81**, 505-512.

CINES, D.B., POLLAK, E.S., BUCK, C.A., LOSCALZO, J., ZIMMERMAN, G.A., MCEVER, R.P., POBER, J.S., WICK, T.M., KONKLE, B.A., SCHWARTZ, B.S., BARNATHAN, E.S., MCCRAE, K.R., HUG, B.A., SCHMIDT, A.M. & STERN, D.M. (1998). Endothelial cells in physiology and in the pathophysiology of vascular disorders. [Review] [467 refs]. *Blood* **91**, 3527-3561.

CLARK, E.A. & BRUGGE, J.S. (1995). Integirns and signal transduction pathways: the road taken. *Science* **268**, 233-239.

CLARKE, P.G.H. & CLARKE, S. (1996). Nineteenth century research on naturally occurring cell death and related phenomena. *Anat.Embroyol.* **193**, 81-99.

CLAUSS, M., GERLACH, M., GERLACH, H., BRETT, J., WANG, F., FAMILLETTI, P.C., PAN, Y.C., OLANDER, J.V., CONNOLLY, D.T. & STERN, D. (1990). Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration. *Journal of Experimental Medicine* **172**, 1535-1545.

CLEARY, M.L., SMITH, S.D. & SKLAR, J. (1986). Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation. *Cell* **47**, 19-28.

CLEMMONS, D.R. & VAN WYK, J.J. (1985). Evidence for a functional role of endogenously produced somatomedin-like peptides in the regulation of DNA synthesis in cultured human fibroblasts and porcine smooth muscle cells. *J.Clin.Invest.* **75**, 1914-1918.

CLOWES, A.W., REIDY, M.A. & CLOWES, M.M. (1983). Kinetics of cellular proliferation after arterial injury. I. Smooth muscle growth in the absence of endothelium. *Laboratory Investigation* **49**, 327-333.

CLYMAN, R.I., MAURAY, F. & KRAMER, R.H. (1992). Beta 1 and beta 3 integrins have different roles in the adhesion and migration of vascular smooth muscle cells on extracellular matrix. *Experimental Cell Research* **200**, 272-284.

COBB, B.S., SCHALLER, M.D., LEU, T.H. & PARSONS, J.T. (1994). Stable association of pp60src and pp59fyn with the focal adhesion-associated protein tyrosine kinase, pp125FAK. *Molecular & Cellular Biology* **14**, 147-155.

CONNOLLY, D.T., OLANDER, J.V., HEUVELMAN, D., NELSON, R., MONSELL, SIEGEL, N., HAYMORE, B.L., LEIMGRUBER, R. & FEDER, J. (1989). Human vascular permeability factor. Isolation from U937 cells. *Journal of Biological Chemistry* **264**, 20017-20024.

COOKE, J.P. (1996). Role of nitric oxide in progression and regression of atherosclerosis. [Review] [52 refs]. *Western Journal of Medicine* **164**, 419-424.

COORAY, P., YUAN, Y., SCHOENWAELDER, S.M., MITCHELL, C.A., SALEM, H.H. & JACKSON, S.P. (1996). Focal adhesion kinase (pp125FAK) cleavage and regulation by calpain. *Biochemical Journal* **318**, 41-47.

COSPEDAL, R., ABEDI, H. & ZACHARY, I. (1999). Platelet-derived growth factor-BB (PDGF-BB) regulation of migration and focal adhesion kinase phosphorylation in rabbit aortic vascular smooth muscle cells: roles of phosphatidylinositol 3-kinase and mitogen-activated protein kinases. *Cardiovascular Research* **41**, 708-721.

COWLEY, S., PATERSON, H., KEMP, P. & MARSHALL, C.J. (1994). Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. *Cell* 77, 841-852.

CREAGER, M.A., GALLAGHER, S.J., GIRERD, X.J., COLEMAN, S.M., DZAU, VJ & COOKE, J.P. (1992). L-arginine improves endothelium-dependent vasodilation in hypercholesterolemic humans. *Journal of Clinical Investigation* **90**, 1248-1253.

CROALL, D.E. & DEMARTINO, G.N. (1991). Calcium-activated neutral protease (calpain) system: structure, function and regulation. *Physiol.Rev.* **71**, 813-847.

CROUCH, D.H., FINCHAM, V.J. & FRAME, M.C. (1996). Targeted proteolysis of the focal adhesion kinase pp125 FAK during c-MYC-induced apoptosis is suppressed by integrin signalling. *Oncogene* **12**, 2689-2696.

CRYNS, V.L., BERGERON, L., ZHU, H., LI, H. & YUAN, J. (1996). Specific cleavage of α-fodrin during FAS- and tumour necrosis factor-induced apoptosis is mediated by interleukin-1β–converting enzyme/CED-3 protease distinct from the poly(ADP-ribose) polymerase protease. *J.Biol.Chem.* **271**, 31277-31282.

CUGNO, M., UZIEL, L., FABRIZI, I., BOTTASSO, B., MAGGIOLINI, F. & AGOSTONI, A. (1989). Fibrinolytic response in normal subjects to venous occlusion and DDAVP infusion. *Thrombosis Research* **56**, 625-634.

CUVILLIER, O., PIRIANOV, G., KLEUSER, B., VANEK, P.G., COSO, O.A., GUTKIND, J.S. & SPIEGEL, S. (1996). Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. *Nature* 800-803.

DANKER, K., GABRIEL, B., HEIDRICH, C. & REUTTER, W. (1998). Focal adhesion kinase pp125FAK and the beta 1 integrin subunit are constitutively complexed in HaCaT cells. *Experimental Cell Research* **239**, 326-331.

DARTSCH, P.C., BAURIEDEL, G., SCHINKO, I., WEISS, H.D., HOFLING, B. & BETZ, E. (1989). Cell constitution and characteristics of human atherosclerotic plaques selectively removed by percutaneous atherectomy. *Atherosclerosis* **80**, 149-157.

DATTA, S.R., DUDEK, H., TAO, X., MASTERS, S., FU, H., GOTOH, Y. & GREENBERG, M.E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* **91**, 231-241.

DAVIES, C.H., DAVIA, K., BENNETT, G.J., PEPPER, J.R., POOLE-WILSON, P.A. & HARDING, S.E. (1995). Reduced contraction and altered frequency response of isolated ventricular myocytes from patients with heart failure. *Circulation* **92**, 2540-2549.

DAVIES, M.J. & THOMAS, A. (1984). Thrombosis and acute coronary-artery lesions in sudden cardiac ischemic death. *New England Journal of Medicine* **310**, 1137-1140.

DAVIES, P.F., REMUZZI, A., GORDON, E.J., DEWEY, C.F.J. & GIMBRONE, M.A., Jr. (1986). Turbulent fluid shear stress induces vascular endothelial cell turnover in vitro. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 2114-2117.

DAVIS, R.J. (1993). The mitogen-activated protein kinase signal transduction pathway. [Review] [55 refs]. *Journal of Biological Chemistry* **268**, 14553-14556.

DBAIBO, G.S., OBEID, L.M. & HANNUN, Y.A. (1993). Tumour necrosis factor-α (TNF-α) signal transduction through ceramide - dissociation of growth inhibitory effects of TNF-α from activation of nuclear factor κ-B. *J.Biol.Chem.* **268**, 17762-17766.

DE CATERINA, R., LIBBY, P., PENG, H.B., THANNICKAL, V.J., RAJAVASHISTH, T.B., GIMBRONE, M.A.J., SHIN, W.S. & LIAO, J.K. (1995). Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *Journal of Clinical Investigation* **96**, 60-68.

DE VRIES, C., ESCOBEDO, J.A., UENO, H., HOUCK, K., FERRARA, N. & WILLIAMS, L.T. (1992). The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* **255**, 989-991.

DEL ROSSO, M., ANICHINI, E., PEDERSEN, N., BLASI, F., FIBBI, G., PUCCI, M. & RUGGIERO, M. (1993). Urokinase-urokinase receptor interaction: non-mitogenic signal transduction in human epidermal cells. *Biochemical & Biophysical Research Communications* **190**, 347-352.

DELAFONTAINE, P., LOU, H. & ALEXANDER, R.W. (1991). Insulin-like growth factor 1 gene expression in vascular cells. *Hypertension* **18**, 742-747.

DELAFONTAINE, P. & LOU, H. (1993). Angiotensin 11 regulates insulin-like growth factor expression in vascular cells. *J.Biol.Chem.* **268**, 16866-16870.

DEWEY, C.F.J., BUSSOLARI, S.R., GIMBRONE, M.A.J. & DAVIES, P.F. (1981). The dynamic response of vascular endothelial cells to fluid shear stress. *Journal of Biomechanical Engineering* **103**, 177-185.

DEWEY, C.F., Jr. (1984). Effects of fluid flow on living vascular cells. *Journal of Biomechanical Engineering* **106**, 31-35.

DICORLETO, P.E. & BOWEN-POPE, D.F. (1983). Cultured endothelial cells produce a platelet-derived growth factor-like protein. *Proc.Natl.Acad.Sci.U.S.A.* **80**, 1919-1923.

DIMMELER, S., HAENDELER, J., RIPPMANN, V., NEHLS, M. & ZEIHER, A.M. (1996). Shear stress inhibits apoptosis of human endothelial cells. *FEBS Letters* **399**, 71-74.

DIMMELER, S., HAENDELER, J., NEHLS, M. & ZEIHER, A.M. (1997a). Suppression of apoptosis by nitric oxide via inhibition of interleukin-1beta-converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases. *Journal of Experimental Medicine* **185**, 601-607.

DIMMELER, S., HAENDELER, J., NEHLS, M. & ZEIHER, A.M. (1997b). Oxidized low-density lipoprotein induces apoptosis of human endothelial cells by activation of CPP32-like proteases: a mechanistic clue to the "response to injury" hypothesis. *Circulation* **95**, 1760-1763.

DISATNIK, M.H. & RANDO, T.A. (1999). Integrin-mediated muscle cell spreading. The role of protein kinase c in outside-in and inside-out signaling and evidence of integrin cross-talk. *Journal of Biological Chemistry* **274**, 32486-32492.

DORMANDY, T.L. (1989). Free radical pathology and medicine. A review. *J.R.Coll.Phys.Lond.* **23**, 221-227.

DRAKE, T.A., MORRISSEY, J.H. & EDGINGTON, T.S. (1989). Selective cellular expression of tissue factor in human tissues. Implications for disorders of hemostasis and thrombosis. *American Journal of Pathology* **134**, 1087-1097.

DUMLER, I., PETRI, T. & SCHLEUNING, W.D. (1993). Interaction of urokinase-type plasminogenactivator (u-PA) with its cellular receptor (u-PAR) induces phosphorylation on tyrosine of a 38 kDa protein. *FEBS Letters* **322**, 37-40.

DUMLER, I., WEIS, A., MAYBORODA, O.A., MAASCH, C., JERKE, U., HALLER, H. & GULBA, D.C. (1998). The Jak/Stat pathway and urokinase receptor signaling in human aortic vascular smooth muscle cells. *Journal of Biological Chemistry* **273**, 315-321.

EIDE, B.L., TURCK, C.W. & ESCOBEDO, J.A. (1995). Identification of Tyr-397 as the primary site of tyrosine phosphorylation and pp60src association in the focal adhesion kinase, pp125FAK. *Molecular & Cellular Biology* **15**, 2819-2827.

ELLIS, P.A., SMITH, I.E. & DOWSETT, M. (1996). Apoptosis: its role in tumour growth and therapy. *Cytopathology* 7, 201-203.

ENARI, M., TALANIAN, R.V.W.W.W. & NAGATA, S. (1996). Sequential actvation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. *Nature* **380**, 723-726.

ESCARGUEIL-BLANC, I., MEILHAC, O., PIERAGGI, M.T., ARNAL, J.F., SALVAYRE, R. & NEGRE-SALVAYRE, A. (1997). Oxidized LDLs induce massive apoptosis of cultured human endothelial cells through a calcium-dependent pathway:prevention by aurintricarboxylic acid. *Arterioscler.Thromb.Vasc.Biol.* **17**, 331-339.

FABRICANT, C.G., FABRICANT, J., MINICK, C.R. & LITRENTA, M.M. (1983). Herpesvirus-induced atherosclerosis in chickens. *Fed.Proc.* **42**, 2476-2479.

FADOK, V.A., SAVILL, J.S., HASLETT, C., BRATTON, D.L., DOHERTY, D.E., CAMPBELL, P.A. & HENSON, PM. (1992). Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognise and remove apoptotic cells. *J.Immunol.* **149**, 4029-4035.

FAGGIOTTO, A., ROSS, R. & HARKER, L. (1984a). Studies of hypercholesterolemia in the non-human primate:1. Changes that lead to fatty streak formation. *Arteriosclerosis* **4**, 323-340.

FAGGIOTTO, A. & ROSS, R. (1984b). Studies of hypercholesterolemia in the non-human primate:11. Fatty streak conversion to fibrous plaque. *Arteriosclerosis* **4**, 341-356.

FEINBERG, R.N., LATKER, C.H. & BEEBE, D.C. (1986). Localized vascular regression during limb morphogenesis in the chicken embryo. I. Spatial and temporal changes in the vascular pattern. *Anatomical Record* **214**, 405-409.

FERNANDES-ALNEMRI, T., ARMSTRONG, R.C., KREBS, J., SRINIVASULA, S.M., WANG, L., BULLRICH, F., FRITZ, L.C., TRAPANI, J.A., TOMASELLI, K.J., LITWACK, G. & ALNEMRI, E.S. (1996). In vitro activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. *Proc.Natl.Acad.Sci.U.S.A.* **93**, 7464-7469.

FERNS, G.A.A., RAINES, E.W., SPRUGEL, K.H., MOTAIN, A.S., REIDY, M.A. & ROSS, R. (1991). Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF. *Science* **253**, 1129-1132.

FERNS, G.A.A., STEWART-LEE, A.L. & ANGGARD, E.E. (1992). Arterial responses to mechanical injury: balloon catheter de-endothelialisation. *Atherosclerosis* **92**, 89-104.

FERRARA, N., HOUCK, K., JAKEMAN, L. & LEUNG, D.W. (1992). Molecular and biological properties of the vascular endothelial growth factor family of proteins. [Review] [171 refs]. *Endocrine Reviews* **13**, 18-32.

FERRARA, N., CARVER-MOORE, K., CHEN, H., DOWD, M., LU, L., O'S, KS, POWELL-BRAXTON, L., HILLAN, K.J. & MOORE, M.W. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* **380**, 439-442.

FISCHER, U., HUBER, J., BOELENS, W.C., MATTAJ, I.W. & LUHRMANN, R. (1995). The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. *Cell* **82**, 475-483.

FLISS, H. & GATTINGER, D. (1996). Apoptosis in ischaemic and reperfused rat myocardium. *Circ.Res.* **79**, 949-956.

