REGULATION OF MMP-9

IN

HUMAN OVARIAN CANCER

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A thesis submitted for the degree of Doctor of Philosophy

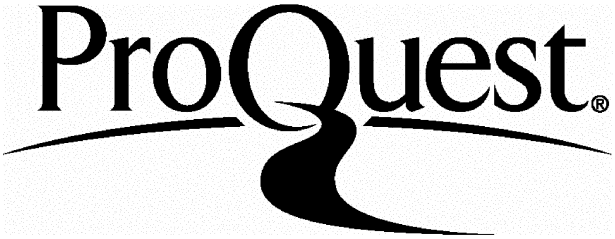
University College London

May 1998

Imperial Cancer Research Fund

Lincoln's Inn Fields

London UK



Diese Doktorarbeit widme ich meiner Mutter.

Abstract

Matrix metalloproteases (MMPs) are a family of structurally and functionally related endopeptidases. They have a zinc ion at their active site and are released as inactive pro-forms. Activation enables MMPs to degrade components of the extracellular matrix and this been associated with tumour growth and metastasis. The aim of this thesis was to establish the pattern of MMPs and tissue inhibitor of metalloproteinases (TIMPs) present in human ovarian cancer and to investigate further one MMP, MMP-9.

MMP/TIMP gene expression was assessed by RT-PCR in biopsies of normal, benign and malignant human ovary and compared to ovarian cancer cell lines and xenograft models of human ovarian cancer. Expression of MMP-2, -11, MT-1-MMP, TIMP-1, -2 and -3 was detectable in all tissue samples. MMP-1, -10 and MT-2-MMP were rarely detectable and MMP-3, -7, -9, -13 and MT-3-MMP were variably expressed. MMP/TIMP expression in the malignant biopsies was similar to that in ovarian cancer cell lines and xenograft models of human ovarian cancer.

MMP-9 production was further investigated in an *in vitro* model of tumour cell macrophage interaction. In human ovarian cancer MMP-9 localises to infiltrating macrophages but little is known of its regulation. Co-culture of ovarian cancer cells (PEO1) and a monocytic cell line (THP-1) led to production of 92kDa proMMP-9. PEO1 conditioned medium (CM) also stimulated THP-1 cells or isolated peripheral blood monocytes to produce proMMP-9. Expression of TIMP-1, however, remained unaffected. The metalloprotease stimulating factor (MMPSF) present in CM was not TNF-α, but acted in a synergistic fashion with autocrine monocyte-derived TNF-α to stimulate release of monocytic proMMP-9. MMPSF was further characterised.

In all experiments only proMMP-9 was released. Cell extracts of THP-1 cells contained a 92 and 85kDa form of MMP-9. N-terminal sequencing revealed that both proteins contained the full pro-peptide and were therefore proteolytically inactive pro-forms of MMP-9.

Acknowledgements

I would like to acknowledge the help and advise I obtained throughout this thesis from all members of the Biological Therapies Laboratory. In particular I would like to thank Dr. Frances R. Balkwill for including me in her team here at the ICRF. I very much appreciate her support, the space she gave me to develop my own thoughts and the time she spent to discuss our views. Furthermore, I would like to thank my co-PhDs Dr. Rupert Negus and Sergio Dias for their help and friendship over the past years. I will miss the conversations we had over the "swift ones down The George" on Friday nights. I also would like to mention Dr. Al Stewart for his advise during the characterisation of MMPSF and his always encouraging "Hang in, man! Hang in!".

Finally I would like to thank my wife *in spe* Isabella who had the patience to endure the neglect she had to suffer from during this thesis and the writing period in particular.

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Abbreviations

APMA	aminophenyl mercuric acetate
BSA	bovine serum albumin
CM	conditioned medium
DTT	dithiothreitol
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
FCS	foetal calf serum
FGF	fibroblast growth factor
FPLC/HPLC	fast protein liquid chromatography/high pressure liquid chromatography
IFN	interferon
IL	interleukin
M	molar (mol per litre)
M-CSF	macrophage colony stimulating factor
MMP	matrix metalloproteinase
MMPI	(synthetic) matrix metalloproteinase inhibitor
MMPSF	matrix metalloproteinase stimulating factor
PAF	platelet activating factor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PHA	phytohemagglutinin or phaseolus vulgaris agglutinin
PVDF	polyvinylidene fluoride
SDS	sodium dodecyl sulphate
TEA	triethanolamine
TGF-α	transforming growth factor-alpha
TIMP	tissue inhibitor of matrix metalloproteinases
TNF-α	tumour necrosis factor-alpha
WGA	wheat germ agglutinin

1. Introduction

1.1 Ovarian Cancer

1.1.1 The ovary and ovarian tumours

The ovary consists of a central medulla surrounded by a cortex which in turn is surrounded by a monolayer of epithelium. These epithelial cells stem from the coelomic endoderm and are separated from the stromal cells by basement membrane. The cortex, which consists of dense stroma, ovarian follicles and corpora lutea, is the functional part of the ovary with respect to oocyte production (1).

Ovarian cancer is the sixth most common cancer among women in the US (2). The empirical lifetime risk of developing ovarian cancer is 1:70 and the median age at diagnosis is 62 years (3). The incidence of ovarian cancer is highest in the highly industrialised countries, in particular Western and Northern Europe and North America (3). Ovarian tumours can arise from any component of the ovary but most (65-70%) are derived from the epithelial layer (4). This type of tumour usually affects women older than 20 years and its incidence increases with age peaking at approximately 50 cases per 100 000 women in the age group 70-75 (2). The second most common ovarian tumours (15-20%) arise from germ cells present in the ovary. In contrast to the epithelial tumours, germ cell tumours tend to affect women of young age (0-25 years). Finally, tumours can also arise from the cortex stroma (5-10%) or may be derived from metastases (5%) of a non ovarian primary tumour (4).

1.1.2 Clinical description of ovarian cancer - stage

More than 50% of patients present with advanced stage disease (stage III-IV, Table 1.1) characterised by metastasis beyond the pelvic cavity (5). Stage at presentation is strongly correlated to the 5-year survival rate (Table 1.1) and therefore cure. Late presentation is thought to be the main reason for the poor prognosis (Table 1.1). The cellular and molecular changes associated with ovarian cancer are poorly understood. Therefore, despite important advances in treatment strategies over the past decades,

there has been no significant improvement in overall survival for patients with advanced stage disease.

Stage	incidence	5-year survival rate	Clinical description
Stage I	26.1%		Growth limited to the ovaries.
Ia		85-100%	Growth limited to one ovary; no ascites. No tumour on external surface; intact capsule.
Ib		85-100%	Growth limited to both ovaries; no ascites. No tumour on external surface; intact capsule.
Ic		85-100%	Tumour either stage Ia or Ib, but with ascites or positive peritoneal washings.
Stage II	15.4%		Growth involving one or both ovaries with pelvic extension.
IIa		40-50%	Extension and/or metastases to the uterus and/or fallopian tubes.
IIb		40-50%	Extension to other pelvic tissues including peritoneum and uterus.
IIc		40-50%	Tumour either stage IIa or IIb, but with ascites or positive peritoneal washings.
Stage III	39.1%		Growth involving one or both ovaries with intraperitoneal metastases outside the pelvis and/or positive retroperitoneal nodes. Tumour limited to the true pelvis with histologically proven malignant extension to the small bowel or omentum.
IIIa		40-50%	Tumour grossly limited to the true pelvis with negative nodes but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces.
IIIb		20%	Tumour involving one or both ovaries with histologically confirmed implants of abdominal peritoneal surfaces, none exceeding 2cm in diameter. Nodes are negative.
IIIc		5-10%	Abdominal implants greater than 2cm in diameter. Nodes are negative.
Stage IV	16.3%	5-10%	Growth involving one or both ovaries with distant metastases. Pleural effusion must have positive cytology. Parenchymal liver metastases equal stage IV.

Table 1.1 Definition of clinical stages, their incidences and associated 5-year survival rates
The parameters defining the clinical stage of human ovarian cancer were established by the International Federation of Gynaecology and Obstetrics (FIGO) (5). The 5-year survival rate declines with advanced stage (3). More then 50% of women present with advanced stage disease (stage III-IV) (5).

1.1.3 Tumour histology

Tumours derived from the epithelial cell monolayer have been classified histologically into several sub-categories on the basis of parameters such as cell shape, formation of cysts, colour and density of cystic fluid (Table 1.2). Serous tumours are most common (40%). They are cystic neoplasms which are lined by tall, columnar, ciliated epithelial cells and filled with serous fluid (4). Only 25% of serous and 10% of mucinous tumours are malignant whereas the remaining are either benign or borderline (4). This distinction is important in view of the associated 10-year survival rates (Table 1.2). Malignant

tumours are characterised by having invaded through the epithelial basement membrane whereas in benign and borderline tumours, the basement membrane remains intact (4). The composition of tissue sections of human ovarian cancer with respect to the cellular infiltrate and amount of tumour versus tumour stroma has been analysed recently (6). In this study which was mainly based on serous ovarian carcinomas, the sections were found to consist of on average 43% tumour, 37% stroma and 4% necrosis. The cellular infiltrate was primarily composed of $CD68^+$ macrophages and $CD8^+/CD45RO^+$ T cells and localised mainly to tumour stroma rather than tumour areas (6).

Type	Incidence	10- year survival rate
Serous	40%	
Benign (60%)		
Borderline (15%)		75%
Malignant (25%)		10-20%
Mucinous	10%	
Benign (80%)		
Borderline (10%)		95%
Malignant (10%)		60%
Endometrioid carcinoma	20%	na.
Undifferentiated carcinoma	10%	na.
Clear cell carcinoma	6%	na.
others	14%	na.

Table 1.2 Incidence and survival rates of histological subtypes of human ovarian cancer Modified from (4), na. = not available.

1.1.4 Risk factors associated with epithelial ovarian cancer

Although the specific causes of ovarian cancer remain unclear, there are, however, factors which have been associated with an increased or a decreased risk. Age, race, the use of talcum powder on the perineum, a history of endometrial or breast cancer are associated with an increase in risk whereas the use of oral contraceptives, a history of breast feeding, tubal ligation and hysterectomy were associated with a reduction in risk (7). Pregnancy also has a protective effect with the risk of nulliparous women being 1.5-3.3 times greater than that of parous women (8). The "Fathalla Hypothesis" suggests that this effect may be due to the rest that pregnancy offers from incessant ovulation (9). Among the risk factors mentioned, family history and nulliparity are considered to be the two most important ones (10, 11). A single family member with ovarian cancer increases the relative risk 2.3 fold for another to develop this malignancy (12).

In 1990 the first major breast cancer susceptibility gene BRCA1 was mapped to the long arm of chromosome 17 (13). Mutations in this region were subsequently reported to be associated with some ovarian cancer cases and an international study showed that the BRCA1 gene was often implicated when there was an inherited susceptibility to both breast and ovarian cancer (14, 15). The BRCA1 gene is mutated in the germline and the normal allele is lost in tumour tissue from hereditary breast and ovarian cancer. Women who carry a mutation in this gene have a 59% risk of developing breast or ovarian cancer by the age of 50 and 82% by the age of 70 (16). The overall population frequency of a BRCA1 mutation was estimated to be 0.0007 (17). Based on this estimation BRCA1 would account for an approximately 2.6% of ovarian cancer cases below the age of 70 and 4.7% of the cases below the age of 50 (18).

1.1.5 Current treatment of ovarian cancer

As mentioned earlier, patients with ovarian cancer usually present at advanced stage of the disease. Initial treatment is therefore debulking surgery often combined with total abdominal hysterectomy, bilateral salpingo-oophorectomy and/or omentectomy (19). Survival of patients is directly related to the residual tumour mass (20). Surgery is usually followed by multi-agent therapy including cisplatin and paclitaxel (taxol) or cyclophosphamide (3). In spite of these treatments less then 30% of patients survive for more then 5 years (3).

More recently, some progress in ovarian cancer therapy has been made through a novel approach in cancer treatment which aims to reduce/prevent tissue remodelling and angiogenesis involved in tumour growth and metastasis (21). Industrial research has produced a series of low molecular weight compounds targeted against the enzymes implicated in these processes, the matrix metalloproteases (MMPs). They have been tested successfully in several animal models of human malignancy including ovarian cancer (22) and are currently in clinical trials (23-28).

1.2 Matrix Metalloproteases - An overview

1.2.1 Tissue architecture and MMPs

A tissue is an organised structure consisting of both cells and extracellular matrix (ECM). The cellular compartment can be divided into epithelial and stromal cells. The epithelial cells of the ovary are derived from the coelomic endoderm and the stromal cells from the mesoderm (29). The ECM is a dense matrix of mostly fibrillar proteins and glycans. Collagens, non collagenous glycoproteins and proteoglycans represent the main constituents (30). These proteins are produced by both epithelial and stromal cells. The matrix is permeable for ions and other small molecules but impermeable for most cells and its integrity is maintained by a finely tuned process of synthesis and degradation. Cells are closely attached to the ECM by transmembrane adhesion molecules (integrins) which themselves bind to the cytoskeleton (30). Besides providing mechanical stability to the cells, the ECM is also important for functions such as cell survival, morphogenesis, tissue specific functions and cell migration (31). Turnover of extracellular matrices is an essential component of tissue homeostasis and is thought to involve the co-ordinated interaction of enzymes from the serine, cysteine, aspartyl and matrix metalloprotease (MMP) families, and their specific endogenous inhibitors (32).

This thesis will concentrate on matrix metalloproteases and on MMP-9 in particular. Matrix metalloproteases are a family of endopeptidases which are able to degrade all major components of the ECM. They have been associated with tissue remodelling and invasion in many physiological processes such as embryonic growth, blastocyst/trophoblast implantation, mammary development and involution, endometrial turnover during the menstrual cycle, tooth eruption, wound healing, angiogenesis and bone growth/remodelling (33, 34). MMPs are also thought to be critical in pathological processes such as periodontal disease, rheumatoid arthritis, tumour invasion and metastasis [for review (35-37)].

1.2.2 MMPs in tumour growth and invasion

Development of a malignant tumour and the formation of metastasis requires the breakdown of the basement membrane, invasion of the surrounding tissue, separation of tumour cells from the primary tumour, intravasation into blood or lymph vessels, extravasation out of the vessel and invasion of a secondary tissue. In addition, the formation of new blood vessels leading into the tumour is also required. All these processes are associated with ECM remodelling and MMP activity (33, 37). Proteolytic activity has been analysed in different malignancies and correlated with tumour progression. In colorectal cancer, for example, high MMP-1 protein levels detected by immunohistochemistry were associated with poor prognosis (38). Increased MMP-2 and MMP-9 protein activity was also detected in stage A and C colorectal tumours as compared with normal or benign tissue samples (39). In breast and bladder cancer increased MMP-2 and -9 proteolytic activity correlated with tumour progression (40, 41). MMP-11 expression was also increased in malignant biopsies if compared with precursor lesions in breast, bladder and carcinomas of the uterine cervix (42).

The proteolytic activity required for these processes, however, may not necessarily be produced by the tumour cells themselves but may be derived from stromal or infiltrating cells. In ovarian cancer, for example, MMP-2 gene expression has been localised by *in situ* hybridisation to stromal fibroblasts whereas MMP-9 expression was associated with infiltrating macrophages (43). Similarly, MMP-2 and -9 gene expression was detected in stromal cells or at the tumour/stroma interface by *in situ* hybridisation in bladder cancer (41). In breast cancer, expression of MMP-1, MMP-2, MMP-3, MMP-11 and MT-1-MMP was localised to stromal fibroblasts whereas MMP-9 expression was primarily associated with endothelial cells. Only MMP-13 and MMP-7 expression was localised to tumour cells (44). Finally, the expression of MMP-11 in skin, head and neck, breast, bladder, colorectal, stomach and many other tumours was also exclusively associated with fibroblast-like cells (42). This led to the hypothesis that the induction of MMP gene expression in tumours may be a tumour-induced host response (44). This will be the subject of chapter 4 of this thesis.

The association of increased malignancy with high proteolytic activity was complemented by studies showing that enhanced production of tissue inhibitors of metalloproteases (TIMPs) reduced the formation of metastasis and tumour take [for

review (45, 46)]. Taken together these data suggest that tumour growth and metastasis requires an imbalance between proteolytic and inhibitory activity towards excess proteolysis (47). Based on this information, considerable industrial efforts led to the development of synthetic MMP inhibitors (MMPIs) with the aim of reducing tumour growth and metastasis [for review (21-23, 25-27)].

1.2.3 The MMP family

MMPs are a family of structurally and functionally related endopeptidases. To date 20 members of the matrix metalloprotease family have been cloned. Sequence comparison suggests that they are derived from a single ancestor gene by gene duplication (48, 49). MMPs have a Zn^{2+} in the active centre and require binding of Ca^{2+} for enzyme stability. All MMPs are produced in a proteolytically inactive pro-form and the enzymes degrade, once activated, all major components of the extracellular matrix (ECM) at physiological pH (pH 5-8) (50).

Sub-classification of MMPs was originally based on both structural similarities and *in vitro* substrate specificity. This led to the definition of four major groups (collagenases, gelatinases, stromelysins and others). Further analysis, however, showed that there is a considerable overlap of substrate specificity between these groups. Powell and Matrisian therefore suggested a classification based on structural similarities only (Fig. 1.1, Table 1.3) (51).

1.2.4 The protein structure of matrix metalloproteases

MMPs are structurally related and a domain structure can be assigned (49, 50). The prototype MMP consists of a signal peptide, which directs the protease for export and is removed in the endoplasmatic reticulum; a pro-peptide; the catalytic domain and the C-terminal hemopexin/vitronectin-like domain. All MMPs can be derived from this structure by insertions, deletions or additions of further domains (Fig. 1.1). MMP-7 for example, does not possess the C-terminal hemopexin/vitronectin-like domain, MMP-2 and -9 have a fibronectin-like domain inserted in the catalytic domain and the MT-MMPs have transmembrane and intracellular domains added to their C-termini.

Enzymes	MMP No.	Precursor (kDa)	Active (kDa)	Matrix substrates
minimal domain MMPs				
Matrilysin (EC 3.4.24.23)	MMP-7	28	19	Aggrecan, Fibronectin, Laminin, Gelatins, Collagen IV, Elastin, Entactin, Small Tesascin-C
hemopexin domain MMPs				
Interstitial collagenase (EC 2.4.24.7)	MMP-1	52 56*	41 45*	Collagen I, II, III, VII, X, Gelatins, Entactin, Aggrecan, Cartilage link protein
Neutrophil collagenase (EC 3.4.14.34)	MMP-8	75*	65	Collagen I, II, III, Proteoglycan, Cartilage link protein
Collagenase 3	MMP-13	65	55	Collagen I
Stromelysin 1 (EC 3.4.24.17)	MMP-3	57 59*	45 28	Aggrecan, Gelatins, Fibronectin, Laminin, Collagen III, IV, IX, X, Large Tesascin-C
Stromelysin 2 (EC 3.4.24.22)	MMP-10	57	45 28	Aggrecan, Fibronectin, Laminin, Collagen IV
Stromelysin 3 (EC 3.4.24)	MMP-11	55	45 28	Fibronectin, Laminin, Collagen IV, Aggrecan, Gelatin
Metalloelastase (EC 3.4.24.65)	MMP-12	53	45 22	Elastin
fibronectin domain MMPs				
Gelatinase A (EC 3.4.24.24)	MMP-2	72	67	Gelatins, Collagens I, IV, V, VII, XI, Fibronectin, Laminin, Aggrecan, Elastin, Large Tesascin-C
Gelatinase B (EC 3.4.24.35)	MMP-9	92*	82	Gelatins, Collagen III, IV, V, XIV, Aggrecan, Elastin, Entactin
transmembrane domain MMPs				
MT-1-MMP	MMP-14	66		Collagen I, II, III, Gelatin, Fibronectin, Vitronectin, Laminin [1]
MT-2-MMP	MMP-15 [2]	72		
MT-3-MMP	MMP-16 [3]	64		
MT-4-MMP	MMP-17 [4]			
others				
	MMP-18 [5]			
	MMP-19 [6]			
	MMP-20 [7]			

Table 1.3 Matrix metalloproteases and their substrates
Table modified from (49, 51, 52). Some MMPs have not been biochemically characterised in depth to date. [1](53), [2](54), [3](55), [4](56), [5](57), [6](58), [7](59) (* glycosylated)

The N-terminal pro-peptide consists of approximately 80 amino acids and contains the highly conserved PRCG(V/N)PDV sequence (49). This pro-peptide plays a crucial role in maintaining the protease in its proteolytically inactive form and is removed in the tightly controlled process of proMMP activation. The structure of the pro-peptide has been determined for MMP-3. It is an extended structure which covers the active site cleft stabilised by an interaction between the central cysteine of the conserved sequence with the Zn^{2+} ion in the active centre (60).

The catalytic domain comprises approximately 165 amino acids and mediates substrate hydrolysis and autolytic cleavages. The conserved sequence HEXGHXXGXXHS within the catalytic domain contains three histidine residues which complex the Zn^{2+} ion in the active centre (49). This model has been confirmed by X-ray crystallography for MMP-1 (61, 62), MMP-3 (60), MMP-7 (63) and MMP-8 (64). These studies also suggested that a second, "structural" Zn^{2+} ion may be bound to the catalytic domain and one or more Ca^{2+} ions contribute to the proteolytic activity by stabilising the tertiary structure of the enzyme (62, 65-68).

The C-terminal hemopexin-like domain can be found in all MMPs apart from MMP-7 (Fig. 1.1). This domain contains four repeats which show strong sequence homology to hemopexin, vitronectin and other members of the hemopexin family (50). The first and the fourth repeat are linked by a disulphide bridge which must be intact to maintain the properties of the domain (Fig. 1.1). For the collagenases (MMP-1, -8, -13) the hemopexin-like domain has been shown to bind collagens (69-72). Truncated collagenases loose their ability to cleave native triple helical collagen suggesting that the hemopexin-like domain plays a role in substrate binding and processing (70-73). It is not clear, however, whether this is also true for other members of the MMP family. A further function of the hemopexin-like domain is the binding of the naturally occurring tissue inhibitors of metalloproteases (TIMPs). Some evidence suggests that initial "docking" of TIMP to the hemopexin-like domain accelerates TIMP binding to the active site and therefore MMP inhibition (74). Finally, a novel function of the hemopexin domain of MMP-2 was discovered recently (75). Brooks *et al.* (75) showed that MMP-2 binds via its hemopexin domain to the $\alpha v\beta 3$ integrin present on endothelial cells leading to an increase in cell associated collagenolytic activity. In addition Brooks *et al.* provided data showing that the hemopexin domain may be a natural degradation product of MMP-2 and that this domain may reduce the cell associated collagenolytic activity by competing with MMP-2 with respect to the $\alpha v\beta 3$ integrin binding site (75).

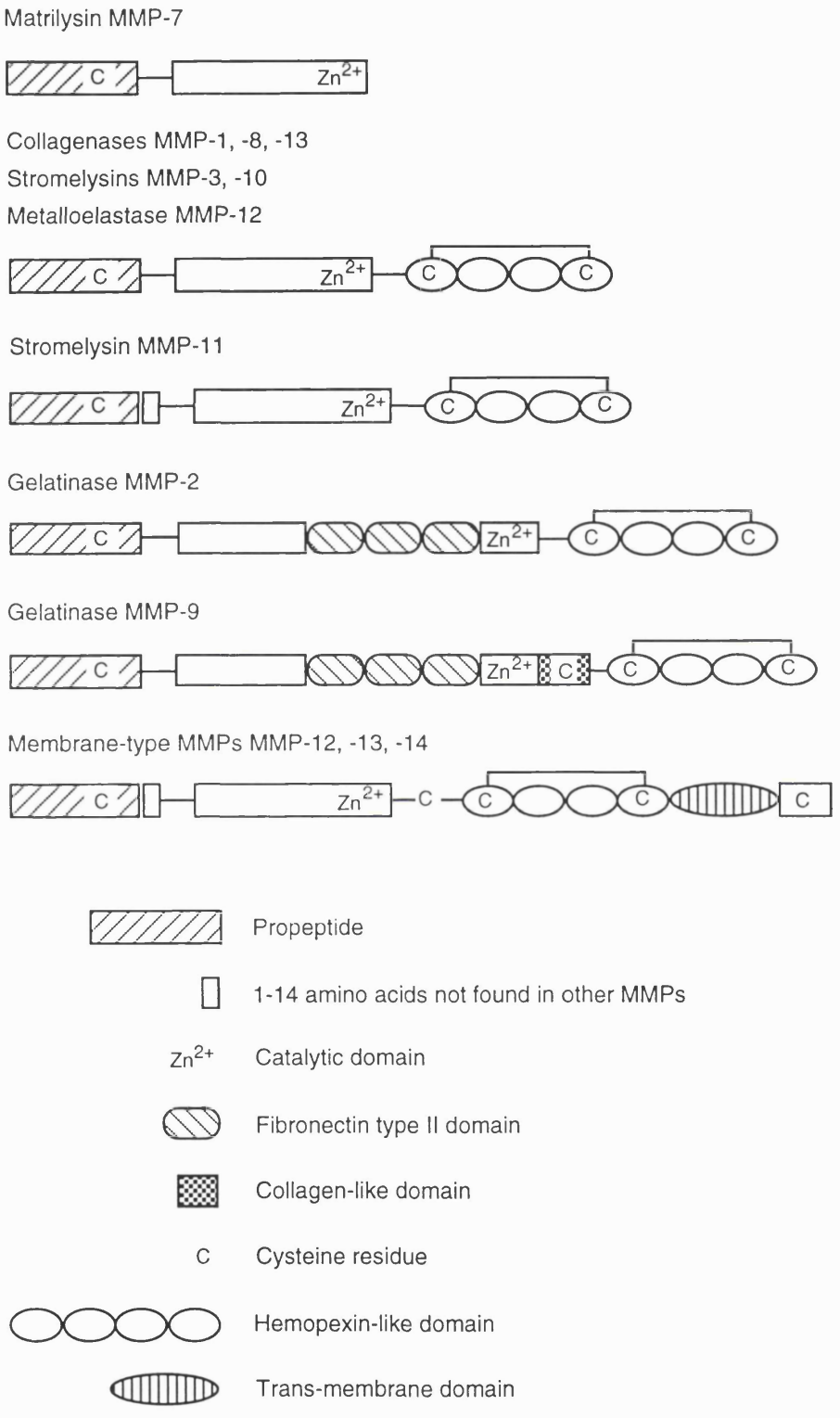


Fig. 1.1 Domain structure of the MMP family
Modified from (50, 52, 76).