FLYNN, P.D., BYRNE, C.D.B.T.P., WEISSBERG, P.L. & BENNETT, M.R. (1997). Thrombin generation by apoptotic vascular smooth cells. *Blood* **89**, 4378-4384.

FRIEDLANDER, P., HAUPT, Y., PRIVES, C. & OREN, M. (1996). A mutant p53 that discriminates between p53-responsive genes cannot induce apoptosis. *Molecular & Cellular Biology* **16** , 4961-4971.

FRISCH, S.M., VUORI, K., RUOSLAHTI, E. & CHAN-HUI, P.Y. (1996). Control of adhesion-dependent cell survival by focal adhesion kinase. *Journal of Cell Biology* **134**, 793-799.

FRITZ, K.E., JARMOLYCH, J. & DAOUD, A.S. (1970). Association of DNA synthesis and apparent dedifferentiation of aortic smooth muscle cells in vitro. *Experimental & Molecular Pathology* **12**, 354-362.

FRY, D.L. (1968). Acute vascular endothelial changes associated with increased blood velocity gradients. *Circulation Research* **22**, 165-197.

FUJIMOTO, J., SAWAMOTO, K., OKABE, M., TAKAGI, Y., TEZUKA, T., YOSHIKAWA, S., RYO, OKANO, H. & YAMAMOTO, T. (1999). Cloning and characterization of Dfak56, a homolog of focal adhesion kinase, in Drosophila melanogaster. *Journal of Biological Chemistry* **274** , 29196-29201.

FUJITA, H., KAMIGUCHI, K., CHO, D., SHIBANUMA, M., MORIMOTO, C. & TACHIBANA, K. (1998). Interaction of Hic-5, A senescence-related protein, with focal adhesion kinase. *Journal of Biological Chemistry* **273**, 26516-26521.

FURUTA, Y., ILIC, D., KANAZAWA, S., TAKEDA, N., YAMAMOTO, T. & AIZAWA, S. (1995). Mesodermal defect in late phase of gastrulation by a targeted mutation of focal adhesion kinase, FAK. *Oncogene* **11**, 1989-1995.

GABBIANI, G., KOCHER, O., BLOOM, W.S., VANDEKERCKHOVE, J. & WEBER (1984). Actin expression in smooth muscle cells of rat aortic intimal thickening, human atheromatous plaque, and cultured rat aortic media. *Journal of Clinical Investigation* **73**, 148-152.

GARG, U.C. & HASSID, A. (1989). Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *Journal of Clinical Investigation* **83**, 1774-1777.

GENG, G.Y. & LIBBY, P. (1995). Evidence for apoptosis in advanced human atheroma. Colocalisation with interleukin-1β converting enzyme. *Am.J.Pathol.* **147**, 251-266.

GENG, G.Y., HENDERSON, L.E., LEVESQUE, E.B., MUSZYNSKI, M. & LIBBY, P. (1997). Fas is expressed in human atherosclerotic intima and promotes apoptosis of cytokine-primed human vascular smooth muscle cells. *Arterioscler.Thromb.Vasc.Biol.* **17**, 2200-2208.

GEORGE, E.L., GEORGES-LABOUESSE, E.N., PATEL-KING, R.S., RAYBURN, H. & HYNES, R.O. (1993). Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. *Development* **119**, 1079-1091.

GERBER, H.P., DIXIT, V. & FERRARA, N. (1998a). Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. *Journal of Biological Chemistry* **273**, 13313-13316.

GERBER, H.P., MCMURTREY, A., KOWALSKI, J., YAN, M., KEYT, B.A., DIXIT, V. & FERRARA, N. (1998b). Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *Journal of Biological Chemistry* **273**, 30336-30343.

GERVAIS, F.G., THORNBERRY, N.A., RUFFOLO, S.C., NICHOLSON, D.W. & ROY, S. (1998). Caspases cleave focal adhesion kinase during apoptosis to generate a FRNK-like polypeptide. *Journal of Biological Chemistry* **273**, 17102-17108.

GILMORE, A.P. & ROMER, L.H. (1996). Inhibition of focal adhesion kinase (FAK) signaling in focal adhesions decreases cell motility and proliferation. *Molecular Biology of the Cell* **7**, 1209-1224.

GIMBRONE, M.A., Jr. (1976). Culture of vascular endothelium. [Review] [193 refs]. *Progress in Hemostasis & Thrombosis* **3**, 1-28.

GLAGOV, S. (1972). Haemodynamic risk factors: mechanical stress, mural architecture, medial nutrition and the vulnerability of arteries to atherosclerosis. In *The pathogenesis of atherosclerosis.*, eds. WISSLER, R.W. & GEER, J.C., pp. 164-199. Baltimore: Williams and Watkins.

GLAGOV, S., ZARINS, C., GIDDENS, D.P. & KU, D.N. (1988). Hemodynamics and atherosclerosis. Insights and perspectives gained from studies of human arteries. [Review] [160 refs]. *Archives of Pathology & Laboratory Medicine* **112**, 1018-1031.

GLENNEY, J.R.J. & ZOKAS, L. (1989). Novel tyrosine kinase substrates from Rous sarcoma virus-transformed cells are present in the membrane skeleton. *Journal of Cell Biology* **108**, 2401-2408.

GLUCKSMANN, A. (1951). Cell death in normal vertebrate ontogeny. *Biol.Rev.* **26**, 5986

GOBE, G., BROWNING, J., HOWARD, T., HOGG, N., WINTERFORD, C. & CROSS, R. (1997). Apoptosis occurs in endothelial cells during hypertension-induced microvascular rarefaction. *Journal of Structural Biology* **118**, 63-72.

GOBE, G.C., BUTTYAN, R., WYBURN, K.R., ETHERIDGE, M.R. & SMITH, P.J. (1995). Clusterin expression and apoptosis in tissue remodeling associated with renal regeneration. *Kidney International* **47**, 411-420.

GOLDEN, M.A., AU, Y.P.T., KIRKMAN, T.R., WILCOX, J.N., RAINES, E.W., ROSS, R. & CLOWES, A.W. (1991). Platelet-derived growth factor activity and mRNA expression in healing vascular grafts in baboons. *J.Clin.Invest.* **87**, 406-414.

GOLL, D.E., THOMPSON, V.F., TAYLOR, R.G. & ZALEWSKA, T. (1992). Is calpain activity regulated by membranes and autolysis or by calcium and calpastatin? *Bioessays* **14**, 549-556.

GOLUBEV, A.G. (1996). Accidental necessity, initiation of transcription, cell differentiation, and necessary accidents. *Biochemistry-Moscow* **61**, 928-938.

GORSKI, D.H. & WALSH, K. (1995). Mitogen-responsive nuclear factors that mediate growth control signals in vascular myocytes. [Review] [114 refs]. *Cardiovascular Research* **30**, 585-592.

GOSPODAROWICZ, D., FERRARA, N., HAAPARANTA, T. & NEUFELD, G. (1988). Basic fibroblast growth factor: expression in cultured bovine vascular smooth muscle cells. *Eur.J.Cell Biol.* **46**, 144-151.

GOSPODAROWICZ, D., ABRAHAM, J.A. & SCHILLING, J. (1989). Isolation and characterization of a vascular endothelial cell mitogen produced by pituitary-derived folliculo stellate cells. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 7311-7315.

GOTTLIEB, R.A., BURLESON, K.O., KLONER, R.A., BABIOR, B.M. & ENGLER, R.L. (1994). Repurfusion injury induces apoptosis in rabbit cardiomyocytes. *J.Clin.Invest.* **94**, 1621-1628.

GRANT, S.G., KARL, K.A., KIEBLER, M.A. & KANDEL, E.R. (1995). Focal adhesion kinase in the brain: novel subcellular localization and specific regulation by Fyn tyrosine kinase in mutant mice. *Genes & Development* **9**, 1909-1921.

GRAVES, L.M., BORNFELDT, K.E., RAINES, E.W., POTTS, B.C., MACDONALD, S.G., ROSS, R. & KREBS, E.G. (1993). Protein kinase A antagonizes platelet-derived growth factor-induced signalling by mitogen-activated protein kinase in human arterial smooth muscle cells. *Proc.Natl.Acad.Sci.U.S.A.* **90**, 10300-10304.

GRAYSTON, J.T. (1993). Chlamydia in atherosclerosis [editorial; comment]. *Circulation* **87**, 1408-1409.

GRONWALD, R.G.K., GRANT, F.J., HALDEMAN, B.A., HART, C.E., OHARA, P.J., HAGEN, F.S., ROSS, R., BOWEN-POPE, D.F. & MURRAY, M.J. (1988). Cloning and expression of a cDNA coding for the human platelet-derived growth factor receptor - evidence for more than one receptor class. *Proc.Natl.Acad.Sci.U.S.A.* **85**, 3435-3439.

GUAN, J.L. & SHALLOWAY, D. (1992). Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. *Nature* **358**, 690-692.

GUINEBAULT, C., PAYRASTRE, B., RACAUD-SULTAN, C., MAZARGUIL, H., BRETON, M., MAUCO, PLANTAVID, M. & CHAP, H. (1995). Integrin-dependent translocation of phosphoinositide 3-kinase to the cytoskeleton of thrombin-activated platelets involves specific interactions of p85 alpha with actin filaments and focal adhesion kinase. *Journal of Cell Biology* **129**, 831-842.

GYETKO, M.R., TODD, R.F., WILKINSON, C.C. & SITRIN, R.G. (1994). The urokinase receptor is required for human monocyte chemotaxis in vitro. *Journal of Clinical Investigation* **93**, 1380-1387.

HAMET, P., RICHARD, L., DAM, T.V., TEIGER, E., ORLOV, S.N., GABOURY, L., GOSSARD, F. & TREMBLAY, J. (1995). Apoptosis in target organs of hypertension. *Hypertension* **26**, 642-648.

HAN, D.K., HAUDENSCHILD, C.C., HONG, M.K., TINKLE, B.T., LEON, M.B. & LIAU, G. (1995). Evidence for apoptosis in human atherogenesis and in a rat vascular injury model. *Am.J.Pathol.* **147**, 267-277.

HAN, J., LEE, J.D., BIBBS, L. & ULEVITCH, R.J. (1994). A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* **265**, 808-811.

HANKS, S.K., CALALB, M.B., HARPER, M.C. & PATEL, S.K. (1992). Focal adhesion protein-tyrosine kinase phosphorylated in response to cell attachment to fibronectin. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 8487-8491.

HART, C.E., FORSTROM, J.W., KELLY, J.D., SEIFERT, R.A., SMITJ, R.A., ROSS, R., MURRAY, M.J. & BOWEN-POPE, D.F. (1988). 2 classes of PDGF receptor recognise different isoforms of PDGF. *Science* **240**, 1529-1531.

HARTE, M.T., HILDEBRAND, J.D., BURNHAM, M.R., BOUTON, A.H. & PARSONS, J.T. (1996). p130Cas, a substrate associated with v-Src and v-Crk, localizes to focal adhesions and binds to focal adhesion kinase. *Journal of Biological Chemistry* **271**, 13649-13655.

HAYRY, P., MYLLARNIEMI, M., AAVIK, E., ALATALO, S., AHO, P., YILMAZ, S., RAISANEN-SOKOLOWSKI, A., COZZONE, G., JAMESON, B.A. & BASERGA, R. (1995). Stable D-peptide analogue of insulin-like growth factor-1 inhibits smooth muscle cell proliferation after carotid balloon injury in the rat. *FASEB J.* **9**, 1336-1344.

HAZLERIGG, D.G., THOMPSON, M., HASTINGS, M.H. & MORGAN, P.H. (1996). Regulation of mitogen-activated protein kinase in the pars tuberalis of the ovine pituitary: interactions between melatonin, insulin-like growth factor-1 and forskolin. *Endocrinology* **137**, 210-218.

HEDIN, U., THYBERG, J., ROY, J., DUMITRESCU, A. & TRAN, P.K. (1997). Role of tyrosine kinases in extracellular matrix-mediated modulation of arterial smooth muscle cell phenotype. *Arteriosclerosis, Thrombosis & Vascular Biology* **17**, 1977-1984.

HENNEY, A.M., WAKELEY, P.R., DAVIES, M.J., FOSTER, K., HEMBRY, R., MURPHY, G. & HUMPHRIES, S. (1991). Localization of stromelysin gene expression in atherosclerotic plaques by in situ hybridization. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 8154-8158.

HENRY, P.D. (1994). Hyperlipidemic endothelial injury and angiogenesis. [Review] [53 refs]. *Basic Research in Cardiology* **89 Suppl 1**, 107-114.

HERNANDEZ-SANCHEZ, C., BLAKESLEY, V., KALEBIC, T., HELMAN, L. & LEROITH, D. (1995). The role of the tyrosine kinase domain of the insulin-like growth factor-1 receptor in intracellular signalling, cellular proliferation and tumorigenesis. *J.Biol.Chem.* **270**, 29176-29181.

HERREN, B., LEVKAU, B., RAINES, E.W. & ROSS, R. (1998). Cleavage of β-Catenin and plakglobin and shedding of VE-Cadherin during endothelial apoptosis: evidence for a role of caspases and metalloproteinases. *Mol.Biol.Cell* **9**, 1589-1601.

HILDEBRAND, J.D., SCHALLER, M.D. & PARSONS, J.T. (1993). Identification of sequences required for the efficient localization of the focal adhesion kinase, pp125FAK, to cellular focal adhesions. *Journal of Cell Biology* **123**, 993-1005.

HILDEBRAND, J.D., SCHALLER, M.D. & PARSONS, J.T. (1995). Paxillin, a tyrosine phosphorylated focal adhesion-associated protein binds to the carboxyl terminal domain of focal adhesion kinase. *Molecular Biology of the Cell* **6**, 637-647.

HIRAI, S., KAWASAKI, H., YANIV, M. & SUZUKI, K. (1991). Degradation of transcription factors c-jun and c-fos by calpain. *FEBS.Lett.* **287**, 57-61.

HIRATA, K., KIKUCHI, A., SASAKI, T., KURODA, S., KAIBUCHI, K., MATSUURA, Y., SEKI, H., SAIDA, K. & TAKAI, Y. (1992). Involvement of rho p21 in the GTP-enhanced calcium ion sensitivity of smooth muscle contraction. *Journal of Biological Chemistry* **267**, 8719-8722.

HIRSH, J., SALZMAN, E.W., MARDER, V.J. & COLMAN, R.W. (1994). Overview of the thrombotic process and its therapy. In *Hemostasis and Thrombosis: Basic Principles.*, eds. COLMAN, R.W., HIRSH, J., MARDER, V.J. & SALZMAN, E.W., pp. 1151Philadelphia: Lippincott.

HONDA, H., ODA, H., NAKAMOTO, T., HONDA, Z., SAKAI, R., SUZUKI, T., SAITO, T., NAKAMURA, K., NAKAO, K., ISHIKAWA, T., KATSUKI, M., YAZAKI, Y. & HIRAI, H. (1998). Cardiovascular anomaly, impaired actin bundling and resistance to Src-induced transformation in mice lacking p130Cas [see comments]. *Nature Genetics* **19**, 361-365.