1.2.5 Regulation of matrix metalloproteases

MMP activity is controlled at several levels. Gene expression can be regulated by cytokines or components of the ECM. Some regulation might also be associated with protein glycosylations which have been detected on some MMPs (Table 1.3). Finally, activation of MMPs can be triggered by proteases and their proteolytic activity is counterbalanced by tissue inhibitors of metalloproteases (TIMPs).

a. Transcriptional regulation

De novo MMP protein synthesis can be manipulated in cell culture with cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interferon-γ (IFN-γ), epidermal growth factor (EGF) and platelet derived growth factor (PDGF) and both up or down regulation of gene expression can be observed [for review (77-79)]. Some members of the MMP family respond in a similar fashion to a particular cytokine. TNF-α, for example, up regulates whilst IFN-γ down regulates the gene expression of MMP-1, -3, -9 (78). Promoter analysis of MMPs revealed several consensus sequences. All promoters analysed (MMP-1, -2, -3, -7, -9, -10, -12) contained a TATA box close to position -30, an AP-1 binding site near position -70 and adjacent to it at least one PEA3 binding site (80). The exception to this is the MMP-2 promoter which contains none of these three elements. Instead, two SP-1 (-69, -89) and one AP-2 (-1650) binding site are present (80). These are characteristics shared by "housekeeping genes" and is consistent with the observation that MMP-2 is often constitutively expressed (80, 81).
In addition to cytokines, ECM derived proteins (e.g. collagen I) also play a role in the regulation of MMP gene expression (82-84). This is in keeping with data showing that signalling through transmembrane ECM binding molecules (integrins) can also modulate MMP gene expression (85-87).

b. Post transcriptional regulation

This type of regulation, which includes alternative splicing and mRNA stability, has not been investigated in great detail yet. Extended mRNA half-life of MMP-1 (88) and MMP-2 (89) was found in response to the cytokines IL-1β and TGF-β, respectively.

MT-3-MMP is the only MMP for which alternative splicing has been reported. In addition to the membrane-bound protease, a modified splicing pattern seems to lead to a soluble form of MT-3-MMP which lacks the transmembrane domain (90).

c. Post translational regulation

Two levels of post translational regulation can be distinguished; zymogen activation and inhibition of the active protease by tissue inhibitors of metalloproteases (TIMPs). In addition, Toth *et al.* reported MMP-9 targeting to the cell surface by a modification of its glycosylation (91). This may represent a new type of post translational regulation of MMPs.

Protease activation - the "cysteine switch"

MMPs are produced as a proteolytically inactive pro-form (zymogen). The latency of the protease is maintained by the N-terminal pro-domain of approximately 80 amino acids which covers the active site cleft (60). The pro-peptide is held in place by the sulfhydryl group (-SH) of the central cysteine residue present in the conserved sequence PRCG(V/N)PD which acts as a fourth ligand complexed to the Zn^{2+} ion in the active centre. In an initial step of zymogen activation, the pro-peptide undergoes proteolytic processing N-terminal of the conserved PRCG(V/N)PD sequence leading to the loss of one part of the pro-peptide and the formation of an intermediate form. This destabilises the co-ordinate bond between the Zn^{2+} and the sulfhydryl group leading to structural rearrangements of the protease and subsequently loss of the remaining pro-peptide by an autocatalytic process (50, 60). The mechanism of proMMP activation has been termed "cysteine switch" (92).

It is thought that MMPs become activated as part of a tightly controlled cascade of proteolytic activation which may involve proteases like plasmin, cathepsin B and G, furins and other MMPs [for review (46, 50)]. *In vitro* experiments have shown, for example, that proMMP-2 and proMMP-13 can be activated by MT-1-MMP (93, 94).

Regulation of MMP activity by TIMPs

TIMPs are small glycoproteins with a molecular weight between 21 and 28kDa. Four (TIMP-1 to -4) have been cloned to date and biochemically characterised [for review (36,

45, 95)]. TIMPs consist of two domains with distinct functions. The N-terminal domain binds to the active centre of the protease blocking its proteolytic activity while the C-terminal domain can bind to the hemopexin domain of MMPs (72, 96-100). Interestingly, initial binding of TIMPs to the hemopexin domain seems to accelerate MMP/TIMP interaction at the active site (74). TIMP-1 and -2 have been detected in tissue extracts and body fluids and are capable of inhibiting proteolytic activity of all known MMPs (36). TIMP-3, in contrast, has exclusively been found in association with connective tissue and the ECM (101, 102). TIMP-1, -2 and -3 can form high affinity, non covalent complexes with the active forms of MMPs in a 1:1 stoichiometry (36). No data are available about the inhibitory activity of TIMP-4. Interestingly, however, its expression is strongly tissue specific and highest in the heart but low or not detectable in other tissues (103).

Although TIMPs are thought to be essential in balancing MMP related proteolytic activity, other protease inhibitors such as α_2-macroglobulin may also play a role. Indeed, competition studies have shown that MMP-1 binds preferentially α_2-macroglobulin and not TIMP-1 (95, 104).

1.2.6 Synthetic inhibitors of matrix metalloproteases

As mentioned earlier, the association of tumour progression and increased MMP related proteolytic activity led to considerable industrial effort to develop synthetic inhibitors for these proteases. Most of the compounds were designed as peptide analogues including an hydroxamic acid as Zn^{2+} binding group. Additional side chains define the compound's specificity and affinity [for review (25, 26)]. Inhibitory constants (IC_{50}) in the low nanomolar range to MMPs such as MMP-1, -2 and -3 have been reported for some compounds (e.g. BB-94) (25). Further research involving these compounds revealed that the shedding of several cytokines including TNF-α (105, 106), TGF-α (107) and the Fas Ligand (107, 108) from their membrane spanning precursors could be blocked. This led to the suggestion that an MMP like enzyme was involved in their release (105, 106). However, cloning of the TNF-α precursor converting enzyme (TACE) showed that the protease was not a member of the MMP family but a disintegrin metalloprotease belonging to the family of adamalysins (ADAMs) (109, 110).

1.3 MMP-9

1.3.1 Protein structure of MMP-9

MMP-9 is the largest MMP, with a molecular weight of 92kDa for the zymogen. It is translated from an mRNA of approximately 2400bp and its gene is encoded on chromosome 20q11.2-q13.1 (111, 112). MMP-9 has N- and O-linked glycosylations (111) and belongs, together with MMP-2, to the fibronectin domain MMPs (Table 1.3). Both have, in addition to the basic structure of propeptide, catalytic and hemopexin-like domain, three tandem repeats of a fibronectin type II-like domain inserted in the catalytic domain (Fig. 1.1 and 1.2). This fibronectin domain is unique to MMP-2 and -9 (49). The function of the domain is not fully understood but studies of it in isolation showed that it can bind to native and denatured type I collagen, denatured type IV and V collagens and elastin (113-115). It seems, however, that binding of denatured collagens (or gelatin) by the fibronectin like domain is not rate limiting for its proteolysis and that other binding sites for gelatin might exist (113). The gelatin binding site can, however, be efficiently used for purification of gelatinases from cell culture supernatant by affinity chromatography (116).
A domain of 54 amino acids is inserted C-terminal adjacent to the catalytic domain of MMP-9. This domain is unique to MMP-9 and shares homology to a portion of the helical region of α2(V) collagen (111). The function of this domain remains unknown.
MMP-9 also has the C-terminal hemopexin-like domain. For the collagenases MMP-1, -8 and -13 this domain has been shown to bind native collagens (69-72) but no evidence is available as to whether such a substrate binding activity also exists for the hemopexin domain of MMP-9. Goldberg *et al.*, however, provided data showing that the MMP-9 hemopexin domain can bind TIMP-1, MMP-1 and MMP-9 (117, 118). In the absence of TIMP-1, the proteolytically inactive pro-form of MMP-9 can therefore form a homodimer or a heterodimer with pro or active MMP-1. The presence of TIMP prevents the formation of the proMMP-9 homodimer and the proMMP-9/proMMP-1. Activation of proMMP-9 by MMP-3 (stromelysin) is also inhibited in presence of TIMP-1. The heterocomplex proMMP-9/proMMP-1 can be activated by MMP-3 in absence of TIMP-1 to yield proteolytic activity against both gelatin and fibrillar type I collagen, suggesting a mechanism for co-operative action of the two enzymes (118).

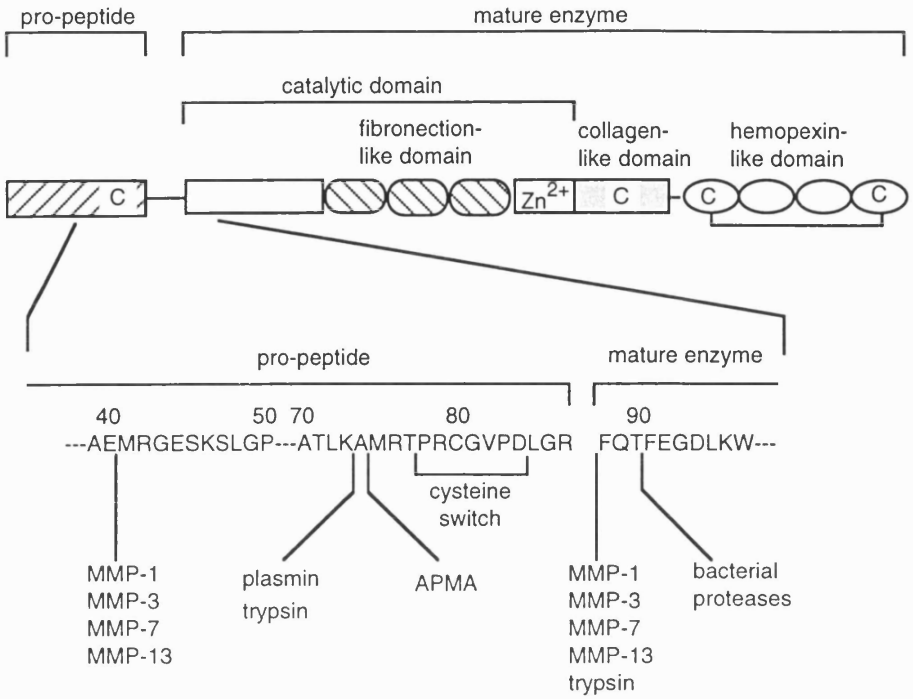


Fig. 1.2 Domain structure of proMMP-9 and sites of activation
Taken from (119-125).

1.3.2 Role of MMP-9 in tumours growth and invasion

In vivo data obtained by *in situ* hybridisation performed on tissue sections showed that MMP-9 mRNA was associated with endothelial cells in breast cancer, and with tumour infiltrating macrophages in ovarian cancer, but not with tumour cells or stromal fibroblasts (43, 44). Increased amounts of MMP-9 protein have been described in various tumours such as brain (126), bladder (41), colorectal (39) and breast (127). Correlation analysis of MMP-9 levels with disease stage or tumour progression showed that MMP-9 expression correlated with the histologic grade of human malignant lymphomas (128) and the aggressiveness of prostatic adenocarcinoma (129). In addition, Davies *et al.* showed by zymography that MMP-9 protein closely correlated with tumour grade and invasiveness in bladder carcinoma (41).

1.3.3 Regulation of gene expression

The MMP-9 promoter has been analysed to approximately 600 base pairs up stream of the point of transcription initiation. It contains the typical TATA box (-29), two AP-1 binding sites (-79, -533), one Sp-1 (-558), several PEA3 binding sites in the region from -80 to -600 and one NFκB (-600) binding site (80). Within the MMP family, MMP-9 is the only one known to have a NFκB binding site.

MMP-9 gene expression can be stimulated by many cytokines such as TNF-α, TGF-α, EGF, IL-1α, IL-1β and TGF-β [for review (79)]. In addition, cell lines treated with agents such as phorbol esters (116) also produce MMP-9. Down regulation of MMP-9 gene expression has been observed with all-trans-retinoic acid (130), vitamin D_3 (131) and IFN-γ (78).

1.3.4 Activation of MMP-9

Activation of MMP-9 has been studied extensively *in vitro*. Proteases such as MMP-2 (132), MMP-3 (118, 122, 133), MMP-7 (134, 135), trypsin (133, 135, 136), cathepsin G (133, 137) and plasmin (137, 138) activate proMMP-9. In addition, several bacterial proteases activate proMMP-9, which might be of particular importance at sites of bacterial infection (120). In the two step process of proMMP-9 activation, the site of the initial proteolytic pro-peptide cleavage depends on the acting protease (Fig. 1.2). Furthermore, some of the proteases (e.g. MMP-3) also process proMMP-9 at its C-terminus leading to a further loss in molecular weight and the formation of a 50kDa protein (121, 135). Interestingly, the intermediate form resulting from initial processing N-terminal of the consensus sequence PRCG(V/N)PDV is very stable and can be detected if, for example, MMP-2, MMP-7 or trypsin are used for *in vitro* activation of MMP-9 (119, 132, 136). In addition to proteolytic activation of proMMP-9, chemical activation has also been observed using sulfhydryl group binding mercurials such as aminophenyl mercuric acetate (APMA) or $HgCl_2$ (121, 135, 139).

Natural occurrence of activated MMP-9 remains controversial. Analysis of tissue samples of colorectal, bladder, breast or gastric carcinomas by zymography did not lead to the detection of activated form MMP-9 (39-41, 140). The authors concluded that activated MMP-9 was not detected because of insufficient resolution of the gel. *In vitro* studies

using stimulated cell lines revealed at least two forms of MMP-9 (92 and 85kDa) which were associated with the surface of cells or vesicles (91, 141, 142). The 92kDa form was consistently identified as proMMP-9 but conflicting data were reported with respect to a 85kDa form. Mazzieri *et al.* and Ginestra *et al.* (141, 142) concluded that the 85kDa band represented an activated species of MMP-9 whereas Toth *et al.* (91) presented data indicating it might be a glycosylation variant of proMMP-9 modified to bind to the cell membrane. This discrepancy will be the subject of chapter 6 of this thesis.

1.3.5 Regulation of MMP-9 activity by TIMPs

The proteolytic activity exerted by MMP-9 can be inhibited by TIMP-1, -2 and -3 through the formation of a tight, non covalent complex in a 1:1 stoichiometry [for review (143)]. TIMP-1, however, binds at a much faster rate to active MMP-9 than TIMP-2 (144). This is dependent on the presence of the MMP-9's C-terminal hemopexin domain suggesting that initial binding of TIMP-1 to this domain facilitates subsequent interaction with the active site (144). In contrast, TIMP-2 binding to active MMP-9 was independent of the hemopexin domain (99). These findings suggested that TIMP-1 was the natural inhibitor for MMP-9.

1.4 Aims of thesis

It was the aim of this thesis to investigate the role of matrix metalloproteases in human ovarian cancer. Previous work of the laboratory had shown that MMP-2 and -9 were present in tissue biopsies of ovarian cancer and their expression was localised to the tumour stroma by *in situ* hybridisation. The objective of the first part of the project was to extend current knowledge of MMP and TIMP gene expression ovarian cancer tissue and to compare this pattern with ovarian cancer cell lines and xenograft models of human ovarian cancer. The aim was then to select one MMP and to study its regulation and activation in an *in vitro* model of the *in vivo* situation.

2. PCR-screen for MMPs and TIMPs in biopsies of human ovarian cancer

2.1 Introduction

MMP and TIMP mRNA and protein have been analysed in both normal and malignant ovary. Most studies, however, assessed only one or two proteins resulting in an incomplete profile of MMPs/TIMPs present in this tissue. For instance, MMP-2 and MMP-9 mRNAs and proteins were detected in ovarian tumours by *in situ* hybridisation and zymography (43). MMP-2 expression localised to stromal areas and was maximal in cells adjacent to neoplastic areas. MMP-9 expression, in contrast, was focal and detectable in both tumour areas and tumour stroma. In another study, MMP-11 (stromelysin-3) gene expression was detectable by *in situ* hybridisation in biopsies of human ovarian cancer and was associated with fibroblastic cells of the tumour stroma (42). The presence of MMP-18 (57), MMP-19 (58), MT-1-MMP (54) and MT-4-MMP (56) mRNA was detected by northern blotting in the normal ovary. MT-1-MMP has also been detected by immunoprecipitation in short term cultures of human primary epithelial ovarian carcinoma cells (145). The expression of the tissue inhibitors of metalloproteases TIMP-1 and TIMP-2, was analysed by *in situ* hybridisation in tumour biopsies of human ovarian cancer. Expression of TIMP-1 was found in stromal areas adjacent to tumour epithelial cells and, in some cases, in epithelial cells (43). TIMP-2 expression localised to stromal areas and resembled that of MMP-2 (43). In normal ovary, MMP-13 (146) and MT-2-MMP (54) mRNA was not detected by RT-PCR and northern blotting, respectively.

The aim of the work described in this chapter was to obtain a broader picture of MMP/TIMP expression in tissue biopsies of the normal and malignant human ovary and to compare this pattern with that of ovarian cancer cell lines and xenograft models of human ovarian cancer. RT-PCR was chosen because it is a very sensitive and specific method. In addition, a large number of samples could be processed simultaneously. PCR primers for a panel of human and murine MMPs and TIMPs were designed and

cDNA from tissue biopsies of the normal and malignant human ovary, ovarian cancer cell lines and xenografts models of human ovarian cancer was analysed.

2.2 Material and Methods

Biopsy material

Biopsy material was collected by Dr. Rupert Negus (Biological Therapies Laboratory, Imperial Cancer Research Fund, London) from St. Thomas Hospital (London, UK). Tissue samples were cut into small pieces (approximately $5mm^3$) at the time of surgery and snap frozen in isopropanol, which was cooled down to near freezing point (- 88°C) with liquid nitrogen. The samples selected are listed in Table 2.1.

ICRF code	tissue type	histopathological description	patient age (years)	stage
ST-6F	normal		43	
ST-3	normal		47	
ST-11	normal		44	
ST-12	normal		70	
ST-16	benign	benign follicular cyst	44	
ST-19 ov-l	benign	benign cystadenoma	69	
ST-19 ov-r	benign	benign cystadenoma	69	
ST-20	benign	benign cystadenoma	54	
ST-22 ov-r	benign	benign mucinous cystadenoma	68	
SAM-9	benign	benign fibroadenoma	40	
SAM-3	carcinoma	mucinous adenocarcinoma	75	III
SAM-6	carcinoma	serous adenocarcinoma	75	I
SAM-7	carcinoma	serous papillary adenocarcinoma	60	III
ST-29 ov-r	carcinoma	serous cystadenocarcinoma	68	I
ST-26 ov-l	carcinoma	serous adenocarcinoma	73	III
ST-27	carcinoma	papillary adenocarcinoma	59	III
ST-23	carcinoma	serous cystadenocarcinoma	80	I
ST-26 om	metastasis (from omentum)	adenocarcinoma (as primary tumour)	73	III
SAM-7 om	metastasis (from omentum)	adenocarcinoma (as primary tumour)	60	III
SAM-7 met	metastasis (not from omentum)	adenocarcinoma (as primary tumour)	60	III

Table 2.1 Tissue biopsies included in PCR screen for human MMPs and TIMPs

Ovarian cancer xenografts

Frozen (-70°C) tissue material from three ovarian cancer xenografts was kindly provided by Dr. Frances Burke (Biological Therapies Laboratory, Imperial Cancer Research Fund, London, UK). Ovarian cancer xenografts OS, LA and HUA were established from primary human tumours as described previously (147) and maintained by serial passage (147). Cells were passaged intraperitoneally (i.p.) and mice maintained strictly

according to ICRF ethics committee and Home Office guidelines. The xenograft OS originated from ascites of a 51-year old woman with a moderately differentiated serous cystadenocarcinoma. LA was derived from ascites of a 72-year old woman with a poorly differentiated mucinous cystadenocarcinoma. HUA is a subline of HU which was derived from a solid tumour obtained from a 23 year old woman with a moderately differentiated serous cystadenocarcinoma. These xenografts grew as free floating ascitic tumours surrounded by mucin. Mice were killed when abdomens were swollen or the mice had any other outward sign of discomfort.

Cell lines

Five ovarian cancer cell lines were studied. PEO1 and PEO4 were derived from ascites of the same patient with a poorly differentiated adenocarcinoma. PEO1, however, was established from a tumour prior to chemotherapy and PEO4 after chemotherapy. PEO2 was also obtained from a patient with a poorly differentiated adenocarcinoma after chemotherapy. PEO14 was obtained prior to chemotherapy from a patient with a well differentiated serous adenocarcinoma. These cell lines were obtained from and described previously by Langdon *et al.* (148). OVCAR-3 was derived from malignant ascites of a patient with a poorly differentiated papillary adenocarcinoma. The cell line was obtained from and described previously by Hamilton *et al.* (149). SKOV-3 originated from ascites in a patient with an ovarian adenocarcinoma and was obtained from the American Tissue Culture Collection (Rockville MD, USA, No. HTB77).

All cell lines were grown in a humidified atmosphere at 37°C (5% CO_2) under pyrogen free conditions. Tissue culture medium and foetal calf serum (both from Gibco, Paisley, UK) were chosen for their low endotoxin content (< 100pg/ml) and plastic tubes and pipettes were used at all times. All ovarian cancer cell lines were adherent. Cells were harvested by aspirating culture medium, washing cells with 5-10ml 0.2% trypsin/0.05% EDTA solution, and incubating the flasks at 37°C (5% CO_2) with trypsin/EDTA solution for 1-5 mins. Detached cells were aspirated and suspended in the appropriate serum containing medium, centrifuged at 200g for 5 minutes, and resuspended in medium. Cell counts were determined using an improved Neubauer haemocytometer. Cells were grown in either RPMI 1640 or DMEM (SKOV-3) containing 10% foetal calf serum (FCS).

With the exception of SKOV-3 cell lines were also grown in the presence of bovine insulin (5µg/ml).

Design of PCR primers

PCR primers were designed using the computer program PRIMER 2.2 (Stephen E. Lincoln, Mark J. Daly and Eric S. Lander, MIT Center for Genome Research and Whitehead Institute for Biomedical Research, Cambridge, Massachusetts, USA). cDNA sequences for human and murine MMPs/TIMPs were retrieved from the EMBL data base and converted into PIR and STADEN file formats. The files in PIR format were further converted into a file format suitable for the PRIMER program with the program shown below. This program was written in PASCAL.