HONDA, H., NAKAMOTO, T., SAKAI, R. & HIRAI, H. (1999). p130(Cas), an assembling molecule of actin filaments, promotes cell movement, cell migration, and cell spreading in fibroblasts. *Biochemical & Biophysical Research Communications* **262**, 25-30.

HOWARD EVANS, W. (1990). Preparative Centrifugation: Isolation and characterisation of membranes and cell organelles. In *PA Series* (Editors: Rickwood and Hames), Oxford University Press.

HSU, H., XIONG, J. & GOEDDEL, D.V. (1995). The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. *Cell* **81**, 495-504.

HSU, Y.T., WOLTER, K.G. & YOULE, R.J. (1997). Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 3668-3672.

HUNG, D.T., VU, T.K., WHEATON, V.I., ISHII, K. & COUGHLIN, S.R. (1992). Cloned platelet thrombin receptor is necessary for thrombin-induced platelet activation. *Journal of Clinical Investigation* **89**, 1350-1353.

HUNGERFORD, J.E., COMPTON, M.T., MATTER, M.L., HOFFSTROM, B.G. & OTEY, C.A. (1996). Inhibition of pp125FAK in cultured fibroblasts results in apoptosis. *Journal of Cell Biology* **135**, 1383-1390.

HYNES, R.O. (1992). Integrins - versatility, modulation and signalling in cell adhesion. *Cell* **69**, 11-25.

ILIC, D., FURUTA, Y., KANAZAWA, S., TAKEDA, N., SOBUE, K., NAKATSUJI, N., NOMURA, S., FUJIMOTO, J., OKADA, M. & YAMAMOTO, T. (1995a). Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature* **377**, 539-544.

ILIC, D., FURUTA, Y., SUDA, T., ATSUMI, T., FUJIMOTO, J., IKAWA, Y., YAMAMOTO, T. & AIZAWA, S. (1995b). Focal adhesion kinase is not essential for in vitro and in vivo differentiation of ES cells. *Biochemical & Biophysical Research Communications* **209**, 300-309.

ILIC, D., KANAZAWA, S., FURUTA, Y., YAMAMOTO, T. & AIZAWA, S. (1996). Impairment of mobility in endodermal cells by FAK deficiency. *Experimental Cell Research* **222**, 298-303.

ILIC, D., DAMSKY, C.H. & YAMAMOTO, T. (1997). Focal adhesion kinase: at the crossroads of signal transduction. [Review] [72 refs]. *Journal of Cell Science* **110**, 401-407.

ILIC, D., ALMEIDA, E.A., SCHLAEPFER, D.D., DAZIN, P., AIZAWA, S. & DAMSKY, C.H. (1998). Extracellular matrix survival signals transduced by focal adhesion kinase suppress p53-mediated apoptosis. *Journal of Cell Biology* **143**, 547-560.

INGHAM, R.J., KREBS, D.L., BARBAZUK, S.M., TURCK, C.W., HIRAI, H., MATSUDA, M. & GOLD, M.R. (1996). B cell antigen receptor signaling induces the formation of complexes containing the Crk adapter proteins. *Journal of Biological Chemistry* **271**, 32306-32314.

IP, J.H., FUSTER, V., BADIMON, L., BADIMON, J., TAUBMAN, M.B. & CHESEBRO, J.H. (1990). Syndromes of accelerated atherosclerosis: role of vascular injury and smooth muscle cell proliferation. [Review] [222 refs]. *Journal of the American College of Cardiology* **15**, 1667-1687.

ISNER, J.M., KEARNEY, M., BORTMAN, S. & PASSERI, J. (1995). Apoptosis in human atherosclerosis and restenosis. *Circulation* **91**, 2703-2711.

ISNER, J.M., PIECZEK, A., SCHAINFELD, R., BLAIR, R., HALEY, L., ASAHARA, T., ROSENFIELD, K., RAZVI, S., WALSH, K. & SYMES, J.F. (1996). Clinical evidence of angiogenesis after arterial gene transfer of phVEGF165 in patient with ischaemic limb [see comments]. *Lancet* **348**, 370-374.

ITOH, N. & NAGATA, S. (1993). A novel protein domain required for apoptosis: mutational analysis of human Fas antigen. *J.Biol.Chem.* **268**, 10932-10937.

JACKSON, C.L. & REIDY, M.A. (1992). The role of plasminogen activation in smooth muscle cell migration after arterial injury. *Annals of the New York Academy of Sciences* **667**, 141-150.

JAIN, R.K., SAFABAKHSH, N., SCKELL, A., CHEN, Y., JIANG, P., BENJAMIN, L., YUAN, F. & KESHET, E. (1998). Endothelial cell death, angiogenesis, and microvascular function after castration in an androgen-dependent tumor: role of vascular endothelial growth factor. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 10820-10825.

JAMES, T.N. (1994). Normal and abnormal consequences of apoptosis in the human heart: from postnatal morphogenesis to paroxysmal arrhythmias. *Circulation* **90**, 556-573.

JANICKE, R.U., WALKER, P.A., LIN, X.Y. & PORTER, A.G. (1996). Specific cleavage of the retinoblastoma protein by an ICE-like protease in apoptosis. *EMBO Journal* **15**, 6969-6978.

JAWIEN, A., LINDNER, V., SCHWARTZ, S.M. & CLOWES, A.W. (1992). Platelet-derived growth factor promotes smooth muscle migration and intimal thickening in a rat model of balloon angioplasty. *J.Clin.Invest.* **89**, 507-511.

JIANG, Y.X., CHAN, J.L.K., ZONG, C.S. & WANG, L.H. (1996). Effect of tyrosine mutations on the kinase activity and transforming potential of an oncogenic human insulin-like growth factor-1 receptor. *J.Biol.Chem.* **271**, 160-167.

JO, H., SIPOS, K., GO, Y.M., LAW, R., RONG, J. & MCDONALD, J.M. (1997). Differential effect of shear stress on extracellular signal-regulated kinase and N-terminal Jun kinase in endothelial cells. Gi2- and Gbeta/gamma-dependent signaling pathways. *Journal of Biological Chemistry* **272**, 1395-1401.

JOVE, R. & HANAFUSA, H. (1987). Cell transformation by the viral src oncogene. [Review] [150 refs]. *Annual Review of Cell Biology* **3**, 31-56.

KAISER, D., FREYBERG, M.A. & FRIEDL, P. (1997). Lack of hemodynamic forces triggers apoptosis in vascular endothelial cells. *Biochemical & Biophysical Research Communications* **231**, 586-590.

KAJSTURA, J., ZHANG, X., LIU, Y., SZOKE, E., CHENG, W., OLIVETTI, G., HINTZE, T.H. & ANVERSA, P. (1995). The cellular basis of pacing-induced dilated cardiomyopathy: myocyte cell loss and myocyte cellular reactive hypertrophy. *Circulation* **92**, 2306-2317.

KANAZAWA, S., ILIC, D., NOUMURA, T., YAMAMOTO, T. & AIZAWA, S. (1995). Integrin stimulation decreases tyrosine phosphorylation and activity of focal adhesion kinase in thymocytes. *Biochemical & Biophysical Research Communications* **215**, 438-445.

KANG, S.S., GOSSELIN, C., REN, D. & GREISLER, H.P. (1995). Selective stimulation of endothelial cell proliferation with inhibition of smooth muscle cell proliferation by fibroblast growth factor-1 plus heparin derived from fibrin glue suspensions. *Surgery* **118**, 280-286.

KANNER, S.B., REYNOLDS, A.B., VINES, R.R. & PARSONS, J.T. (1990). Monoclonal antibodies to individual tyrosine-phosphorylated protein substrates of oncogene-encoded tyrosine kinases. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 3328-3332.

KANNER, S.B., REYNOLDS, A.B., WANG, H.C., VINES, R.R. & PARSONS, J.T. (1991). The SH2 and SH3 domains of pp60src direct stable association with tyrosine phosphorylated proteins p130 and p110. *EMBO Journal* **10**, 1689-1698.

KANNER, S.B. (1996). Focal adhesion kinase-related fakB is regulated by the integrin LFA-1 and interacts with the SH3 domain of phospholipase C gamma 1. *Cellular Immunology* **171**, 164-169.

KATOH, Y. & PERIASAMY, M. (1996). Growth and differentiation of smooth muscle cells during vascular development. *Trends in Cardiovascular Medicine* **6**, 100-106.

KEARNEY, M., PIECZEK, A., HALEY, L., LOSORDO, D.W., ANDRES, V., SCHAINFELD, R., ROSENFIELD, K. & ISNER, J.M. (1997). Histopathology of in-stent restenosis in patients with peripheral artery disease. *Circulation* **95**, 1998-2002.

KENAGY, R.D., VERGEL, S., MATTSSON, E., BENDECK, M., REIDY, M.A. & CLOWES, A.W. (1996). The role of plasminogen, plasminogen activators, and matrix metalloproteinases in primate arterial smooth muscle cell migration. *Arteriosclerosis, Thrombosis & Vascular Biology* **16**, 1373-1382.

KERR, J.F., GOBE, G.C., WINTERFORD, C.M. & HARMON, B.V. (1995). Anatomical methods in cell death. [Review] [29 refs]. *Methods in Cell Biology* **46**, 1-27.

KERR, J.F.R. (1971). Shrinkage necrosis: a distinct mode of cellular death. *J.Pathology* **105**, 13-21.

KERR, J.F.R., WYLLIE, A.H. & CURRIE, A.R. (1972). Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. *Br.J.Cancer* **26**, 239-257.

KHORSANDI, M.J., FAGIN, J.A., GIANNELLA-NETO, D., FORRESTER, J.S. & CERCEK, B. (1992). Regulation of insulin-like growth factor-1 and its receptor in rat aorta after balloon denudation: evidence for local bioactivity. *J.Clin.Invest.* **90**, 1926-1931.

KING, G.L., GOODMAN, A.D., BUZNEY, S., MOSES, A. & KAHN, C.R. (1985). Receptors and growth-promoting effects of insulin and insulin-like growth factors on cells from bovine retinal capillaries and aorta. *J.Clin.Invest.* **75**, 1028-1036.

KIRSCHENLOHR, H.L., METCALFE, J.C., WEISSBERG, P.L. & GRAINGER, D.J. (1995). Proliferation of human aortic vascular smooth muscle cells in culture is modulated by active TGF beta. *Cardiovascular Research* **29**, 848-855.

KLAGSBRUN, M. & EDELMAN, E.R. (1989). Biological and biochemical properties of basic fibroblast growth factors: implications for the pathogenesis of atherosclerosis. *Atherosclerosis* **9**, 269-278.

KLUCK, R.M., BOSSY-WETZEL, E., GREEN, D.R. & NEWMEYER, D.D. (1997). The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* **275**, 1132-1136.

KO, L.J. & PRIVES, C. (1996). p53: puzzle and paradigm. [Review] [245 refs]. *Genes & Development* **10**, 1054-1072.

KOCKX, M.M., CAMBIER, B.A., BORTIER, H.E., DE MEYER, G.R., DECLERQ, S.C., VAN CAUWELAERT, P.A. & BULTINCK, J. (1994). Foam cell replication and smooth muscle cell apoptosis in human saphenous vein grafts. *Histopathology* **25**, 365-371.

KORNBERG, L., EARP, H.S., PARSONS, J.T., SCHALLER, M. & JULIANO, R.L. (1992). Cell adhesion or integrin clustering increases phosphorylation of a focal adhesion-associated tyrosine kinase. *Journal of Biological Chemistry* **267**, 23439-23442.

KOTHAKOTA, S., AZUMA, T., REINHARD, C., KLIPPEL, A., TANG, J., CHU, K., MCGARRY, T.J., KIRSCHNER, M.W., KOTHS, K., KWIATKOWSKI, D.J. & WILLIAMS, L.T. (1997). Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science* **278**, 294-298.

KOYAMA, N., KOSHIKAWA, T., MORISAKI, N., SAITO, Y. & YOSHIDA, S. (1990). Bifunctional effects of transforming growth factor-beta on migration of cultured rat aortic smooth muscle cells. *Biochemical & Biophysical Research Communications* **169**, 725-729.

KOYAMA, N., MORISAKI, N., SAITO, Y. & YOSHIDA, S. (1992). Regulatory effects of platelet-derived growth factor-aa homodimer on migration of vascular smooth muscle cells. *J.Biol.Chem.* **267**, 22806-22812.

KOYAMA, N., HART, C.E. & CLOWES, A.W. (1994). Different functios of the platelet-derived growth factor-alpha and factor-beta receptors for the migration and proliferation of cultured baboon smooth muscle cells. *Circ.Res.* **75**, 682-691.

KRATZSCHMAR, J., HAENDLER, B., KOJIMA, S., RIFKIN, D.B. & SCHLEUNING, W.D. (1993). Bovine urokinase-type plasminogen activator and its receptor: cloning and induction by retinoic acid. *Gene* **125**, 177-183.

KUBBUTAT, M.G.H. & VOUSDEN, K.H. (1997). Proteolytic cleavage of human p53 by calpain: a potential regulator of protein stability. *Mol.Cell.Biol.* **17**, 460-468.

KUBES, P., SUZUKI, M. & GRANGER, D.N. (1991). Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 4651-4655.

KYRIAKIS, J.M., BANERJEE, P., NIKOLAKAKI, E., DAI, T., RUBIE, E.A., AHMAD, M.F., AVRUCH, J. & WOODGETT, J.R. (1994). The stress-activated protein kinase subfamily of c-Jun kinases. *Nature* **369**, 156-160.

LAI, K., WANG, H., LEE, W.S., JAIN, M.K., LEE, M.E. & HABER, E. (1996). Mitogen-activated protein kinase phosphatase-1 in rat arterial smooth muscle cell proliferation. *Journal of Clinical Investigation* **98**, 1560-1567.

LAKKAKORPI, P.T., HELFRICH, M.H., HORTON, M.A. & VAANANEN, H.K. (1993). Spatial organization of microfilaments and vitronectin receptor, alpha v beta 3, in osteoclasts. A study using confocal laser scanning microscopy. *Journal of Cell Science* **104**, 663-670.

LAUFFENBURGER, D.A. & HORWITZ, A.F. (1996). Cell migration: a physically integrated molecular process. *Cell* **84**, 359-369.

LAW, S.F., ESTOJAK, J., WANG, B., MYSLIWIEC, T., KRUH, G. & GOLEMIS, E.A. (1996). Human enhancer of filamentation 1, a novel p130cas-like docking protein, associates with focal adhesion kinase and induces pseudohyphal growth in Saccharomyces cerevisiae. *Molecular & Cellular Biology* **16**, 3327-3337.

LAZAROVICI, P., RASOULY, D., FRIEDMAN, L., TABEKMAN, R., OVADIA, H. & MATSUDA, Y. (1996). K252a and staurosporine microbial alkaloid toxins as prototype of neurotropic drugs. [Review] [32 refs]. *Advances in Experimental Medicine & Biology* **391**, 367-377.

LEE, W.S., HARDER, J.A., YOSHIZUMI, M., LEE, M.E. & HABER, E. (1997). Progesterone inhibits arterial smooth muscle cell proliferation. *Nature Medicine* **3**, 1005-1008.

LEFER, A.M. & MA, X.L. (1993). Cytokines and growth factors in endothelial dysfunction. [Review] [38 refs]. *Critical Care Medicine* **21**, S9-14.

LEHOUX, S. & TEDGUI, A. (1998). Signal transduction of mechanical stresses in the vascular wall. [Review] [103 refs]. *Hypertension* **32**, 338-345.

LEV, S., MORENO, H., MARTINEZ, R., CANOLL, P., PELES, E., MUSACCHIO, J.M., PLOWMAN, GD, RUDY, B. & SCHLESSINGER, J. (1995). Protein tyrosine kinase PYK2 involved in Ca(2+)-induced regulation of ion channel and MAP kinase functions [see comments]. *Nature* **376** , 737-745.

LEVIN, E.G., MAROTTI, K.R. & SANTELL, L. (1989). Protein kinase C and the stimulation of tissue plasminogen activator release from human endothelial cells. Dependence on the elevation of messenger RNA. *Journal of Biological Chemistry* **264**, 16030-16036.

LEVINE, A.J. (1997). p53, the cellular gatekeeper for growth and division. [Review] [55 refs]. *Cell* **88**, 323-331.

LEVKAU, B., HERREN, B., KOYAMA, H., ROSS, R. & RAINES, E.W. (1998). Caspase-mediated cleavage of focal adhesion kinase pp125FAK and disassembly of focal adhesions in human endothelial cell apoptosis. *Journal of Experimental Medicine* **187**, 579-586.

LI, Y.S., SHYY, J.Y., LI, S., LEE, J., SU, B., KARIN, M. & CHIEN, S. (1996). The Ras-JNK pathway is involved in shear-induced gene expression. *Molecular & Cellular Biology* **16** , 5947-5954.

LIAW, L., ALMEIDA, M., HART, C.E., SCHWARTZ, S.M. & GIACHELLI, C.M. (1994). Osteopontin promotes vascular cell adhesion and spreading and is chemotactic for smooth muscle cells in vitro. *Circulation Research* **74**, 214-224.

LINDNER, V. & REIDY, M.A. (1991). Proliferation of smooth muscle cells after vascular injury is inhibited by an antibody against basic fibroblast growth factor. *Proc.Natl.Acad.Sci.U.S.A.* **88**, 3739-3743.

LINDNER, V. & REIDY, M.A. (1993). Expression of basic fibroblast growth factor and its receptor by smooth muscle cells and endothelium in injured rat arteries: an *en face* study. *Circ.Res.* **73**, 589-595.

LIPFERT, L., HAIMOVICH, B., SCHALLER, M.D., COBB, B.S., PARSONS, JT & BRUGGE, J.S. (1992). Integrin-dependent phosphorylation and activation of the protein tyrosine kinase pp125FAK in platelets. *Journal of Cell Biology* **119**, 905-912.

LIPSKY, B.P., BEALS, C.R. & STAUNTON, D.E. (1998). Leupaxin is a novel LIM domain protein that forms a complex with PYK2. *Journal of Biological Chemistry* **273**, 11709-11713.

LISTON, P., ROY, N., TAMAI, K., LEFEBVRE, C., BAIRD, S., CHERTON-HORVAT, G., FARAHANI, R., MCLEAN, M., IKEDA, J.E., MACKENZIE, A. & KORNELUK, R.G. (1996). Suppression of apoptosis in mammalian cells by NIAP and a related family of IAP genes. *Nature* **379**, 349-353.

LIU, S., THOMAS, S.M., WOODSIDE, D.G., ROSE, D.M., KIOSSES, W.B., PFAFF, M., GINSBERG & MH (1999). Binding of paxillin to alpha4 integrins modifies integrin-dependent biological responses. *Nature* **402**, 676-681.

LIU, X., KIM, C.N., YANG, J., JEMMERSON, R. & WANG, X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome C. *Cell* **86**, 147-157.

LUDMER, P.L., SELWYN, A.P., SHOOK, T.L., WAYNE, R.R., MUDGE, G.H., ALEXANDER, R.W. & GANZ, P. (1986). Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. *New England Journal of Medicine* **315**, 1046-1051.

LUDWIG, R.L., BATES, S. & VOUSDEN, K.H. (1996). Differential activation of target cellular promoters by p53 mutants with impaired apoptotic function. *Molecular & Cellular Biology* **16**, 4952-4960.

MAJACK, R.A., MILBRANDT, J. & DIXIT, V.M. (1990). Role of PDGF-A expression in the control of vascular smooth muscle cell growth by transforming growth factor-beta. *J.Cell Biol.* **111**, 239-247.

MAJESKY, M.W., REIDY, M.A., BOWEN-POPE, D.F., HART, C.E., WILCOX, J.N. & SCHWARTZ, S.M. (1990). PDGF ligand and receptor gene expression during repair of arterial injury. *J.Cell Biol.* **111**, 2149-2158.

MALEK, A.M. & IZUMO, S. (1995). Control of endothelial cell gene expression by flow. *Journal of Biomechanics* **28**, 1515-1528.

MANIE, S.N., BECK, A.R., ASTIER, A., LAW, S.F., CANTY, T., HIRAI, H., DRUKER, B.J., AVRAHAM, H., HAGHAYEGHI, N., SATTLER, M., SALGIA, R., GRIFFIN, J.D., GOLEMIS, E.A. & FREEDMAN, A.S. (1997). Involvement of p130(Cas) and p105(HEF1), a novel Cas-like docking protein, in a cytoskeleton-dependent signaling pathway initiated by ligation of integrin or antigen receptor on human B cells. *Journal of Biological Chemistry* **272**, 4230-4236.

MANSOUR, S.J., MATTEN, W.T., HERMANN, A.S., CANDIA, J.M., RONG, S., FUKASAWA, K., VANDE, W.G. & AHN, N.G. (1994). Transformation of mammalian cells by constitutively active MAP kinase kinase. *Science* **265**, 966-970.

MARKS, D.S., VITA, J.A., FOLTS, J.D., KEANEY, J.F.J., WELCH, G.N. & LOSCALZO, J. (1995). Inhibition of neointimal proliferation in rabbits after vascular injury by a single treatment with a protein adduct of nitric oxide. *Journal of Clinical Investigation* **96**, 2630-2638.

MARSTERS, S.A., SHERIDAN, J.P., PITTI, R.M., BRUSH, J., GODDARD, A. & ASHKENAZI, A. (1998). Identification of a ligand for the death-domain containing receptor APO3. *Curr.Biol.* **8**, 525-528.

MARTIN, J.F., BOOTH, R.F. & MONCADA, S. (1990). Arterial wall hypoxia following hypoperfusion through the *vasa vasorum* is an initial lesion in atherosclerosis. *Eur.J.Clin.Invest.* **20**, 588-592.

MARTIN, S.J., REUTERLINGSPERGER, C.P., MCGAHON, A.J., RADER, J.A., VAN SCHIE, R.C., LAFACE, D.M. & GREEN, D.R. (1995a). Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J.Exp.Med.* **182**, 1545-1556.

MARTIN, S.J., O'BRIEN, G.A., NISHIOKA, W.A., MCGAHON, A.J., MAHBOUBI, A., SAIDO, T.C. & GREEN, D.R. (1995b). Proteolysis of fodrin (non-erythroid spectrin) during apoptosis. *J.Biol.Chem.* **270**, 6425-6428.

MARTINOU, J.C., DUBOIS-DAUPHIN, M., STAPLE, J.K., RODRIGUEZ, I., FRANKOWSKY, H., MISSOTTEN, M., ALBERTINI, P., TALBOT, D., CATSICAS, S. & PIETRA, C. (1994). Overexpression of BCL-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischaemia. *Neuron* **13**, 1017-1030.

MASON, I.J. (1994). The ins and outs of fibroblast growth factors. *Cell* **78**, 547-552.

MATSUDA, M., MAYER, B.J., FUKUI, Y. & HANAFUSA, H. (1990). Binding of transforming protein, P47gag-crk, to a broad range of phosphotyrosine-containing proteins. *Science* **248**, 1537-1539.

MATSUMOTO, K., NAKAMURA, T. & KRAMER, R.H. (1994). Hepatocyte growth factor/scatter factor induces tyrosine phosphorylation of focal adhesion kinase (p125FAK) and promotes

migration and invasion by oral squamous cell carcinoma cells. *Journal of Biological Chemistry* **269**, 31807-31813.

MATTHEWS, W., JORDAN, C.T., GAVIN, M., JENKINS, N.A., COPELAND, N.G. & LEMISCHKA, I.R. (1991). A receptor tyrosine kinase cDNA isolated from a population of enriched primitive hematopoietic cells and exhibiting close genetic linkage to c-kit. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 9026-9030.

MAZAKI, Y., HASHIMOTO, S. & SABE, H. (1997). Monocyte cells and cancer cells express novel paxillin isoforms with different binding properties to focal adhesion proteins. *Journal of Biological Chemistry* **272**, 7437-7444.

MCGILL JR, H.C. (1984). George Lyman Duff memorial lecture. Persistent problems in the pathogenesis of atherosclerosis. *Arteriosclerosis* **4**, 443-451.

MCGORISK, G.M. & TREASURE, C.B. (1996). Endothelial dysfunction in coronary heart disease. [Review] [109 refs]. *Current Opinion in Cardiology* **11**, 341-350.

MCLENACHAN, J.M., VITA, J., FISH, D.R., TREASURE, C.B., COX, D.A., GANZ, P. & SELWYN, A.P. (1990). Early evidence of endothelial vasodilator dysfunction at coronary branch points. *Circulation* **82**, 1169-1173.

MCMURRAY, H.F., PARROTT, D.P. & BOWYER, D.E. (1991). A standardised method of culturing aortic explants, suitable for the study of factors affecting the phenotypic modulation, migration and proliferation of aortic smooth muscle cells. *Atherosclerosis* **86**, 227-237.

MCNAMARA, C.A., SAREMBOCK, I.J., GIMPLE, L.W., FENTON, J.W., COUGHLIN, S.R. & OWENS, G.K. (1993). Thrombin stimulates proliferation of cultured rat aortic smooth muscle cells by a proteolytically activated receptor [see comments]. *Journal of Clinical Investigation* **91**, 94-98.

MELAMED, I., TURNER, C.E., AKTORIES, K., KAPLAN, D.R. & GELFAND, E.W. (1995). Nerve growth factor triggers microfilament assembly and paxillin phosphorylation in human B lymphocytes. *Journal of Experimental Medicine* **181**, 1071-1079.

MENDELSOHN, M.E., O'NEILL, S., GEORGE, D. & LOSCALZO, J. (1990). Inhibition of fibrinogen binding to human platelets by S-nitroso-N-acetylcysteine. *Journal of Biological Chemistry* **265**, 19028-19034.

MEREDITH JR, J., MU, Z., SAIDO, T. & DU, X. (1998). Cleavage of the cytoplasmic domain of the integrin β_3-subunit during endothelial cell apoptosis. *J.Biol.Chem.* **273**, 30530-30536.

METCALF, D., LINDEMAN, G.J. & NICOLA, N.A. (1995). *Blood* **85**, 2364-2370.

MINEGISHI, M., TACHIBANA, K., SATO, T., IWATA, S., NOJIMA, Y. & MORIMOTO, C. (1996). Structure and function of Cas-L, a 105-kD Crk-associated substrate-related protein that is involved in beta 1 integrin-mediated signaling in lymphocytes. *Journal of Experimental Medicine* **184**, 1365-1375.

MITCHISON, T.J. & CRAMER, L.P. (1996). Actin-based cell motility and cell locomotion. *Cell* **84**, 371-379.

MIURA, M., ZHU, H., ROTELLO, R., HARTWIG, E.A. & YUAN, J. (1993). Induction of apoptosis in fibroblasts by IL-1β-converting enzyme, a mammalian homologue of the C. elegans cell death gene ced-3. *Cell* **75**, 653-660.

MIYASHITA, T. & REED, J.C. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* **80**, 293-299.

MOGI, A., HATAI, M., SOGA, H., TAKENOSHITA, S., NAGAMACHI, Y., FUJIMOTO, J., YAMAMOTO, T., YOKOTA, J. & YAOI, Y. (1995). Possible role of protein kinase C in the regulation of intracellular stability of focal adhesion kinase in mouse 3T3 cells. *FEBS Letters* **373**, 135-140.

MONCADA, S. & HIGGS, E.A. (1991). Endogenous nitric oxide: physiology, pathology and clinical relevance. [Review] [190 refs]. *European Journal of Clinical Investigation* **21**, 361-374.

MORA, R., LUPU, F. & SIMIONESCU, N. (1987). Prelesional events in atherogenesis. Colocalisation of apolipoprotein B, unesterified cholesterol and extracellular phospholipid liposomes in the aorta of the hyperlipidemic rabbit. *Atherosclerosis* **67**, 143-154.

MORRISON, D.K. & CUTLER, R.E. (1997). The complexity of Raf-1 regulation. [Review] [62 refs]. *Current Opinion in Cell Biology* **9**, 174-179.

MUNRO, J.M. & COTRAN, R.S. (1988). The pathogenesis of atherosclerosis: atherogenesis and inflammation. *Lab.Invest.* **58**, 249-261.

MURPHY, J.D., RABINOVITCH, M., GOLDSTEIN, J.D. & REID, L.M. (1981). The structural basis of persistent pulmonary hypertension of the newborn infant. *Journal of Pediatrics* **98**, 962-967.

MUZIO, M., CHINNAIYAN, A.M., KISCHKEL, F.C., O'ROURKE, K., SCHEVCHENKO, A., NI, J., SCAFFIDI, C., BRETZ, J.D., ZHANG, M., GENTZ, R., MANN, M., KRAMMER, P.H., PETER, M.E. & DIXIT, V.M. (1996). FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signal complex. *Cell* **85**, 817-827.

NAGATA, S. & GOLSTEIN, P. (1995). The Fas death factor. *Science* **267**, 1449-1456.

NAITO, M., HAYASHI, T., FUNAKI, C., KUZUYA, M., ASAI, K., YAMADA & KUZUYA, F. (1991). Vitronectin-induced haptotaxis of vascular smooth muscle cells in vitro. *Experimental Cell Research* **194**, 154-156.

NAKAMOTO, T., SAKAI, R., OZAWA, K., YAZAKI, Y. & HIRAI, H. (1996). Direct binding of C-terminal region of p130Cas to SH2 and SH3 domains of Src kinase. *Journal of Biological Chemistry* **271**, 8959-8965.