Computer program to convert files of PIR format into PRIM format

```
Program FileConversion (Input, Output, FileIn, FileOut);

Var     ch                      : Char;
        index, StopLabel        : integer;
        FileIn, FileOut         : TEXT;
        FileNameIn, FileNameOut : String( 20 );

BEGIN
Write('Enter name of file to be converted (*.pir): ');
readln(FileNameIn);

Write('Enter name of file containing the converted data: ');
readln(FileNameOut);

Reset(FileIn, FileNameIn);
Rewrite(FileOut, FileNameOut);
write(FileOut,'*sequence: ');
writeln(FileOut,FileNameOut);

StopLabel := 0;
readln(FileIn);
readln(FileIn);
read(FileIn, ch);

IF ch = '*' THEN StopLabel := 1;

WHILE ((NOT EOF(FileIn)) AND (StopLabel <> 1)) DO
        BEGIN
        FOR index := 1 TO 10 DO read(FileIn, ch);
        IF ch = '*' THEN StopLabel := 1;
        WHILE ((NOT EOLN(FileIn)) AND (StopLabel <>1)) DO
                BEGIN
                If ch = ' ' THEN
                        BEGIN
```

```
                write(FileOut, ch);
                write(FileOut, ch);
                read(FileIn, ch);
                IF ch = '*' THEN StopLabel := 1;
                END
        ELSE
                BEGIN
                write(FileOut, ch);
                read(FileIn, ch);
                IF ch = '*' THEN StopLabel := 1;
                END;
        END;

    readln(FileIn);
    read(FileIn, ch);
    IF ch = '*' THEN StopLabel := 1;
    writeln(FileOut);
    END;

writeln('File format converted');
Close(FileIn);
Close(FileOut)
END.
```

Within the program PRIMER 2.2 the following parameters were used for the automated primer selection process:

OPTIMAL primer length:	20 base pairs
MINIMUM primer length:	18 base pairs
MAXIMUM primer length:	22 base pairs
OPTIMAL primer melting temperature:	60.0°C
MINIMUM acceptable primer melting temperature:	57.0°C
MAXIMUM acceptable primer melting temperature:	63.0°C
MINIMUM acceptable primer GC%:	40%
MAXIMUM acceptable primer GC%:	60%
Salt concentration:	50.0mM
DNA concentration:	50.0nM
MAXIMUM number of unknown bases allowed in a primer:	0 bases
MAXIMUM acceptable primer self-complementarity:	4 bases
MAXIMUM acceptable 3' end primer self-complementarity:	8 bases
GC clamp how many 3' bases:	0 bases

Using the computer program STADEN, the PCR primers selected were tested for homology against all cDNAs for human and murine MMPs/TIMPs available in the EMBL data base. Primer pairs were accepted if at least one of the two primers showed homology above 70% only to the specific binding site. In addition, a restriction enzyme leading to a single cut within the theoretical PCR product was determined. PCR primers close to the 3' end of the mRNA were preferentially selected.

PCR

All PCR reactions were carried out using the Perkin-Elmer Gene-Amp kit (Perking-Elmer, Norwalk, USA). Two µl of cDNA (the equivalent of 300ng of total RNA) was used for each amplification. A master mix consisting of all reagents except the cDNA was prepared for each primer pair. A final volume of 25µl per reaction was overlaid with mineral oil (Sigma, Poole, UK) and the following protocol used in a Gene-E thermal cycler (Techne, Cambridge, UK):

1 cycle: 94°C (5 min), 60°C (1 min), 72°C (30 s);

35 cycles: 94°C (45 s), 60°C (45 s), 72°C (2 min);

1 cycle: 72°C (7 min).

Fifteen µl of reaction mixture was subsequently electrophoresed on a 1.2-1.5% agarose gel containing 5% (v/v) ethidium bromide (Sigma). Bands were visualised under UV illumination and band sizes estimated in comparison to a co-migrated 123bp-markers (Gibco). A sample without addition of cDNA was used as negative control in the PCR reaction. Contamination of the master mix was never detected. All PCR reactions were set up in a flow cabinet and filter tips were used at all times. Repeat reactions showed reproducibility of the results.

Restriction digest of PCR products

Restriction enzyme digests were performed as previously described (150). Enzymes and buffers were from Promega (Southampton, UK) or NBL Gene Sciences (Cramlington, UK).

Isolation and sequencing of PCR products

PCR products were isolated from agarose gels using the QIAquick gel extraction kit (QIAGEN, Hilden, Germany) according to the manufacturers instructions. The isolated PCR products were sequenced from either end using the ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Warrington, UK) according to the manufacturers instruction. Sequencing products were analysed on a ABI Prism 377 Sequencer (Perkin Elmer)[1] and compared against the EMBL data base.

---

[1] Sequencing gels were prepared and run by Graham Clark and his staff in the ICRF equipment park.

RNA isolation

Total RNA was isolated from tissue material and cell lines using Tri-Reagent (Molecular Research Center, Cincinnati, Ohio, USA) according to the manufacturers instructions. Frozen tissue material was ground at 4°C, 14000rpm using the Ultra-Turrax T25 (Janke & Kunkel, Staufen, Germany).

DNAse treatment and cDNA synthesis

DNAse buffer (5x Transcription buffer, Promega, Southampton, UK), 2 units (U) of RNAse-free DNAse-1 (Promega) and 2U RNAse inhibitor (RNAsin, Promega) were added to 15µg total RNA. The volume was adjusted to 50µl with DEPC treated water and the sample incubated at 37°C for 1 hour to digest any contaminating genomic DNA. DNA-free RNA was extracted and precipitated as described before using Tri-Reagent and resuspended in 15µl DEPC treated water. cDNA synthesis was performed using the ReadyToGo oligo-dT cDNA synthesis kit (Pharmacia, Uppsala, Sweden) using 5µg total RNA per reaction.

Positive controls for PCR

cDNA for mouse MMP-11 was used as a positive control for PCR. The cDNA was excised with Sal I from a plasmid kindly provided by Prof. Pierre Chambon (Institut de Génétique et de Biologie Moleculaire et Cellulaire, Illkirch, France).

cDNA derived from total RNA of the human breast cancer xenograft 1068 was found positive for mouse MMP-3 expression and used as positive control.

2.3 Results

2.3.1 Specificity of PCR primers

A PCR product was considered specific if two criteria were fulfilled. First, the size of the PCR product had to correspond to the predicted size. Second, a restriction digest of the PCR product was performed and the fragments had to show the expected sizes. Alternatively the PCR product was sequenced and, when compared to the EMBL data base, the sequence had to be homologous to the correct cDNA. Table 2.2 shows the expected sizes for both the PCR products and restriction digest fragments. Typical examples of PCR products and their restriction digest fragments are shown in Fig. 2.1. The annealing of sequences obtained for some PCR products against the EMBL data base are shown in Fig. 2.2.

Based on these results cDNA derived from tissue biopsies, cell lines and xenograft models was screened for MMP/TIMP expression. Typical results are shown in Fig. 2.3.

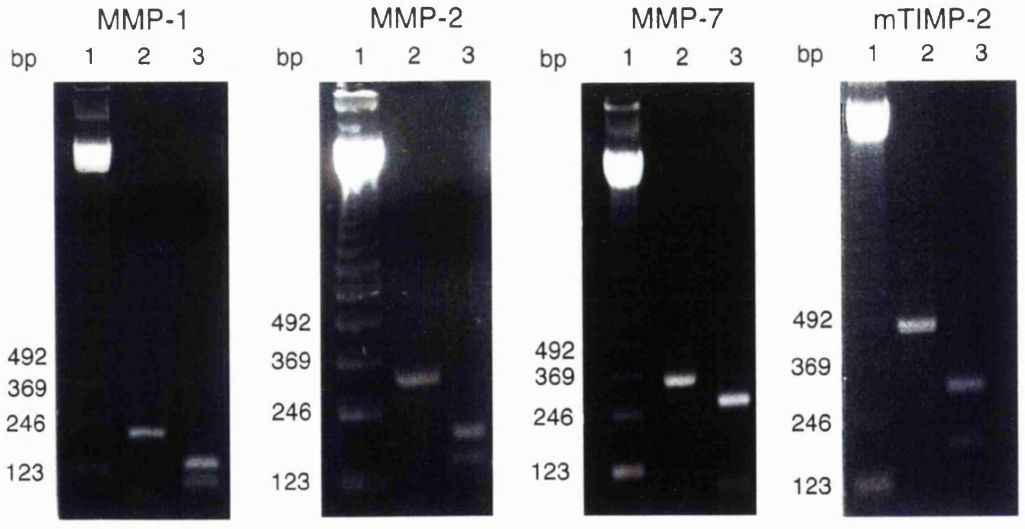


Fig. 2.1 Restriction digests of PCR products
PCR products were tested for specificity by a restriction digest which leads to fragments of defined size. Shown are typical examples. Alternatively the PCR product was sequenced. (The letter "m" stands for mouse)

target	5' primer 3' primer	PCR product	enzyme	digest products
human MMP-1	AATTACACGCCAGATTTGCC CTGGTTGAAAAGCATGAGCA	205bp	EcoRV	125bp 80bp
human MMP-2	TCCTTTCACAACCTTCTGTGG GGGAACCATCACTATGTGGG	316bp	BstX1	131bp 185bp
human MMP-3	GTACCTCATTTCCTCTGATGGC TGCTTTGTCCTTTGATGCTG	230bp	Sequence determined	
human MMP-7	GAGCTACAGTGGGAACAGGC ATGCAGGGGGATCTCTTTG	345bp	AvaI	266bp 79bp
human MMP-9	ACCGCTATGGTTACACTCGG GCAGGCAGAGTAGGAGCG	584bp	Sequence determined	
human MMP-10	GGCTCTTTCACTCAGCCAAC TCAGATCCCGAAGGAACAGA	181bp	HindIII	156bp 25bp
human MMP-11	TGACTTCTTTGGCTGTGCC GTTGTCATGGTGGTTGTACCC	199bp	Sequence determined	
human MMP-13	TGGAGTAACCGTATTGTTCGC GTGTGGGAATGATCATCAACCA	321bp	Sequence determined	
human MT-1-MMP	CACTGCCTACGAGAGGAAGG TGAATGACCCTCTGGGAGAC	296bp	Sequence determined	
human MT-2-MMP	CGTGTCCTGCTTTACTGCAA CTCCAACTGGGCAAAGAGAG	430bp	MboII	375bp 55bp
human MT-3-MMP	CAGGGTGATGGATGGATACC CCTTGAGGATGGATCTTGGA	470bp	Sequence determined	
human TIMP-1	CCAAGTTCGTGGGGACAC TGCAGTTTTCCAGCAATGAG	208bp	Sequence determined	
human TIMP-2	AGAAGAACATCAACGGGCAC CTTGGAGGCTTTTTTTGCAG	172bp	Sequence determined	
human TIMP-3	CCTGCTACTACCTGCCTTGC TCAGGGGTCTGTGGCATT	191bp	Sequence determined	
murine MMP-2	GACGTGGGGTACAGGTGTG TAAAGGGAGAAGCAAGAAGGC	320bp	AvaI	166bp 155bp
murine MMP-3	TGGAAACCTGAGACATCACCA TCTTCTTCACGGTTGCAGG	318bp	BalI	163bp 155bp
murine MMP-7	CGGAGATGCTCACTTTGACA CATCCACAGCACAAGGAAGA	358bp	NciI	235bp 123bp
murine MMP-9	GGCGTGTGAGTTTCCAAAAT CAAAGTTGTCTTCTTGCTCGG	389bp	PstI	298bp 91bp
murine TIMP-1	ATTCAAGGCTGTGGGAAATG AGGCAAGCAAAGTGACGG	354bp	PstI	155bp 199bp
murine TIMP-2	GTAGTGGGTCTGGAGGGACA CGCTGAAGTCTGTGGATTCA	469bp	KpnI	292bp 177bp
murine TIMP-3	CCAGACAAGAGCATCAGCAA GGGCCAGGAAGGTATTTCC	207bp	MboII	120bp 87bp

Table 2.2 PCR primers for a panel of human and murine MMPs and TIMPs
PCR primers were designed for a panel of human and murine MMPs/TIMPs. Target specificity was checked by the size of the PCR product obtained and a restriction digest with the enzyme listed. Alternatively the sequence of the PCR product was determined by cycle sequencing.

PCR product MMP-3 vs data base
gblJ03209IHUMMMP3A Human matrix metalloproteinase-3 (MMP-3) mRNA, complete cds
Score = 925 (255.6 bits), P = 2.9e-69
Identities = 185/185 (100%), Positives = 185/185 (100%), Strand = Minus / Plus

```
Query:   185 GCTTTGTCCTTTGATGCTGTCAGCACTCTGAGGGGAGAAATCCTGATCTTTAAAGACAGG 126
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   877 GCTTTGTCCTTTGATGCTGTCAGCACTCTGAGGGGAGAAATCCTGATCTTTAAAGACAGG 936

Query:   125 CACTTTTGGCGCAAATCCCTCAGGAAGCTTGAACCTGAATTGCATTTGATCTCTTCATTT 66
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   937 CACTTTTGGCGCAAATCCCTCAGGAAGCTTGAACCTGAATTGCATTTGATCTCTTCATTT 996

Query:    65 TGGCCATCTCTTCCTTCAGGCGTGGATGCCGCATATGAAGTTACTAGCAAGGACCTCGTT 6
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   997 TGGCCATCTCTTCCTTCAGGCGTGGATGCCGCATATGAAGTTACTAGCAAGGACCTCGTT 1056

Query:     5 TTCAT 1
             |||||
Sbjct:  1057 TTCAT 1061
```

PCR product human MMP-9 vs data base
gblJ05070IHUM4COLA Human type IV collagenase mRNA, complete cds.
Score = 1423 (393.2 bits), P = 9.8e-111
Identities = 299/324 (92%), Positives = 299/324 (92%), Strand = Plus / Plus

```
Query:     1 TGCCCGAGACCGGTGAGCTGGATAGCGCCNCGCTGAAGGCCATGCNAACCCCACGGTGCG 60
             |||||||||||||||||||||||||||| ||||||||||||| ||||||||||||||
Sbjct:   258 TGCCCGAGACCGGTGAGCTGGATAGCGCCACGCTGAAGGCCATGCGAACCCCACGGTGCG 317

Query:    61 GGGTCCCAAACCTGGGCAAATTCCAAACCTTTGAGGGCNACCTCAATTGGCACCACCACA 120
             |||||||| ||||||||| ||||||||||||||||||| ||||||| ||||||||||||
Sbjct:   318 GGGTCCCAGACCTGGGCAGATTCCAAACCTTTGAGGGCGACCTCAAGTGGCACCACCACA 377

Query:   121 ACATCACCTATTGGATCCAAAACTACTCGGAAAACTTGCCGCGGGCGGTGATTGACAACN 180
             |||||||||||||||||||||||||||||| |||||||||||||||||||||| ||
Sbjct:   378 ACATCACCTATTGGATCCAAAACTACTCGGAAGACTTGCCGCGGGCGGTGATTGACGACG 437
```

PCR product MMP-11 vs data base
emblX57766IHSSTROL3 Human stromelysin-3 mRNA
Score = 551 (152.3 bits), P = 4.9e-38
Identities = 111/112 (99%), Positives = 111/112 (99%), Strand = Plus / Plus

```
Query:    24 TGGGATGCCCTCAGGGGTGCTGACCCCTGCCAGGCCACGAATATCAGGCTAGAGACCCAT 83
             | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1484 TTGGATGCCCTCAGGGGTGCTGACCCCTGCCAGGCCACGAATATCAGGCTAGAGACCCAT 1543

Query:    84 GGCCATCTTTGTGGCTGTGGGCACCAGGCATGGGACTGAGCCCATGTCTCCT 135
             ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1544 GGCCATCTTTGTGGCTGTGGGCACCAGGCATGGGACTGAGCCCATGTCTCCT 1595
```

Fig. 2.2 Sequence comparison of PCR products with a data base
PCR products were sequenced and the sequence obtained compared with the EMBL data base. The best match is shown. In all cases the PCR product was found to be specific. Alignment for human MMP-13, MT-1-MMP, MT-3-MMP, TIMP-1, TIMP-2 and TIMP-3 showed equally high degrees of homology (data not shown).

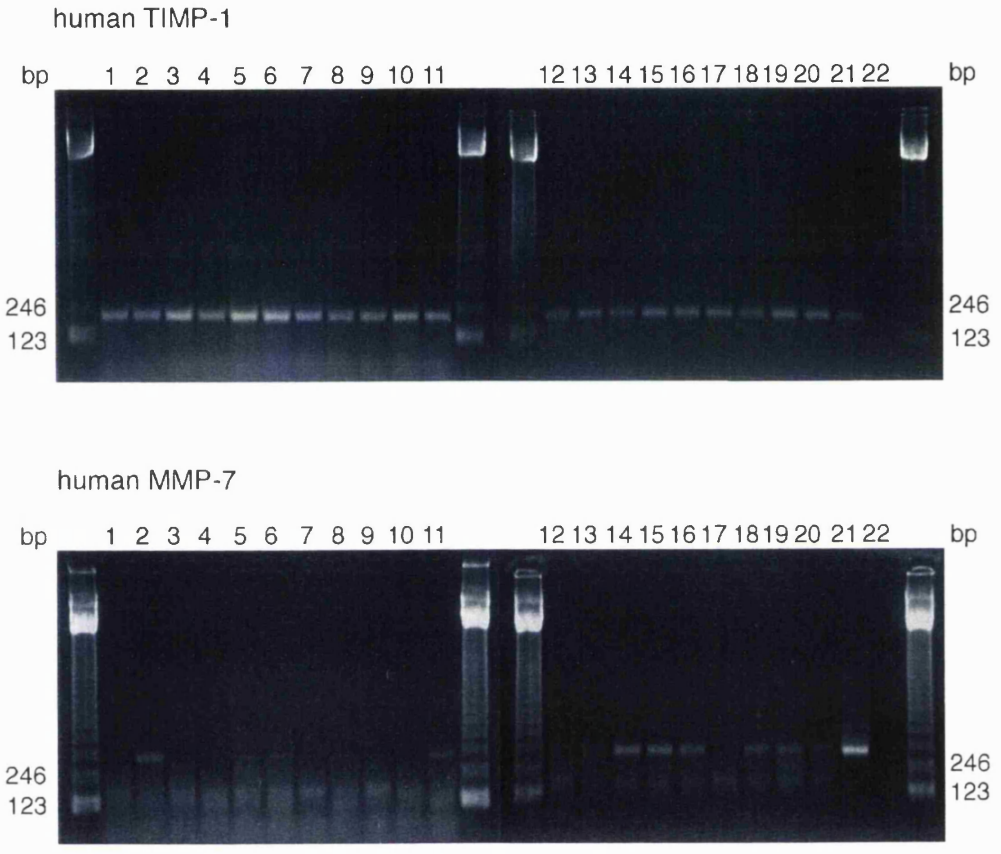


Fig. 2.3 Agarose gel of RT-PCR products
The figure shows typical results obtained by RT-PCR for MMPs/TIMPs using cDNA from human tissue biopsies. Lane 1 - 4: normal tissue biopsies; lane 5 - 10: benign tissue biopsies; lane 11 - 17: malignant tissue biopsies; lane 18 - 20 metastasis; lane 21: positive control for PCR primers; lane 22: negative control

2.3.2 MMP/TIMP expression in biopsies of the normal, benign and malignant human ovary

The results obtained for the human tissue material is shown in Fig. 2.4. Three patterns of expression were observed: MMP-2, -11, MT-1-MMP, TIMP-1, -2, -3, were expressed in all biopsies. MMP-1, -10, and MT-2-MMP were either not detectable or only found in a single sample. MMP-3, -7, -9, -13 and MT-3-MMP showed a variable pattern of expression.

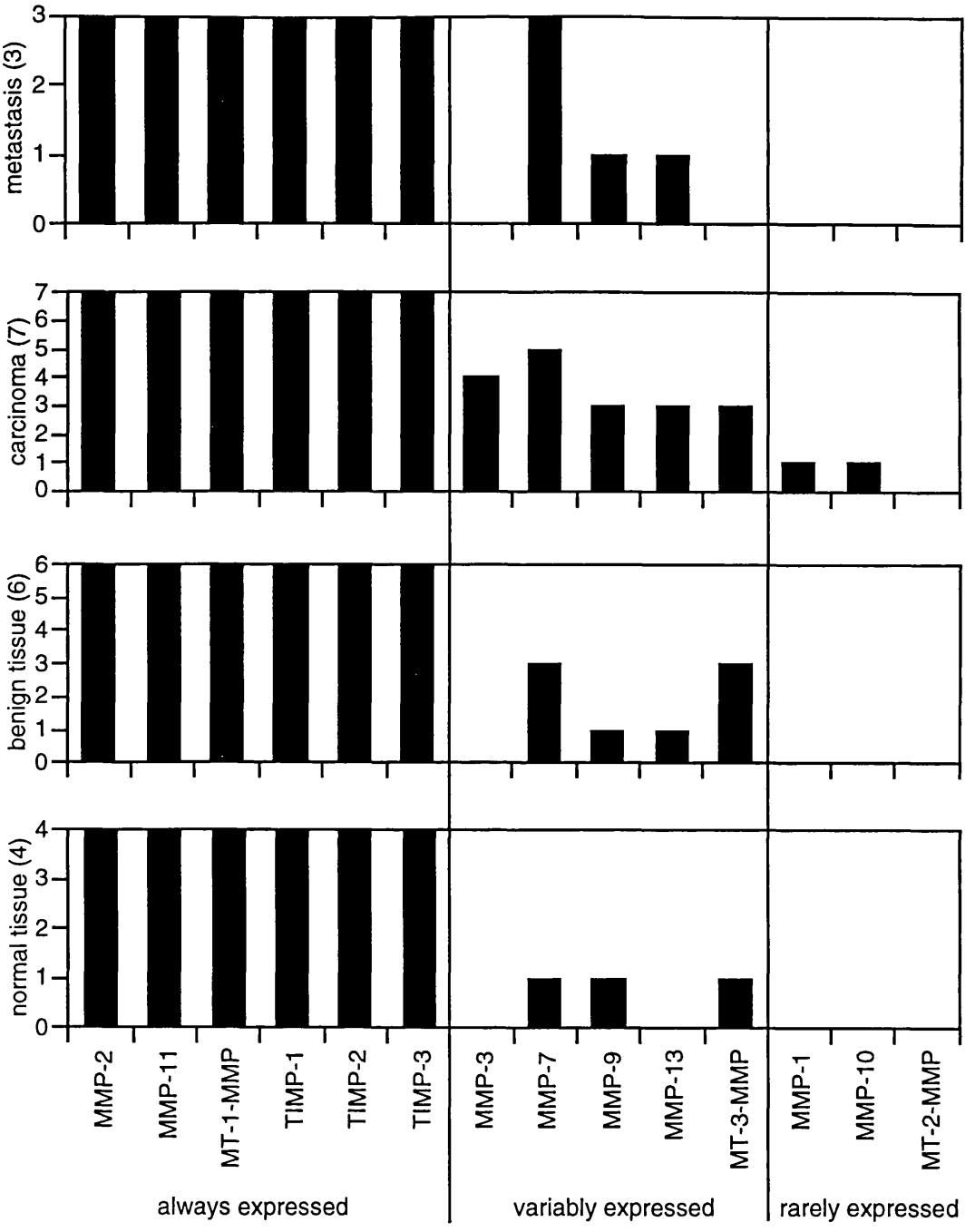


Fig. 2.4 MMP/TIMP expression in tissue biopsies
Graphical representation of MMP/TIMP expression in normal, benign and malignant biopsies of the human ovary. The MMPs/TIMPs were classified according to their frequency of expression (always , variably or rarely).

The observation that some MMPs were variably expressed raised the possibility of an association between the frequency of expression and the type of sample. To evaluate this, biopsies were classified as non-malignant (normal and benign) or malignant

(carcinoma and metastasis). Each group contained 10 samples. The results are shown in Fig. 2.5.

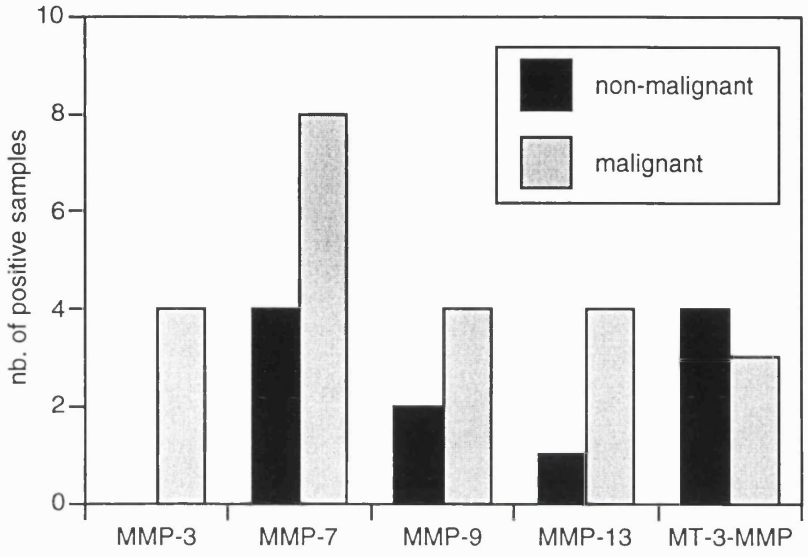


Fig. 2.5 MMP expression in malignant and non-malignant tissue biopsies
Comparison of the MMP expression frequency between malignant and non malignant samples of human ovarian cancer. Each group consisted of 10 samples. All but MT-3-MMP showed an increased frequency of expression in the malignant versus the non-malignant group.