NAKAMOTO, T., SAKAI, R., HONDA, H., OGAWA, S., UENO, H., SUZUKI, T., AIZAWA, S., YAZAKI, Y. & HIRAI, H. (1997). Requirements for localization of p130cas to focal adhesions. *Molecular & Cellular Biology* **17**, 3884-3897.

NARULA, J., HAIDER, N., VIRMANI, R., DISALVO, T.G., KOLODGIE, F.D., HAJJAR, R.J., SCHMIDT, U., SEMIGRAN, M.J., DEC, G.W. & KHAW, B.A. (1996). Apoptosis in myocytes in end-stage heart failure. *N.Engl.J.Med.* **148**, 141-149.

NARUSE, K., SHIMIZU, K., MURAMATSU, M., TOKI, Y., MIYAZAKI, Y., OKUMURA, K., HASHIMOTO, H. & ITO, T. (1994). Long-term inhibition of NO synthesis promotes atherosclerosis in the hypercholesterolemic rabbit thoracic aorta. PGH2 does not contribute to impaired endothelium-dependent relaxation [see comments]. *Arteriosclerosis & Thrombosis* **14**, 746-752.

NARUSE, K., YAMADA, T., SAI, X.R., HAMAGUCHI, M. & SOKABE, M. (1998). Pp125FAK is required for stretch dependent morphological response of endothelial cells. *Oncogene* **17**, 455-463.

NEREM, R.M., HARRISON, D.G., TAYLOR, W.R. & ALEXANDER, R.W. (1993). Hemodynamics and vascular endothelial biology. [Review] [38 refs]. *Journal of Cardiovascular Pharmacology* **21 Suppl 1**, S6-10.

NEWBY, A.C. & GEORGE, S.J. (1993). Proposed roles for growth factors in mediating smooth muscle proliferation in vascular pathologies. [Review] [167 refs]. *Cardiovascular Research* **27**, 1173-1183.

NEWTON, A.C. (1995). Protein kinase C: structure, function, and regulation. [Review] [78 refs]. *Journal of Biological Chemistry* **270**, 28495-28498.

NICHOLSON, D.W. (1996). ICE/CED3-like proteases as therapeutic targets for the control of inappropriate apoptosis. [Review] [91 refs]. *Nature Biotechnology* **14**, 297-301.

NICHOLSON, D.W. & THORNBERRY, N.A. (1997). Caspases: killer proteases. *Trends Biochem.Sci.* **22**, 299-306.

NIGG, E.A. (1997). Nucleocytoplasmic transport: signals, mechanisms and regulation. [Review] [137 refs]. *Nature* **386**, 779-787.

NILSSON, J. (1986). Growth factors and the pathogenesis of atherosclerosis. [Review] [135 refs]. *Atherosclerosis* **62**, 185-199.

NISHIZUKA, Y. (1992). Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. [Review] [107 refs]. *Science* **258**, 607-614.

NISHIZUKA, Y. (1995). Protein kinase C and lipid signaling for sustained cellular responses. [Review] [198 refs]. *FASEB Journal* **9**, 484-496.

NIX, D.A. & BECKERLE, M.C. (1997). Nuclear-cytoplasmic shuttling of the focal contact protein, zyxin: a potential mechanism for communication between sites of cell adhesion and the nucleus. *Journal of Cell Biology* **138**, 1139-1147.

NODA, M., YASUDA-FUKAZAWA, C., MORIISHI, K., KATO, T., OKUDA, T., KUROKAWA, K. & TAKUWA, Y. (1995). Involvement of rho in GTP gamma S-induced enhancement of phosphorylation of 20 kDa myosin light chain in vascular smooth muscle cells: inhibition of phosphatase activity. *FEBS Letters* **367**, 246-250.

NOJIMA, Y., MORINO, N., MIMURA, T., HAMASAKI, K., FURUYA, H., SAKAI, R., SATO, T., TACHIBANA, K., MORIMOTO, C. & YAZAKI, Y. (1995). Integrin-mediated cell adhesion promotes tyrosine phosphorylation of p130Cas, a Src homology 3-containing molecule having multiple Src homology 2-binding motifs. *Journal of Biological Chemistry* **270**, 15398-15402.

NOMOTO, A., MUTOH, S., HAGIHARA, H. & YAMAGUCHI, I. (1988). Smooth muscle cell migration induced by inflammatory cell products and its inhibition by a potent calcium antagonist, nilvadipine. *Atherosclerosis* **72**, 213-219.

NOR, J.E., CHRISTENSEN, J., MOONEY, D.J. & POLVERINI, P.J. (1999). Vascular endothelial growth factor (VEGF)-mediated angiogenesis is associated with enhanced endothelial cell survival and induction of Bcl-2 expression. *American Journal of Pathology* **154**, 375-384.

O'BRIEN, E.R., GARVIN, M.R., DEV, R., STEWART, D.K., HINOHARA, T., SIMPSON, J.B. & SCHWARTZ, S.M. (1994). Angiogenesis in human coronary atherosclerotic plaques. *American Journal of Pathology* **145**, 883-894.

ODEKON, L.E., SATO, Y. & RIFKIN, D.B. (1992). Urokinase-type plasminogen activator mediates basic fibroblast growth factor-induced bovine endothelial cell migration independent of its proteolytic activity. *Journal of Cellular Physiology* **150**, 258-263.

OJANIEMI, M. & VUORI, K. (1997). Epidermal growth factor modulates tyrosine phosphorylation of p130Cas. Involvement of phosphatidylinositol 3'-kinase and actin cytoskeleton. *Journal of Biological Chemistry* **272**, 25993-25998.

OKADA, S.S., GROBMYER, S.R. & BARNATHAN, E.S. (1996). Contrasting effects of plasminogen activators, urokinase receptor, and LDL receptor-related protein on smooth muscle cell migration and invasion. *Arteriosclerosis, Thrombosis & Vascular Biology* **16**, 1269-1276.

OKTAY, M., WARY, K.K., DANS, M., BIRGE, R.B. & GIANCOTTI, F.G. (1999). Integrin-mediated activation of focal adhesion kinase is required for signaling to Jun NH2-terminal kinase and progression through the G1 phase of the cell cycle. *Journal of Cell Biology* **145**, 1461-1469.

OLIVETTI, G., QUAINI, F., SALA, R., LAGRASTA, C., CORRADI, D., BONACINA, E., GAMBERT, S.R., CIGOLA, E. & ANVERSA, P. (1996). Acute myocardial infarction in humans is associated with activation of programmed myocyte cell death in the surviving portion of the heart. *J.Mol.Cell Cardiol.* **28**, 2005-2016.

OLIVETTI, G., ABBI, R., QUAINI, F., KAJSTURA, J., CHENG, W., NITAHARA, J.A., QUAINI, E., DI LORETO, C., BELTRAMI, C.A., KRAJEWSKI, S., REED, J.C. & ANVERSA, P. (1997). Apoptosis in the failing human heart. *N.Engl.J.Med.* **336**, 1131-1141.

OWENS, G.K. & THOMPSON, M.M. (1986). Developmental changes in isoactin expression in rat aortic smooth muscle cells in vivo. Relationship between growth and cytodifferentiation. *Journal of Biological Chemistry* **261**, 13373-13380.

OWENS, G.K. (1995). Regulation of differentiation of vascular smooth muscle cells. [Review] [294 refs]. *Physiological Reviews* **75**, 487-517.

OWENS, L.V., XU, L., CRAVEN, R.J., DENT, G.A., WEINER, T.M., KORNBERG, L., LIU, E.T. & CANCE, W.G. (1995). Overexpression of the focal adhesion kinase (p125FAK) in invasive human tumors. *Cancer Research* **55**, 2752-2755.

PARSONS, J.T. & WEBER, M.J. (1989). Genetics of src: structure and functional organization of a protein tyrosine kinase. [Review] [215 refs]. *Current Topics in Microbiology & Immunology* **147**, 79-127.

PARSONS, J.T. (1996). Integrin-mediated signalling: regulation by protein tyrosine kinases and small GTP-binding proteins. [Review] [47 refs]. *Current Opinion in Cell Biology* **8**, 146-152.

PERLMAN, H., MAILLARD, L., KRASINSKI, K. & WALSH, K. (1997). Evidence for the rapid onset of apoptosis in medial smooth cells after balloon injury. *Circulation* **95**, 981-987.

PETCH, L.A., BOCKHOLT, S.M., BOUTON, A., PARSONS, J.T. & BURRIDGE, K. (1995). Adhesion-induced tyrosine phosphorylation of the p130 src substrate. *Journal of Cell Science* **108**, 1371-1379.

PEXIEDER, T. (1975). Cell death in the morphogenesis and teratogenesis of the heart. *Adv.Anat.Embryol.Cell Biol.* **51**, 3-91.

PFEIFLE, B., DITSCHUNEIT, H.H. & DITSCHUNEIT, H. (1982). Binding and biological actions of insulin-like growth factors on human arterial smooth muscle cells. *Horm.Metab.Res.* **4**, 409-414.

PITTI, R.M., MARSTERS, S.A., RUPPERT, S., DONAHUE, C.J., MOORE, A. & ASHKENAZI, A. (1996). Induction of apoptosis by Apo-2 ligand, a new member of the tumour necrosis factor cytokine family. *J.Biol.Chem.* **271**, 12687-12690.

PLATE, K.H., BREIER, G., WEICH, H.A. & RISAU, W. (1992). Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. *Nature* **359**, 845-848.

PLOUG, M., RONNE, E., BEHRENDT, N., JENSEN, A.L., BLASI, F. & DANO (1991). Cellular receptor for urokinase plasminogen activator. Carboxyl-terminal processing and membrane anchoring by glycosyl-phosphatidylinositol. *Journal of Biological Chemistry* **266**, 1926-1933.

POLTE, T.R. & HANKS, S.K. (1995). Interaction between focal adhesion kinase and Crk-associated tyrosine kinase substrate p130Cas. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 10678-10682.

POLUNOVSKY, V.A., WENDT, C.H., INGBAR, D.H., PETERSON, M.S. & BITTERMAN, P.B. (1994). Induction of endothelial cell apoptosis by TNF alpha: modulation by inhibitors of protein synthesis. *Experimental Cell Research* **214**, 584-594.

PORTER, A.G., NG, P. & JANICKE, R.U. (1997). Death substrates come alive. *Bioessays* **19**, 501-507.

PRITCHARD, K.A.J., O'BANION, M.K., MIANO, J.M., VLASIC, N., BHATIA, UG, YOUNG, D.A. & STEMERMAN, M.B. (1994). Induction of cyclooxygenase-2 in rat vascular smooth muscle cells in vitro and in vivo. *Journal of Biological Chemistry* **269**, 8504-8509.

QIAN, D., LEV, S., VAN OERS, N.S., DIKIC, I., SCHLESSINGER, J. & WEISS, A. (1997). Tyrosine phosphorylation of Pyk2 is selectively regulated by Fyn during TCR signaling. *Journal of Experimental Medicine* **185**, 1253-1259.

RABBANI, S.A., MAZAR, A.P., BERNIER, S.M., HAQ, M., BOLIVAR, I., HENKIN, J. & GOLTZMAN, D. (1992). Structural requirements for the growth factor activity of the amino-terminal domain of urokinase. *Journal of Biological Chemistry* **267**, 14151-14156.

RAFF, M.C., BARRES, B.A., BURNE, J.F., COLES, H.S., ISHIZAKI, Y. & JACOBSON, M.D. (1996). Programmed cell death and the control of cell survival, lessons from the nervous system. *Science* **262**, 695-700.

RAINES, E.W. & ROSS, R. (1993). Smooth muscle cells and the pathogenesis of the lesions of atherosclerosis. *Br.Heart J.* **69**, S30-S37

RANKIN, S. & ROZENGURT, E. (1994). Platelet-derived growth factor modulation of focal adhesion kinase (p125FAK) and paxillin tyrosine phosphorylation in Swiss 3T3 cells. Bell-shaped dose response and cross-talk with bombesin. *Journal of Biological Chemistry* **269**, 704-710.

RANKIN, S., MORII, N., NARUMIYA, S. & ROZENGURT, E. (1994). Botulinum C3 exoenzyme blocks the tyrosine phosphorylation of p125FAK and paxillin induced by bombesin and endothelin. *FEBS Letters* **354**, 315-319.

REED, J.C. (1994). Bcl-2 and the regulation of programmed cell death. *J.Cell Biol.* **124**, 1-6.

RESNATI, M., GUTTINGER, M., VALCAMONICA, S., SIDENIUS, N., BLASI & FAZIOLI, F. (1996). Proteolytic cleavage of the urokinase receptor substitutes for the agonist-induced chemotactic effect. *EMBO Journal* **15**, 1572-1582.

RESNICK, N. & GIMBRONE, M.A., Jr. (1995). Hemodynamic forces are complex regulators of endothelial gene expression. [Review] [55 refs]. *FASEB Journal* **9**, 874-882.

REUNING, U., LITTLE, S.P., DIXON, E.P., JOHNSTONE, E.M. & BANG, N.U. (1993). Molecular cloning of cDNA for the bovine urokinase-type plasminogen activator receptor. *Thrombosis Research* **72**, 59-70.

REYNOLDS, A.B., KANNER, S.B., WANG, H.C. & PARSONS, J.T. (1989). Stable association of activated pp60src with two tyrosine-phosphorylated cellular proteins. *Molecular & Cellular Biology* **9**, 3951-3958.

RICHARDSON, A. & PARSONS, T. (1996). A mechanism for regulation of the adhesion-associated proteintyrosine kinase pp125FAK [published erratum appears in Nature 1996 Jun 27;381(6585):810]. *Nature* **380**, 538-540.

RIMARACHIN, J.A., JACOBSON, J.A., SZABO, P., MACLOUF, J., CREMINON & WEKSLER, B.B. (1994). Regulation of cyclooxygenase-2 expression in aortic smooth muscle cells. *Arteriosclerosis & Thrombosis* **14**, 1021-1031.

ROBAYE, B., MOSSELMANS, R., FIERS, W., DUMONT, J.E. & GALAND, P. (1991). Tumor necrosis factor induces apoptosis (programmed cell death) in normal endothelial cells in vitro. *American Journal of Pathology* **138**, 447-453.

ROSENBERG, R.D. & ROSENBERG, J.S. (1984). Natural anticoagulant mechanisms. [Review] [68 refs]. *Journal of Clinical Investigation* **74**, 1-6.

ROSS, R. & GLOMSET, J.A. (1973). Atherosclerosis and the arterial smooth muscle cell: proliferation of smooth muscle is a key event in the genesis of the lesions of atherosclerosis. *Science* **180**, 1332-1339.

ROSS, R. & GLOMSET, J.A. (1976). The pathogenesis of atherosclerosis (second of two parts). [Review] [12 refs]. *New England Journal of Medicine* **295**, 420-425.

ROSS, R. & GLOMSET, J.A. (1976). The pathogenesis of atherosclerosis (first of two parts). [Review] [119 refs]. *New England Journal of Medicine* **295**, 369-377.

ROSS, R. (1986). The pathogenesis of atherosclerosis: an update. *N.Engl.J.Med.* **314**, 488-500.

ROSS, R. & RAINES, E.W. (1986). The biology of platelet-derived growth factor. *Cell* **46**, 155-169.

ROSS, R. & RAINES, E.W. (1988). Platelet-derived growth factor--its role in health and disease. [Review] [85 refs]. *Advances in Experimental Medicine & Biology* **234**, 9-21.

ROSS, R. (1993). The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 801-809.

ROSS, R. (1997). Cellular and molecular studies of atherogenesis. [Review] [2 refs]. *Atherosclerosis* **131 Suppl**, S3-S4

ROSSI, G.L., ALROY, J. & ROTHENMUND, S. (1973). Morphological studies of cultured swine aorta media explants. *Virchows Archiv* **B. Cell Pathology. 12**, 133-144.

SABE, H., HATA, A., OKADA, M., NAKAGAWA, H. & HANAFUSA, H. (1994). Analysis of the binding of the Src homology 2 domain of Csk to tyrosine-phosphorylated proteins in the suppression and mitotic activation of c-Src. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 3984-3988.

SABE, H., SHOELSON, S.E. & HANAFUSA, H. (1995). Possible v-Crk-induced transformation through activation of Src kinases. *Journal of Biological Chemistry* **270**, 31219-31224.

SACHDEV, S., HOFFMANN, A. & HANNINK, M. (1998). Nuclear localization of IkappaB alpha is mediated by the second ankyrin repeat: the IkappaB alpha ankyrin repeats define a novel class of cis-acting nuclear import sequences. *Molecular & Cellular Biology* **18** , 2524-2534.

SAKAHIRA, H., ENARI, M. & NAGATA, S. (1998). Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature* **391**, 96-99.

SAKAI, R., IWAMATSU, A., HIRANO, N., OGAWA, S., TANAKA, T., NISHIDA, J., YAZAKI, Y. & HIRAI, H. (1994a). Characterization, partial purification, and peptide sequencing of p130,the main phosphoprotein associated with v-Crk oncoprotein. *Journal of Biological Chemistry* **269**, 32740-32746.

SAKAI, R., IWAMATSU, A., HIRANO, N., OGAWA, S., TANAKA, T., MANO, H., YAZAKI, Y. & HIRAI, H. (1994b). A novel signaling molecule, p130, forms stable complexes in vivo with v-Crk and v-Src in a tyrosine phosphorylation-dependent manner. *EMBO Journal* **13**, 3748-3756.

SALGIA, R., UEMURA, N., OKUDA, K., LI, J.L., PISICK, E., SATTLER, M., DE JONG, R., DRUKER, B., HEISTERKAMP, N. & CHEN, L.B. (1995). CRKL links p210BCR/ABL with paxillin in chronic myelogenous leukemia cells. *Journal of Biological Chemistry* **270**, 29145-29150.

SALTIS, J., THOMAS, A.C., AGROTIS, A., CAMPBELL, J.H. & CAMPBELL, G.R. (1995). Expression of growth factor receptors on arterial smooth muscle cell. Dependency on cell phenotype and serum factors. *Atherosclerosis* **118**, 77-87.

SARASTE, A., PULKKI, K., KALLAJOKI, M., HENRIKSEN, K., PARVINEN, M. & VOIPIO-PULKKI, L.M. (1997). Apoptosis in human acute myocardial infarction. *Circulation* **95**, 320-323.

SARIN, A., ADAMS, D.H. & HENKART, P.A. (1993). Protease inhibitors selectively block T-cell receptor-triggered programmed cell death in a murine T-cell hybridoma and activated peripheral T-cells. *J.Exp.Med.* **178**, 1693-1700.

SASAKI, H., NAGURA, K., ISHINO, M., TOBIOKA, H., KOTANI, K. & SASAKI, T. (1995). Cloning and characterization of cell adhesion kinase beta, a novel protein-tyrosine kinase of the focal adhesion kinase subfamily. *Journal of Biological Chemistry* **270**, 21206-21219.

SAVILL, J.S. (1998). Phagocytic docking without shocking. *Nature* **392**, 442-443.

SAWDEY, M.S. & LOSKUTOFF, D.J. (1991). Regulation of murine type 1 plasminogen activator inhibitor gene expression in vivo. Tissue specificity and induction by lipopolysaccharide, tumor necrosis factor-alpha, and transforming growth factor-beta. *Journal of Clinical Investigation* **88**, 1346-1353.

SCHALLER, M.D., BORGMAN, C.A., COBB, B.S., VINES, R.R., REYNOLDS, A.B. & PARSONS, J.T. (1992). pp125FAK a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 5192-5196.

SCHALLER, M.D., BORGMAN, C.A. & PARSONS, J.T. (1993). Autonomous expression of a noncatalytic domain of the focal adhesion-associated protein tyrosine kinase pp125FAK. *Molecular & Cellular Biology* **13**, 785-791.

SCHALLER, M.D. & PARSONS, J.T. (1994). Focal adhesion kinase and associated proteins. [Review] [44 refs]. *Current Opinion in Cell Biology* **6**, 705-710.

SCHALLER, M.D., HILDEBRAND, J.D., SHANNON, J.D., FOX, J.W., VINES, R.R. & PARSONS, J.T. (1994). Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. *Molecular & Cellular Biology* **14**, 1680-1688.

SCHALLER, M.D., OTEY, C.A., HILDEBRAND, J.D. & PARSONS, J.T. (1995). Focal adhesion kinase and paxillin bind to peptides mimicking beta integrin cytoplasmic domains. *Journal of Cell Biology* **130**, 1181-1187.

SCHALLER, M.D. & PARSONS, J.T. (1995). pp125FAK-dependent tyrosine phosphorylation of paxillin creates a high-affinity binding site for Crk. *Molecular & Cellular Biology* **15**, 2635-2645.

SCHALLER, M.D. & SASAKI, T. (1997). Differential signaling by the focal adhesion kinase and cell adhesion kinase beta. *Journal of Biological Chemistry* **272**, 25319-25325.

SCHALLER, M.D., HILDEBRAND, J.D. & PARSONS, J.T. (1999). Complex formation with focal adhesion kinase: A mechanism to regulate activity and subcellular localization of Src kinases. *Molecular Biology of the Cell* **10**, 3489-3505.

SCHLAEPFER, D.D., HANKS, S.K., HUNTER, T. & VAN DER GEER, P. (1994). Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature* **372**, 786-791.

SCHLAEPFER, D.D. & HUNTER, T. (1996). Evidence for in vivo phosphorylation of the Grb2 SH2-domain binding site on focal adhesion kinase by Src-family protein-tyrosine kinases [published erratum appears in Mol Cell Biol 1996 Dec;16(12):7182-4]. *Molecular & Cellular Biology* **16**, 5623-5633.

SCHLAEPFER, D.D., BROOME, M.A. & HUNTER, T. (1997). Fibronectin-stimulated signaling from a focal adhesion kinase-c-Src complex: involvement of the Grb2, p130cas, and Nck adaptor proteins. *Molecular & Cellular Biology* **17**, 1702-1713.

SCHLAEPFER, D.D. & HUNTER, T. (1998). Integrin signalling and tyrosine phosphorylation: just the FAKs?. [Review] [61 refs]. *Trends in Cell Biology* **8**, 151-157.

SCHLESSINGER, J. (1994). SH2/SH3 signaling proteins. [Review] [44 refs]. *Current Opinion in Genetics & Development* **4**, 25-30.

SCHMALZ, D., HUCHO, F. & BUCHNER, K. (1998). Nuclear import of protein kinase C occurs by a mechanism distinct from the mechanism used by proteins with a classical nuclear localization signal. *Journal of Cell Science* **111**, 1823-1830.

SCHMEICHEL, K.L. & BECKERLE, M.C. (1994). The LIM domain is a modular protein-binding interface. *Cell* **79**, 211-219.

SCHMIDT, A., SKALETZ-ROROWSKI, A., BREITHARDT, G. & BUDDECKE, E. (1995). Growth status-dependent changes of bFGF compartmentalisation and heparan sulfate structure in arterial smooth muscle cells. *Eur.J.Cell Biol.* **67**, 130-134.

SCHRAW, W. & RICHMOND, A. (1995). Melanoma growth stimulatory activity signaling through the class II interleukin-8 receptor enhances the tyrosine phosphorylation of Crk-associated substrate, p130, and a 70-kilodalton protein. *Biochemistry* **34**, 13760-13767.

SECRIST, J.P., SEHGAL, I., POWIS, G. & ABRAHAM, R.T. (1990). Preferential inhibition of the platelet-derived growth factor receptor tyrosine kinase by staurosporine. *Journal of Biological Chemistry* **265**, 20394-20400.

SEGER, R., SEGER, D., RESZKA, A.A., MUNAR, E.S., ELDAR-FINKELMAN, H., DOBROWOLSKA, G., JENSEN, A.M., CAMPBELL, J.S., FISCHER, E.H. & KREBS, E.G. (1994). Overexpression of mitogen-activated protein kinase kinase (MAPKK) and its mutants in NIH 3T3 cells. Evidence that MAPKK involvement in cellular proliferation is regulated by phosphorylation of serine residues in its kinase subdomains VII and VIII. *Journal of Biological Chemistry* **269**, 25699-25709.

SEGER, R. & KREBS, E.G. (1995). The MAPK signaling cascade. [Review] [99 refs]. *FASEB Journal* **9**, 726-735.

SEIFERT, R.A., HART, C.E., PHILLIPS, P.E., FORSTROM, J.W., ROSS, R., MURRAY, M.J. & BOWEN-POPE, D.F. (1989). 2 different subunits associate to create isoform-specific platelet-derived growth factor receptors. *J.Biol.Chem.* **264**, 8771-8778.

SEKO, Y., TAKAHASHI, N., TOBE, K., KADOWAKI, T. & YAZAKI, Y. (1999). Pulsatile stretch activates mitogen-activated protein kinase (MAPK) family members and focal adhesion kinase (p125(FAK)) in cultured rat cardiac myocytes. *Biochemical & Biophysical Research Communications* **259**, 8-14.

SERHAN, C.N., HAEGGSTROM, J.Z. & LESLIE, C.C. (1996). Lipid mediator networks in cell signaling: update and impact of cytokines. [Review] [62 refs]. *FASEB Journal* **10**, 1147-1158.

SERVANT, M.J., GIASSON, E. & MELOCHE, S. (1996). Inhibition of growth factor-induced protein synthesis by a selective MEK inhibitor in aortic smooth muscle cells. *Journal of Biological Chemistry* **271**, 16047-16052.

SEUFFERLEIN, T. & ROZENGURT, E. (1994). Lysophosphatidic acid stimulates tyrosine phosphorylation of focal adhesion kinase, paxillin, and p130. Signaling pathways and cross-talk with platelet-derived growth factor. *Journal of Biological Chemistry* **269**, 9345-9351.

SHAHAM, S. & HORVITZ, H.R. (1996). Developing Caenorhabditis elegans neurons may contain both cell-death protective and killer activities. *Genes Dev.* **10**, 578-591.

SHANAHAN, C.M., WEISSBERG, P.L. & METCALFE, J.C. (1993). Isolation of gene markers of differentiated and proliferating vascular smooth muscle cells. *Circulation Research* **73**, 193-204.

SHEN, Y. & SCHALLER, M.D. (1999). Focal adhesion targeting: the critical determinant of FAK regulation and substrate phosphorylation. *Molecular Biology of the Cell* **10**, 2507-2518.

SHI, Y., FARD, A., GALEO, A., HUTCHINSON, H.G., VERMANI, P., DODGE, GR, HALL, D.J., SHAHEEN, F. & ZALEWSKI, A. (1994). Transcatheter delivery of c-myc antisense oligomers reduces neointimal formation in a porcine model of coronary artery balloon injury. *Circulation* **90**, 944-951.

SHIBANUMA, M., MASHIMO, J., KUROKI, T. & NOSE, K. (1994). Characterization of the TGF beta 1-inducible hic-5 gene that encodes a putative novel zinc finger protein and its possible involvement in cellular senescence. *Journal of Biological Chemistry* **269**, 26767-26774.

SHIMOKADO, K., RAINES, E.W., MADTES, D.K., BARRET, T.B., BENDITT, E.P. & ROSS, R. (1985). A significant part of macrophage-derived growth factor consists of at least two forms of PDGF. *Cell* **43**, 277-286.

SHWEIKI, D., ITIN, A., SOFFER, D. & KESHET, E. (1992). Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* **359**, 843-845.

SHWEIKI, D., ITIN, A., NEUFELD, G., GITAY-GOREN, H. & KESHET, E. (1993). Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis. *Journal of Clinical Investigation* **91**, 2235-2243.

SIEG, D.J., ILIC, D., JONES, K.C., DAMSKY, C.H., HUNTER, T. & SCHLAEPFER, D.D. (1998). Pyk2 and Src-family protein-tyrosine kinases compensate for the loss of FAK in fibronectin-stimulated signaling events but Pyk2 does not fully function to enhance FAK- cell migration. *EMBO Journal* **17**, 5933-5947.

SIEG, D.J., HAUCK, C.R. & SCHLAEPFER, D.D. (1999). Required role of focal adhesion kinase (FAK) for integrin-stimulated cell migration. *Journal of Cell Science* **112**, 2677-2691.

SIEGBAHN, A., HAMMACHER, A., WESTERMARK, B. & HELDIN, C.H. (1990). Differential effects of the various isoforms of platelet-derived growth factor on chemotaxis of fibroblasts, monocytes and granulocytes. *J.Clin.Invest.* **85**, 916-920.

SIEGEL, G., RUCKBORN, K., SCHNALKE, F. & MULLER, J. (1993). Endothelial dysfunction in human atherosclerotic coronary arteries. *European Heart Journal* **14 Suppl I**, 99-103.

SINNETT-SMITH, J., ZACHARY, I., VALVERDE, A.M. & ROZENGURT, E. (1993). Bombesin stimulation of p125 focal adhesion kinase tyrosine phosphorylation. Role of protein kinase C, Ca2+ mobilization, and the actin cytoskeleton. *Journal of Biological Chemistry* **268** , 14261-14268.

SJOLUND, M., HEDIN, U., SEJERSEN, T., HELDIN, C.H. & THYBERG, J. (1988). Arterial smooth muscle cells express platelet-derived growth factor (PDGF) A chain mRNA, secrete a PDGF-like mitogen and bind exogenous PDGF in a phenotype and growth state-dependent manner. *J.Cell Biol.* **106**, 403

SJOLUND, M., RAHM, M., CLAESSON-WELSH, L., SEJERSEN, T., HELDIN, C.H. & THYBERG, J. (1990). Expression of PDGF-α and -β receptors in rat arterial smooth muscle cells is phenotype and growth state dependent. *Growth Factors* **3**, 191

SKALETZ-ROROWSKI, A., SCHMIDT, A., BREITHARDT, G. & BUDDECKE, E. (1996). Heparin-induced overexpression of basic fibroblast growth factor, basic fibroblast growth factor receptor, and cell-associated proteoheparan sulfate in cultured coronary artery smooth muscle cells. *Arterioscler.Thromb.Vasc.Biol.* **16**, 1063-1069.