In the malignant group MMP-3, -7, -9 and -13 showed a frequency of expression which was at least twice the one detected in the non-malignant samples. The frequency of expression of MT-3-MMP was comparable in both groups.

2.3.3 *Comparison of the MMP/TIMP expression pattern in malignant human biopsies with cell lines*

The pattern of MMP/TIMP expression observed in malignant biopsies of the human ovary was compared to that obtained from ovarian cancer cell lines. The results are shown in Fig. 2.6. The pattern of expression observed in the cell lines was remarkably similar to that of the malignant biopsies. However, some differences were observed. MT-1-MMP and MMP-2 were expressed in all tissue biopsies but were not in all tumour cell lines. In addition, both MMP-1 and MMP-10 were detected at a higher frequency (3/5) in the cell lines than in the malignant biopsies of human ovarian cancer (1/10).

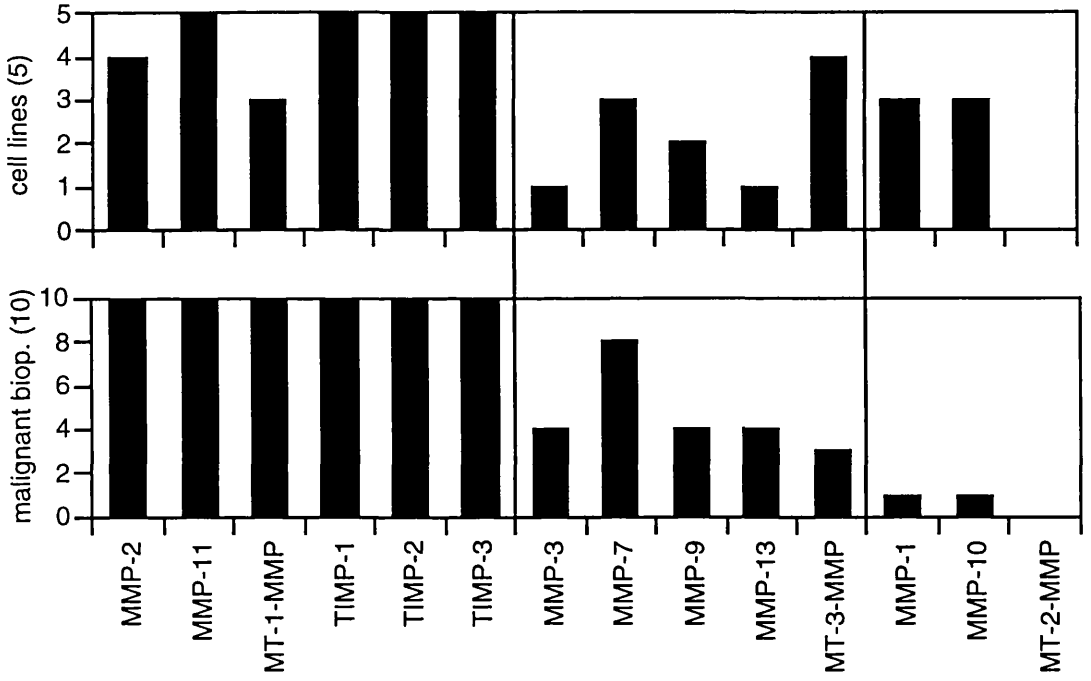


Fig. 2.6 MMP/TIMP expression in malignant biopsies and ovarian cancer cell lines
Comparison of the MMP/TIMP expression frequency in biopsies of human ovarian cancer and ovarian cancer cell lines showed a remarkably similar pattern of expression.

2.3.4 *Comparison of the MMP/TIMP expression pattern in malignant human biopsies with xenograft models of human ovarian cancer*

The MMP/TIMP expression pattern of the malignant biopsy group was finally compared with that of three xenografts models of human ovarian cancer. In this study some murine homologues of human MMPs/TIMPs were included. The results are shown in Fig. 2.7.

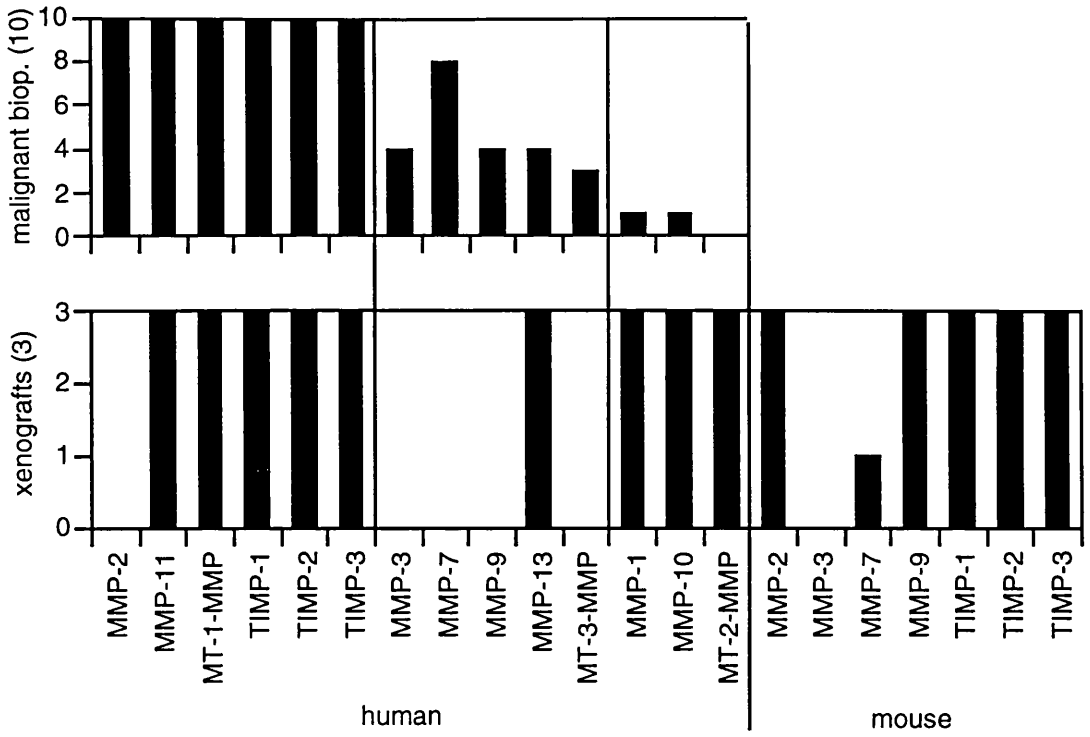


Fig. 2.7 MMP/TIMP expression in malignant tissue biopsies and xenograft models of human ovarian cancer
Comparison of the MMP/TIMP expression frequency observed in biopsy material of human ovarian cancer with xenograft models of this tumour showed some similarities in the pattern of expression.

The three xenografts models showed a very uniform expression pattern for both human and murine MMPs/TIMPs. Apart from mouse MMP-7, all other MMPs/TIMPs analysed were present in all or none of the tumour xenograft models. Human MMP-1, -10, -11, -13, MT-1-MMP, MT-2-MMP, TIMP-1, -2, -3 and murine MMP-2, -9, TIMP-1, -2, -3 were detected in all xenograft samples. Human MMP-2, -3, -7, -9, MT-3-MMP and mouse MMP-3 were not detected in any sample. Murine MMP-7 was detectable in only one of the three xenograft models.

The MMP/TIMP frequency of expression in the malignant biopsies and the xenograft models was compared. Most striking were the results for MMP-2 and MT-2-MMP. Human MMP-2 was detectable in all tissue samples (10/10) but was not detectable in any xenograft model (0/3). Human MT-2-MMP, which was expressed in none (0/10) of the malignant biopsies was detected in all tumour xenografts (3/3). Comparison of the expression pattern of human and mouse homologues in the xenograft model was also interesting. MMP-2, -7 and -9 were detectable only in their murine form but not in their

human form. MMP-3 was not detectable in either form and TIMP-1, -2 and -3 were detectable in both human and mouse forms.

2.4 Discussion

The aim of this part of the project was to establish a broad picture of MMP/TIMP expression in biopsies of normal, benign and malignant human ovary and to compare this with that of cell lines and xenograft models. For this purpose specific PCR primers for a panel of human and murine MMPs/TIMPs were designed and 20 tissue samples, 5 ovarian cancer cell lines and 3 ovarian cancer xenografts were analysed by RT-PCR. The results obtained from the tissue biopsies of human ovarian cancer showed that MMPs/TIMPs could be classified according to their frequency of expression. MMP-2, -11, MT-1-MMP, TIMP-1, -2 and -3 were always expressed. MMP-1, -10 and MT-2-MMP were not detectable and MMP-3, -7, -9, -13 and MT-3-MMP showed a variable frequency of expression. To analyse the latter group further the samples were divided into a malignant and a non malignant group. This led to the identification of four MMPs (MMP-3, -7, -9, -13) which were expressed at least twice as frequently in the malignant compared to the non malignant group. The MMP/TIMP expression frequency in the malignant biopsies was then compared to that detected in 5 ovarian cancer cell lines. Apart from MT-1-MMP and MMP-2 the pattern of expression frequency observed *in vivo* was maintained in the cell lines. MT-1-MMP and MMP-2 were expressed in all tissue biopsies but not in all cell lines. Finally MMP/TIMP expression frequency in the malignant group of the tissue biopsies was compared with that of three xenograft models of human ovarian cancer. The pattern observed within the three xenograft models was very uniform but showed some differences to the pattern observed in the tissue biopsies of human ovarian cancer. Human MMP-2 which was detectable in all tissue samples (10/10) was not detectable in any xenograft model (0/3) and human MT-2-MMP was expressed in all tumour xenografts (3/3) but in no (0/10) malignant biopsy.

For an adequate interpretation of the results obtained in this part of the work the properties of the RT-PCR method have to be considered. RT-PCR is a highly sensitive and qualitative method for detection of mRNA of a particular gene. Hence quantitative differences in gene expression were not analysed. In addition, not all mRNA is necessarily translated into biologically active protein since further control could take place

on the translational and post-translational level. Therefore, further studies of MMP/TIMP mRNA and protein are required to complement the data obtained.

The first result of this project was the classification of the MMPs/TIMPs according to their frequency of expression. The observation that some (MMP-2, -11, MT-1-MMP, TIMP-1, -2, -3) were expressed in all samples independent of their classification as normal, benign, malignant or metastatic tissue was surprising because this suggested continuous synthesis of matrix degrading enzymes and their inhibitors. In principal this finding was in keeping with the biological function of the ovary as the location of egg maturation and ovulation both of which have been associated with tissue modulation and repair (9). The tissue samples in our study, however, were mostly derived from post menopausal women (average age 57.6 years) and were therefore expected to show reduced tissue turnover and also reduced expression of MMPs. These results suggested that - perhaps localised - expression of a particular set of MMPs/TIMPs might be associated with normal cellular functions in the ovary. In addition, MMPs and TIMPs might have also have properties other then their proteolytic/inhibitory activities. For both TIMP-1 (151, 152) and TIMP-2 (153, 154) a growth factor-like activity has been suggested.

A further finding of this study was that expression of some MMPs was very rarely detectable in the tissue biopsies analysed. It was therefore possible to exclude those from further studies. Furthermore, this suggested that there was a set of MMPs/TIMPs which could be associated with the ovary and upon which further investigations should focus. In this respect, the MMPs which showed an increased expression frequency in the malignant samples in comparison to the non malignant ones (MMP-3, -7, -9 and -13) may be of particular interest. This finding suggested an association with tumour progression but the results have to be confirmed using a larger set of samples then the one assessed in this study. However, the results were in keeping with published reports. In human prostate cancer, for example, MMP-7 gene expression was found to be more frequent in malignant then in non-malignant biopsies (155). In addition, MMP-7 gene expression and protein was found to be increased in malignant biopsies in comparison to non malignant colorectal tissue (156, 157). Similarly, MMP-3 gene expression and protein was increased in squamous cell carcinoma as compared to normal epithelium of the oral cavity (158) and expression of MMP-13 was more frequent

in malignant then in non malignant tissue samples of the squamous epithelium of the skin (159), head and neck (160). In addition, expression of MMP-13 has not been detected in normal ovary by northern blotting (146). We confirmed this result by PCR (Fig. 2.4) but detected its expression in benign and malignant biopsies (Fig. 2.4).