SKALETZ-ROROWSKI, A., WALTENBERGER, J., MÜLLER, J.G., PAWLUS, E., PINKERNELL, K. & BREITHARDT, G. (1999). Protein kinase C mediates basic fibroblast growth factor-induced proliferation through mitogen-activated protein kinase in coronary smooth muscle cells. *Arterioscler.Thromb.Vasc.Biol.* **19**, 1608-1614.

SLOWIK, M.R., DE LUCA, L.G., MIN, W. & POBER, J.S. (1996). Ceramide is not a factor for tumour necrosis factor-induced gene expression but does cause programmed cell death in human vascular endothelial cells. *Circ.Res.* **79**, 736-747.

SMITH, S.C. & ALLEN, P.M. (1992). Neutralisation of endogenous tumour necrosis factor ameliorates the severity of myosin-induced myocarditis. *Circ.Res.* **70**, 856-863.

SOKER, S., TAKASHIMA, S., MIAO, H.Q., NEUFELD, G. & KLAGSBRUN, M. (1998). Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* **92**, 735-745.

SORENSEN, K.E., CELERMAJER, D.S., GEORGAKOPOULOS, D., HATCHER, G., BETTERIDGE, D.J. & DEANFIELD, J.E. (1994). Impairment of endothelium-dependent dilation is an early event in children with familial hypercholesterolemia and is related to the lipoprotein(a) level. *Journal of Clinical Investigation* **93**, 50-55.

SOUTHGATE, K.M., DAVIES, M., BOOTH, R.F. & NEWBY, A.C. (1992). Involvement of extracellular-matrix-degrading metalloproteinases in rabbit aortic smooth-muscle cell proliferation. *Biochemical Journal* **288**, 93-99.

SQUIER, M.K., MILLER, A.C., MALKINSON, A.M. & COHEN, J.J. (1994). Calpain activation in apoptosis. *J.Cell.Physiol.* **159**, 229-237.

STARY, H.C., CHANDLER, A.B., DINSMORE, R.E., FUSTER, V., GLAGOV, S., INSULL, W., ROSENFELD, M.E., SCHWARTZ, C.J., WAGNER, W.D. & WISSLER, R.W. (1995). A new definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis - a report from the committee on vascular lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation* **91**, 1355-1374.

STARY, H.C. (1989). Evolution and progression of atherosclerotic lesions in coronary arteries of children and young adults. *Arteriosclerosis* **9**, 119-132

STEINBERG, H.O., CHAKER, H., LEAMING, R., JOHNSON, A., BRECHTEL & BARON, A.D. (1996). Obesity/insulin resistance is associated with endothelial dysfunction. Implications for the syndrome of insulin resistance. *Journal of Clinical Investigation* **97**, 2601-2610.

STELLER, H. (1995). Mechanisms and genes of cellular suicide. *Science* **258**, 1955-1957.

STEPHENS, L.E., SUTHERLAND, A.E., KLIMANSKAYA, I.V., ANDRIEUX, A., MENESES, J., PEDERSEN, R.A. & DAMSKY, C.H. (1995). Deletion of beta 1 integrins in mice results in inner cell mass failure and peri-implantation lethality. *Genes & Development* **9**, 1883-1895.

SUDA, T., TAKAHASHI, T., GOLSTEIN, P. & NAGATA, S. (1993). Molecular cloning and expression of the Fas ligand, a novel member of the tumour necrosis factor family. *Cell* **75**, 1169-1178.

SUSIN, S.A., ZAMZAMI, N., CASTEDO, M., HIRSCH, T., MARCHETTI, P., MACHO, A., DAUGAS, E., GEUSKENS, M. & KROEMER, G. (1996). Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. *J.Exp.Med.* **184**, 1331-1341.

TACHIBANA, K., SATO, T., D'AVIRRO, N. & MORIMOTO, C. (1995). Direct association of pp125FAK with paxillin, the focal adhesion-targeting mechanism of pp125FAK. *Journal of Experimental Medicine* **182**, 1089-1099.

TACHIBANA, K., URANO, T., FUJITA, H., OHASHI, Y., KAMIGUCHI, K., IWATA, S., HIRAI & MORIMOTO, C. (1997). Tyrosine phosphorylation of Crk-associated substrates by focal adhesion kinase. A putative mechanism for the integrin-mediated tyrosine phosphorylation of Crk-associated substrates. *Journal of Biological Chemistry* **272**, 29083-29090.

TAKAHASHI, M., ISHIDA, T., TRAUB, O., CORSON, M.A. & BERK, B.C. (1997). Mechanotransduction in endothelial cells: temporal signaling events in response to shear stress. [Review] [55 refs]. *Journal of Vascular Research* **34**, 212-219.

TAKAHASHI, N., SEKO, Y., NOIRI, E., TOBE, K., KADOWAKI, T., SABE, H. & YAZAKI, Y. (1999). Vascular endothelial growth factor induces activation and subcellular translocation of focal adhesion kinase (p125FAK) in cultured rat cardiac myocytes. *Circulation Research* **84**, 1194-1202.

TAKAYAMA, S., CAZALS-HATEM, D.L., KITADA, S., TANAKA, S., MIYASHI, T., HOVER, L.R., HUEN, D., RICKINSON, A., VEERAPANDIAN, P., KRAJEWSKI, S., SAITO, K. & REED, J.C. (1994). Evolutionary conservation of function among mammalian, avian and viral homologues of the bcl-2 oncoprotein: structure-function implications. *DNA Cell Biol.* **13**, 679-692.

TALLETT, A., CHILVERS, E.R., MACKINNON, A.C., HASLETT, C. & SETHI, T. (1996). Neuropeptides stimulate tyrosine phosphorylation and tyrosine kinase activity in small cell lung cancer cell lines. *Peptides* **17**, 665-673.

TAMURA, M., GU, J., DANEN, E.H., TAKINO, T., MIYAMOTO, S. & YAMADA, K.M. (1999a). PTEN interactions with focal adhesion kinase and suppression of the extracellular matrix-dependent phosphatidylinositol 3-kinase/Akt cell survival pathway. *Journal of Biological Chemistry* **274**, 20693-20703.

TAMURA, M., GU, J., TAKINO, T. & YAMADA, K.M. (1999b). Tumor suppressor PTEN inhibition of cell invasion, migration, and growth: differential involvement of focal adhesion kinase and p130Cas. *Cancer Research* **59**, 442-449.

TANAKA, M., ITO, H., ADACHI, S., AKIMOTO, H., KASAJIMA, T., MARUMO, F. & HIROE, M. (1994). Hypoxia induces apoptosis with enhanced expression of Fas antigen messenger RNA in cultured neonatal rat cardiomyocytes. *Circ.Res.* **75**, 426-433.

TARTAGLIA, L.A., AYRES, T.M., WONG, G.H. & GOEDDEL, D.V. (1993). A novel domain within the 55 kD TNF receptor signals cell death. *Cell* **74**, 845-853.

TEIGER, E., THAN, V.D., RICHARD, L., WISNEWSKY, C., TEA, B.S., GABOURY, L., TREMBLAY, J., SCHWARTZ, K. & HAMET, P. (1996). Apoptosis in pressure overload-induced heart hypertrophy in the rat. *J.Clin.Invest.* **97**, 2891-2897.

TERMAN, B.I., DOUGHER-VERMAZEN, M., CARRION, M.E., DIMITROV, D., ARMELLINO, D.C., GOSPODAROWICZ, D. & BOHLEN, P. (1992). Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. *Biochemical & Biophysical Research Communications* **187**, 1579-1586.

THOMAS, J.W., COOLEY, M.A., BROOME, J.M., SALGIA, R., GRIFFIN, J.D., LOMBARDO, C.R. & SCHALLER, M.D. (1999). The role of focal adhesion kinase binding in the regulation of tyrosine phosphorylation of paxillin. *Journal of Biological Chemistry* **274**, 36684-36692.

THOMAS, S.M., HAGEL, M. & TURNER, C.E. (1999). Characterization of a focal adhesion protein, Hic-5, that shares extensive homology with paxillin. *Journal of Cell Science* **112**, 181-190.

THOMAS, W.A., LEE, K.T. & KIM, D.N. (1985). Cell population kinetics in atherogenesis. Cell births and losses in intimal cell mass-derived lesions in the abdominal aorta of swine. *Annals of the New York Academy of Sciences.* **454**, 305-315.

TOBE, K., MATUOKA, K., TAMEMOTO, H., UEKI, K., KABURAGI, Y., ASAI, S., NOGUCHI, T., MATSUDA, M., TANAKA, S. & HATTORI, S. (1993). Insulin stimulates association of insulin receptor substrate-1 with the protein abundant Src homolgy/growth factor receptor-bound protein 2. *J.Biol.Chem.* **268**, 11167-11171.

TOULLEC, D., PIANETTI, P., COSTE, H., BELLEVERGUE, P., GRAND-PERRET, T., AJAKANE, M., BAUDET, V., BOISSIN, P., BOURSIER, E. & LORIOLLE, F. (1991). The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *Journal of Biological Chemistry* **266**, 15771-15781.

TRAN, J., RAK, J., SHEEHAN, C., SAIBIL, S.D., LACASSE, E., KORNELUK, R.G. & KERBEL, R.S. (1999). Marked induction of the IAP family antiapoptotic proteins survivin and XIAP by VEGF in vascular endothelial cells. *Biochemical & Biophysical Research Communications* **264**, 781-788.

TRAVERSE, S., GOMEZ, N., PATERSON, H., MARSHALL, C. & COHEN, P. (1992). Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor. *Biochemical Journal* **288**, 351-355.

TSAO, P.S., BUITRAGO, R., CHAN, J.R. & COOKE, J.P. (1996). Fluid flow inhibits endothelial adhesiveness. Nitric oxide and transcriptional regulation of VCAM-1. *Circulation* **94**, 1682-1689.

TURNER, C.E., GLENNEY, J.R.J. & BURRIDGE, K. (1990). Paxillin: a new vinculin-binding protein present in focal adhesions. *Journal of Cell Biology* **111**, 1059-1068.

TURNER, C.E. & MILLER, J.T. (1994). Primary sequence of paxillin contains putative SH2 and SH3 domain binding motifs and multiple LIM domains: identification of a vinculin and pp125Fak-binding region. *Journal of Cell Science* **107**, 1583-1591.

TURNER, C.E., PIETRAS, K.M., TAYLOR, D.S. & MOLLOY, C.J. (1995). Angiotensin II stimulation of rapid paxillin tyrosine phosphorylation correlates with the formation of focal adhesions in rat aortic smooth muscle cells. *Journal of Cell Science* **108**, 333-342.

TURNER, C.E., BROWN, M.C., PERROTTA, J.A., RIEDY, M.C., NIKOLOPOULOS, S.N., MCDONALD, AR, BAGRODIA, S., THOMAS, S. & LEVENTHAL, P.S. (1999). Paxillin LD4 motif binds PAK and PIX through a novel 95-kD ankyrin repeat, ARF-GAP protein: A role in cytoskeletal remodeling. *Journal of Cell Biology* **145**, 851-863.

UEDA, N. & SHAH, S.V. (1994). Apoptosis. [Review] [85 refs]. *Journal of Laboratory & Clinical Medicine* **124**, 169-177.

ULLRICH, A., BELL, J.R., CHEN, E.Y., HERRERA, R., PETRUZZELLI, L.M., DULL, T.J., GRAY, A., COUSSENS, L., LIAO, Y.C., TSUBOKAWA, M., MASON, A., SEEBURG, P.H., GRUNFELD, C., ROSEN, O.M. & RAMACHANDRAN, J. (1985). Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. *Nature* **313**, 756-761.

ULLRICH, A., GRAY, A., TAM, A.W., YANGFENG, T., TSUBOKAWA, M., COLLINS, C., HENZEL, W.J., LEBON, T., KATHURIA, S., CHEN, E.Y., FRANCKE, U., RAMACHANDRAN, J. & FUJITAYAMAGUCHI, Y. (1986). Insulin-like growth factor-1 receptor primary structure- comparison with insulin receptor suggests structural determinants that define functional specificity. *EMBO J.* **5**, 2503-2512.

ULLRICH, A. & SCHLESSINGER, J. (1990). Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**, 203-212.

UREN, A.G., PAKUSCH, M., HAWKINS, C.J., PULS, K.L. & VAUX, D.L. (1996). Cloning and expression of apoptosis inhibitory protein homologs that function to inhibit apoptosis and/or bind tumour necrosis factor receptor-associated factors. *Proc.Natl.Acad.Sci.U.S.A.* **93**, 4974-4978.

VAN DE WATER, B., NAGELKERKE, J.F. & STEVENS, J.L. (1999). Dephosphorylation of focal adhesion kinase (FAK) and loss of focal contacts precede caspase-mediated cleavage of FAK during apoptosis in renal epithelial cells. *Journal of Biological Chemistry* **274** , 13328-13337.

VANDE, P.S., BROWN, M.C. & TURNER, C.E. (1998). Association of Bovine Papillomavirus Type 1 E6 oncoprotein with the focal adhesion protein paxillin through a conserved protein interaction motif. *Oncogene* **16**, 43-52.

VILLA, P.G., HENZEL, W.J., SENSENBRENNER, M., HENDERSON, C.E. & PETTMANN, B. (1998). Calpain inhibitors, but not caspase inhibitors, prevent actin proteolysis and DNA fragmentation during apoptosis. *J.Cell Sci.* **111**, 713-722.

VOGT, C. (1842). Untersuchungen uber die Entwicklungsgeschichte der Geburtshelferkroete. *Alytes obstetricans*

VUORI, K. & RUOSLAHTI, E. (1995). Tyrosine phosphorylation of p130Cas and cortactin accompanies integrin-mediated cell adhesion to extracellular matrix. *Journal of Biological Chemistry* **270**, 22259-22262.

VUORI, K., HIRAI, H., AIZAWA, S. & RUOSLAHTI, E. (1996). Introduction of p130cas signaling complex formation upon integrin-mediated cell adhesion: a role for Src family kinases. *Molecular & Cellular Biology* **16**, 2606-2613.

WADA, A., FUKUDA, M., MISHIMA, M. & NISHIDA, E. (1998). Nuclear export of actin: a novel mechanism regulating the subcellular localization of a major cytoskeletal protein. *EMBO Journal* **17**, 1635-1641.

WALKER, L.N., BOWEN-POPE, D.F., ROSS, R. & REIDY, M.A. (1986). Production of platelet-derived growth factor-like molecules by cultured arterial smooth muscle cells accompanies proliferation after injury. *Proc.Natl.Acad.Sci.U.S.A.* **83**, 7311-7315.

WALKER, N.I. & GOBE, G.C. (1987). Cell death and cell proliferation during atrophy of the rat parotid gland induced by duct obstruction. *Journal of Pathology* **153**, 333-344.

WALKER, N.I., HARMON, B.V., GOBE, G.C. & KERR, J.F. (1988). Patterns of cell death. [Review] [236 refs]. *Methods & Achievements in Experimental Pathology* **13**, 18-54.

WALKER, N.I., BENNETT, R.E. & KERR, J.F. (1989). Cell death by apoptosis during involution of the lactating breast in mice and rats. *American Journal of Anatomy* **185**, 19-32.

WALTERS, T.K., GOROG, D.A. & WOOD, R.F. (1994). Thrombin generation following arterial injury is a critical initiating event in the pathogenesis of the proliferative stages of the atherosclerotic process. *Journal of Vascular Research* **31**, 173-177.

WALTZ, D.A., SAILOR, L.Z. & CHAPMAN, H.A. (1993). Cytokines induce urokinase-dependent adhesion of human myeloid cells. A regulatory role for plasminogen activator inhibitors. *Journal of Clinical Investigation* **91**, 1541-1552.

WAN, Y., KUROSAKI, T. & HUANG, X.Y. (1996). Tyrosine kinases in activation of the MAP kinase cascade by G-protein-coupled receptors. *Nature* **380**, 541-544.

WANG, W., CHEN, H.J., SCHWARTZ, A., CANNON, P.J. & RABBANI, L.E. (1997). T cell lymphokines modulate bFGF-induced smooth muscle cell fibrinolysis and migration. *Am.J.Physiol.* **272**, 392-398.

WATT, F. & MOLLOY, P.L. (1993). Specific cleavage of transcription factors by the thiol protease *m*-calpain. *Nucleic Acids Res.* **21**, 5092-5100.

WEBER, K.T. & BRILLA, C.G. (1991). Pathological hypertrophy and cardiac interstitium: fibrosis and renin-angiotensin-aldosterone system. *Circulation* **83**, 1849-1865.

WEIL, M., JACOBSON, M.D., COLES, H.S., DAVIES, T.J., GARDENER, R.L., RAFF, K.D. & RAFF, M.C. (1998). Constitutive expression of the machinery for programmed cell death. *J.Cell Biol.* **133**, 1053-1059.

WEN, L.P., FAHRNI, J.A., TROIE, S., GUAN, J.L., ORTH, K. & ROSEN, G.D. (1997). Cleavage of focal adhesion kinase by caspases during apoptosis. *Journal of Biological Chemistry* **272**, 26056-26061.

WEN, W., MEINKOTH, J.L., TSIEN, R.Y. & TAYLOR, S.S. (1995). Identification of a signal for rapid export of proteins from the nucleus. *Cell* **82**, 463-473.

WHITE, G.E., GIMBRONE, M.A.J. & FUJIWARA, K. (1983). Factors influencing the expression of stress fibers in vascular endothelial cells in situ. *Journal of Cell Biology* **97**, 416-424.

WHO (1985). World Health Organisation. *WHO Tech.Rep.Serv.* **143**, 1-20.

WIDMANN, C., GIBSON, S. & JOHNSON, G.L. (1998). Caspase-dependent cleavage of signaling proteins during apoptosis. A turn-off mechanism for anti-apoptotic signals. *Journal of Biological Chemistry* **273**, 7141-7147.

WIGHT, T.N., COOKE, P.H. & SMITH, S.C. (1977). An electron microscopic study of pigeon aorta cell cultures. Cytodifferentiation and intracellular lipid accumulation. *Experimental & Molecular Pathology* **27**, 1-18.

WILCOX, J.N., SMITH, K.M., WILLIAMS, L.T., SCHWARTZ, S.M. & GORDON, D. (1988). Platelet-derived growth factor mRNA detection in human atherosclerotic plaques by *in situ* hybridisation. *J.Clin.Invest.* **82**, 1134-1143.

WILSON, L., CARRIER, M.J. & KELLIE, S. (1995). pp125FAK tyrosine kinase activity is not required for the assembly of F-actin stress fibres and focal adhesions in cultured mouse aortic smooth muscle cells. *Journal of Cell Science* **108**, 2381-2391.

WOLF, B.B., GOLDSTEIN, J.C., STENNICKE, H.R., BEERE, H., AMARANTE-MENDES, G.P., SALVESEN, G.S. & GREEN, D.R. (1999). Calpain functions in a caspase-independent manner to promote apoptosis-like events during platelet activation. *Blood* **94**, 1683-1692.

WOOD, D.E. & NEWCOMB, E.W. (1999). Caspase-dependent activation of calpain during drug-induced apoptosis. *J.Biol.Chem.* **274**, 8309-8315.

WOODS, A. & COUCHMAN, J.R. (1992). Protein kinase C involvement in focal adhesion formation. *Journal of Cell Science* **101**, 277-290.

WU, R., DURICK, K., SONGYANG, Z., CANTLEY, L.C., TAYLOR, S.S., GILL & GN (1996). Specificity of LIM domain interactions with receptor tyrosine kinases. *Journal of Biological Chemistry* **271**, 15934-15941.

XIA, P., AIELLO, L.P., ISHII, H., JIANG, Z.Y., PARK, D.J., ROBINSON, GS, TAKAGI, H., NEWSOME, W.P., JIROUSEK, M.R. & KING, G.L. (1996). Characterization of vascular endothelial growth factor's effect on the activation of protein kinase C, its isoforms, and endothelial cell growth. *Journal of Clinical Investigation* **98**, 2018-2026.

XIA, P., WANG, L., VADAS, M.A. & GARDENER, R.L. (1998). Tumour necrosis factor induces sphingosine kinase activation in human endothelial cells; a possible mechanism for resistance to apoptosis. *(Abstract) Xth International Vascular Biology Meeting, Cairns, Australia.*

XING, Z., CHEN, H.C., NOWLEN, J.K., TAYLOR, S.J., SHALLOWAY, D. & GUAN, J.L. (1994). Direct interaction of v-Src with the focal adhesion kinase mediated by the Src SH2 domain. *Molecular Biology of the Cell* **5**, 413-421.

XIONG, W. & PARSONS, J.T. (1997). Induction of apoptosis after expression of PYK2, a tyrosine kinase structurally related to focal adhesion kinase. *Journal of Cell Biology* **139**, 529-539.

XIONG, W.C., MACKLEM, M. & PARSONS, J.T. (1998). Expression and characterization of splice variants of PYK2, a focal adhesion kinase-related protein. *Journal of Cell Science* **111** , 1981-1991.

XU, J., YEH, C.H., CHEN, S.W., HE, L.M., SENSI, S.L., CANZONIERO, L.M.T., CHOI, D.W. & HSU, C.Y. (1998). Involvement of de novo ceramide biosynthesis in tumour necrosis factor-α cycloheximide-induced cerebral endothelial death. *J.Biol.Chem.* **273**, 16521-16526.

XU, L.H., OWENS, L.V., STURGE, G.C., YANG, X., LIU, E.T., CRAVEN, R.J. & CANCE, W.G. (1996). Attenuation of the expression of the focal adhesion kinase induces apoptosis in tumor cells. *Cell Growth & Differentiation* **7**, 413-418.

XU, L.H., YANG, X., CRAVEN, R.J. & CANCE, W.G. (1998). The COOH-terminal domain of the focal adhesion kinase induces loss of adhesion and cell death in human tumor cells. *Cell Growth & Differentiation* **9**, 999-1005.

YAMAKITA, Y., TOTSUKAWA, G., YAMASHIRO, S., FRY, D., ZHANG, X., HANKS, S.K. & MATSUMURA, F. (1999). Dissociation of FAK/p130(CAS)/c-Src complex during mitosis: role of mitosis-specific serine phosphorylation of FAK. *Journal of Cell Biology* **144**, 315-324.

YANG, J., LIU, X., BHALLA, K., KIM, C.N., IBRADO, A.M., CAI, J., PENG, T.I., JONES, D.P. & WANG, X. (1997). Prevention of apoptosis by Bcl-2: release of cytochrome C from mitochondria blocked. *Science* **275**, 1129-1132.

YANG, J.T., RAYBURN, H. & HYNES, R.O. (1993). Embryonic mesodermal defects in alpha 5 integrin-deficient mice. *Development* **119**, 1093-1105.

YAYON, A., KLAGSBRUN, M., ESKO, J.D., LEDER, P. & ORNITZ, D.M. (1991). Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell* **64**, 841-848.

YOSHIDA, A., ANAND-APTE, B. & ZETTER, B.R. (1996). Differential endothelial migration and proliferation to basic fibroblast growth factor and vascular endothelial growth factor. *Growth Factors* **13**, 57-64.

YU, H., LI, X., MARCHETTO, G.S., DY, R., HUNTER, D., CALVO, B., DAWSON, T.L., WILM, M., ANDEREGG, R.J., GRAVES, L.M. & EARP, H.S. (1996). Activation of a novel calcium-dependent protein-tyrosine kinase. Correlation with c-Jun N-terminal kinase but not mitogen-activated protein kinase activation. *Journal of Biological Chemistry* **271**, 29993-29998.

YUE, T.L., MCKENNA, P.J., OHLSTEIN, E.H., FARACH-CARSON, M.C., BUTLER, W.T., JOHANSON, K., MCDEVITT, P., FEUERSTEIN, G.Z. & STADEL, J.M. (1994). Osteopontin-stimulated vascular smooth muscle cell migration is mediated by beta 3 integrin. *Experimental Cell Research* **214**, 459-464.

ZACHARY, I. & ROZENGURT, E. (1992). Focal adhesion kinase (p125FAK): a point of convergence in the action of neuropeptides, integrins, and oncogenes. [Review] [18 refs]. *Cell* **71** , 891-894.

ZACHARY, I., SINNETT-SMITH, J. & ROZENGURT, E. (1992). Bombesin, vasopressin, and endothelin stimulation of tyrosine phosphorylation in Swiss 3T3 cells. Identification of a novel tyrosine kinase as a major substrate. *Journal of Biological Chemistry* **267**, 19031-19034.

ZACHARY, I., SINNETT-SMITH, J., TURNER, C.E. & ROZENGURT, E. (1993). Bombesin, vasopressin, and endothelin rapidly stimulate tyrosine phosphorylation of the focal adhesion-associated protein paxillin in Swiss 3T3 cells. *Journal of Biological Chemistry* **268** , 22060-22065.

ZAMZAMI, N., SUSIN, S.A., MARCHETTI, P., HIRSCH, T., GOMEZ-MONTEREY, I., CASTEDO, M. & KROEMER, G. (1996). Mitochondrial control of nuclear apoptosis. *J.Exp.Med.* **183**, 1533-1544.

ZHANG, C., LAMBERT, M.P., BUNCH, C., BARBER, K., WADE, W.S., KRAFFT, G.A. & KLEIN, W.L. (1994). Focal adhesion kinase expressed by nerve cell lines shows increased tyrosine phosphorylation in response to Alzheimer's A beta peptide. *Journal of Biological Chemistry* **269**, 25247-25250.

ZHANG, X., WRIGHT, C.V. & HANKS, S.K. (1995). Cloning of a Xenopus laevis cDNA encoding focal adhesion kinase (FAK) and expression during early development. *Gene* **160**, 219-222.

ZHAO, J.H., REISKE, H. & GUAN, J.L. (1998). Regulation of the cell cycle by focal adhesion kinase. *Journal of Cell Biology* **143**, 1997-2008.

ZHENG, C., XING, Z., BIAN, Z.C., GUO, C., AKBAY, A., WARNER, L. & GUAN, J.L. (1998). Differential regulation of Pyk2 and focal adhesion kinase (FAK). The C-terminal domain of FAK confers response to cell adhesion. *Journal of Biological Chemistry* **273**, 2384-2389.

ZHENG, L., FISHER, G., MILLER, R.E., PESCHON, J., LYNCH, D.H. & LENARDO, M.J. (1995). Induction of apoptosis in mature T cells by tumour necrosis factor. *Nature* **377**, 348-351.

ZHIVOTOVSKY, B., BURGESS, D.H., VANAGS, D.M. & ORRENIUS, S. (1997). Involvement of cellular proteolytic machinery in apoptosis. [Review] [74 refs]. *Biochemical & Biophysical Research Communications* **230**, 481-488.

ZHU, W., MURTHA, P.E. & YOUNG, C.Y.F. (1995). Calpain inhibitor-induced apoptosis in human prostate adenocarcinoma cells. *Biochem.Biophys.Res.Commun.* **214**, 1130-1137.

APPENDIX

Antibodies for Experimental Procedures

All antibodies used in the experiments reproduced in this thesis were affinity purified products obtained from commercial sources.

Monoclonal antibodies from Transduction Laboratories Inc. (stock concentrations of 250 μg/ml), were used at the following dilutions: FAK (354-533) 1:1000 for western blotting (WB) and 1:25 for immunofluorescence (IF). Paxillin 1:10000 WB, 1:100 IF. $P130^{Cas}$ 1:1000 WB, nucleoporin 1:1000 WB and GRB-2, 1:5000 WB. Monoclonal antibodies to smooth muscle myosin heavy chain (1:100000 WB) and smooth muscle α-actin (1:100000 WB) and vinculin (1:50000 WB, 1:400 IF), were purified from mouse ascites fluid and purchased from Sigma Chemical Co. Antibodies to the total and activated (stock - 0.2mg/ml) forms of MAP kinase were from New England Biolabs Inc. and used at WB dilution of 1:1000.

Polyclonal antibodies from Santa Cruz Inc. came in stock concentrations of 200 μg/ml and were used at the following dilutions: FAK carboxy terminus, amino acid residues 1033-1052 (FAK-C), 1:1000 WB, 1:20 IF. FAK amino terminus, amino acid residues 1-17 (FAK-A), 1:100 WB, 1:10 IF. The corresponding peptide immunogens to these antibodies, were used at dilutions specified in experimental procedures. Monoclonal antibodies to Rho and PCNA were also from Santa Cruz Inc. and used at dilutions of 1:1000 from stock concentrations of 200 μg/ml. Protein A/G+ agarose and normal rabbit and mouse immunoglobulins came from Santa Cruz Inc. Antibody to human urokinase receptor (No. 398) was purchased from American_Diagnostica Inc. and used at 1 μg/ml following reconstitution. FAK phospho-specific antibodies: Y577 (450 mg/ml), Y861 (640 mg/ml), Y925, Y576, Y407 and Y397 (all at 250 mg/ml) were from Biosource and all used at 0.5μg/ml except for the Y861 product which was used at 1 μg/ml.

HRP-conjugated secondary antibodies to mouse and rabbit immunoglobulins from Amersham International and fluorochrome-conjugated secondary antibodies from DAKO Corp. were used at standard dilutions as recommended by the manufacturer.