Comparison of MMP/TIMP expression in malignant tissue samples of the human ovary with ovarian cancer cell lines showed that the pattern of expression was essentially maintained. This finding was rather surprising for MMP-2, -11 and TIMP-2 since their expression *in vivo* has been associated with the tumour stroma rather than tumour cells (42, 43). Similarly, MMP-9 expression has been localised to tumour infiltrating macrophages *in vivo* (43). These discrepancies might be due to the cell culture environment or genetic alterations in the cell lines.

Inclusion of PCR primers for both human and murine MMPs/TIMPs was of particular interest for the analysis of the three xenograft models of human ovarian cancer. It was expected that the results would provide information about the cellular origin, tumour or tumour stroma, of MMP/TIMP expression. Although the three xenograft models were derived from different patients and different states of disease, the pattern of MMP/TIMP expression was surprisingly similar. In fact, apart from murine MMP-7 which was detectable in one of the three xenografts, the pattern obtained was identical within the three xenografts. These data might suggest that there is a defined set of MMPs/TIMPs involved in the development of an ascitic tumour in nude mice. In addition, our study revealed the cellular origin of some of the MMPs and TIMPs (Fig. 2.7). MMP-2 and MMP-9 were only produced from mouse cells confirming the *in situ* data in human ovarian cancer which suggested stromal origin of these enzymes (43). The TIMPs were detected using both the human and the murine set of PCR primers which is also in keeping with the *in vivo* data (43). The classification, however, of a murine protease as the homologue of a human MMP has been based on sequence homology rather than functional analysis of the protease *in vivo*. For this reason it has to be considered that a direct comparison of mouse and human MMPs/TIMPs might be invalid.

The data obtained in this part of the work have provided useful information about MMPs and TIMPs in human ovarian cancer. More work, however, is required on both MMP/TIMP mRNA and protein to obtain an understanding of the role of MMPs/TIMPs in

human ovarian cancer. Work is ongoing to analyse the total MMP related collagenolytic activity present tissue extracts of human ovarian cancer and normal ovary. The aim of this thesis was, however, to select one MMP and to study its regulation. MMP-9 was chosen because some complementary information about this protease in ovarian cancer and an assay for its detection was available. MMP-9 protein has been detected in tumour samples by zymography and its expression localised in tissue samples of human ovarian cancer (43). In the following chapter a method to detect and quantitate MMP-9 protein, zymography, was optimised with respect to assay linearity and sensitivity.

3. Optimisation of the zymography technique for the quantitation of MMP-9

3.1 Introduction

In chapter 2 the profile of MMP/TIMP gene expression in biopsies of human ovarian cancer was determined by RT-PCR. MMP-9 was selected for further analysis because an assay to detect this protease (zymography) and information on its gene expression in biopsies of human ovarian cancer were available. Zymography is a simple, sensitive, quantifiable and functional assay to analyse proteolytic activity. It has been widely used for research on extracellular matrix degrading enzymes, in particular the matrix metalloproteases MMP-2 and MMP-9 (161).

The standard method is based on SDS-gels impregnated with a protein substrate, in particular gelatin or casein. Proteases which have the ability to renature and exert proteolytic activity on a co-polymerised substrate upon removal of the SDS, can be analysed by this method (162). MMP-2 and MMP-9 are detectable on gelatin zymograms and MMP-7 on casein gels (162, 163). Co-polymerisation of plasminogen together with gelatin allows detection of the plasminogen activators uPA and tPA (164). Fluorescent or radioactively labelled substrates and overlay techniques have also been used (165).

Zymography offers several features which made it particularly useful with respect to alternative methods such as ELISA. No expensive materials are required (e.g. antibodies) and several proteases showing activity on the same substrate can be detected and quantified on a single gel. MMPs are released from cells in a proteolytically inactive pro-form (zymogen) which is about 10kDa larger than the activated forms. These pro-forms become activated during the process of renaturation after gel electrophoresis and are therefore also detectable on zymograms (161, 166). In addition, MMPs are often associated with their corresponding tissue inhibitors of metalloproteases (TIMPs). During electrophoresis the inhibitors dissociates from the MMP and do not interfere with the detection of the enzymatic activity (161). All potential proteolytic activity can therefore determined. Finally, zymography is extremely sensitive. Levels of

less than 10pg of MMP-2 are detectable on gelatin zymograms comparing this technique favourably with ELISAs (161).

Proteolytic activity on zymograms can be quantified by computer supported densitometry scanning and image analysis (161). Within a certain range of the assay a linear relationship exists between the amount of enzyme loaded and the activity detected (161, 162). However, quantitation of zymograms remains difficult because i) the limited number of wells per gel do not allow a full standard curve and several samples to be run on the same gel and ii) the two-step staining/destaining method is not reliable and difficult to reproduce. Traditionally, gels are stained in a 0.5% (w/v) Coomassie-Blue solution for 3 hours (161, 162). This is followed by a destaining step of undefined length, usually several hours, until a satisfactory background/band staining is achieved. There are, however, major drawbacks to these undefined protocols. Over-staining of the gels reduces the assay sensitivity because bands of low activity become undetectable. Excess destaining can also "bleach" the bands so that their intensity is no longer within the linear range of the assay.

Thus, the aim of this part of the work was to develop a more reliable and reproducible staining/destaining protocol. Using this method the linear range, the reproducibility and sensitivity of the assay for proMMP-9 on standard gels (140x130x1mm) was established. Furthermore it was demonstrated that a single point standard was sufficient for the determination of proteolytic activity in the remaining samples loaded on the gel.

3.2 Material and Methods

Materials

Materials required for gel preparation were purchased from National Diagnostics (Atlanta, Georgia, USA; AccuGel 29:1 [40% Acrylamide, 29:1 Acrylamide:Bis-acrylamide]), ProtoGel stacking gel buffer and ProtoGel running gel buffer). Chemicals for the running buffer (25mM Tris, 192mM glycine, 0.1% (w/v) SDS), gelatin (Porcine skin type I, bloom 300), ammonium persulfate, TEMED and Brij-35 were obtained from Sigma (Poole, UK). Highly purified human proMMP-9 was purchased from TCS-Biologicals (Botolph Claydon, UK). The slab gel apparatus was from Genetic Research Instrumentation (Dunmow, UK).

Preparation and running of gels

Zymogram gels, loading buffer (50mM Tris (pH 6.8), 10% (v/v) glycerol, 1% (w/v) SDS, 0.01% (w/v) Bromophenol-Blue) and collagenase buffer (50mM Tris (pH 7.6), 0.2M NaCl, 5mM $CaCl_2$, 0.2% (v/v) Brij-35) were prepared as described elsewhere (167). Briefly, 11% running gels (100x130x1mm) containing 0.12mg/ml gelatin were overlaid with a 4% stacking gel (40x130x1mm), samples loaded and run at 180 volts until the dye front ran off the gel. Gels were removed from glass plates and soaked for 1 hour in 2.5% Triton-X-100 on a shaker followed by two brief washes in collagenase buffer in which the gels were thereafter incubated for 18 hours at 37°C.

Staining of gels

Prior to staining, gels were briefly rinsed in distilled water. PhastBlue tablets were purchased from Pharmacia (Uppsala, Sweden) and a 0.2% stain stock solution prepared according to the manufacturers instructions. Prior to storage at 4°C, stain stock solution was filtered through No. 1 Whatman filter paper to remove undissolved stain particles. A stain working solution (200ml per gel) was prepared by mixing 10ml of stain stock solution with 200ml of destain (1:3:6 glacial acetic acid : methanol : distilled water) and gels were stained for the periods of time indicated on a shaker at room temperature.

After completion of staining, gels were briefly rinsed in distilled water and carefully wrapped in Saran Wrap (Dow Chemicals).

Scanning of gels and quantitation of bands

Gels were scanned using a Umax (MagicScan or Astra 1200S) flat bed scanner driven by NIH Image 1.58 image analysis program on a Macintosh computer. This program, which can be obtained through the National Institute of Health, was also used for quantitation of the bands. Gels were scanned in grey scale (mode: transmissive; gamma = 1; shadow = 0; range = 1-255). The density of each pixel was encoded on a scale ranging from 1 (clear) to 255 (opaque). The resolution was set to 150 dots per inch. Pilot experiments showed that this resolution provided satisfactory results without leading to excessively large files. Further, re-scanning of the same gel did not affect the outcome of the quantitation procedure, which was consistent with observations made by others (161). Determination of band intensity was done as described in the handbook of NIH Image 1.58. Briefly, the scanner was calibrated using the step tablet No. 2 (Kodak, Rochester, NY, USA) and the density measurements linearised using the Rodbard curve fitting function within the NIH Image 1.58 program. The results were stored in a file for later use. Calibration of the scanner proved to be important since there was no linear relationship between the defined optical density of the step tablet and the measurements taken by the flat bed scanner. These findings were reproduced on a different flatbed scanner (Agfa, Arcus II).

To assess a zymogram, the file containing the scan of a gel, the calibration file and the "gel plotting macro" were loaded. Using the rectangular selection tool an area consisting of the band and sufficient background (approximately 2cm) above and below the band was selected. Using the commands offered by the "gel plotting macro" the pixel intensity in each lane was plotted. The default setting of Image 1.58 was modified to obtain plots on which highest densities (opaque) were represented on the bottom end of the y-axis. Using the line tool the area under the curve was closed by joining the horizontal background line before and after the peak. The area under the curve was evaluated by applying the wand tool on the enclosed area to determine the integrated band intensity.

Preparation of proMMP-9

Purified human proMMP-9 (TCS-Biologicals) was diluted to 1000pg/µl in 60mM Tris (pH 7.2), 15mM $CaCl_2$, 80mM $NaCl_2$, 0.1% bovine serum albumin (BSA, Sigma) and stored at -20°C. For zymograms doubling dilutions were prepared in the same buffer and 8µl loaded onto a gel.

3.3 Results

3.3.1 Time course of zymogram staining

Zymogram gels were poured as described, samples of proMMP-9 prepared by doubling dilution from a stock solution (1000pg/µl) and loaded in the range from 8000 to 32pg/well. Gels were scanned after 1, 2, 4.5 and 6 hours of incubation in the staining solution. A typical zymogram at the four time points is shown in Fig 3.1. A darkening of the background staining over time could be observed. It should be noted that bands at the lower end of the dilution curve were barely visible. Although they were detectable by the scanner they did not photograph well. This finding was consistent with those made by others (161). The integrated area measured for each amount of proMMP-9 per time point is shown in Fig. 3.2 A. For a particular amount of proMMP-9 the area increased with time. This was due to the darkening of the background staining. However, this effect diminished between 4.5 and 6 hours of staining indicating that a point of saturation of background staining was approached.

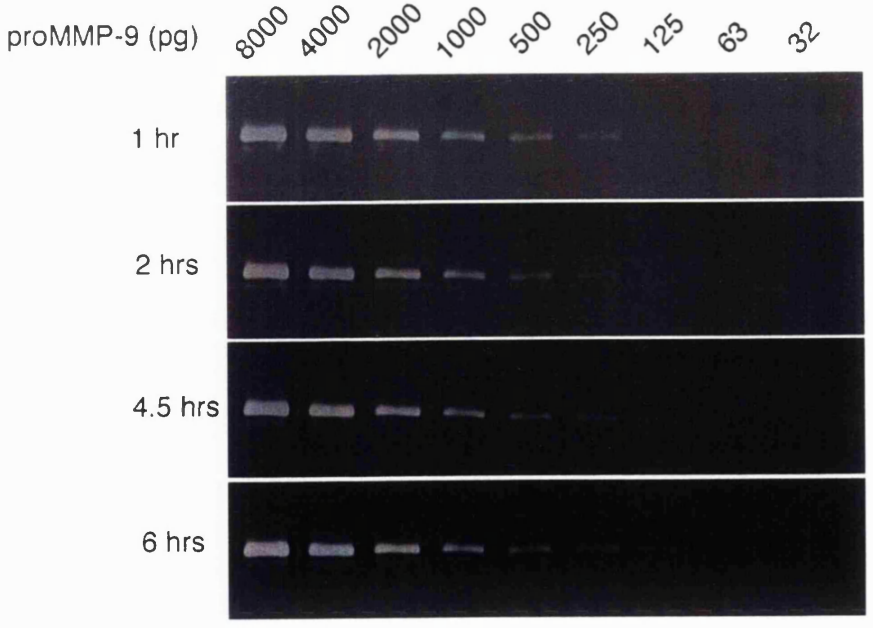


Fig. 3.1 Time course of zymogram staining
Purified human proMMP-9 was diluted in doubling dilutions and loaded in the range between 8000 and 32pg protein per well. Gels were run as described in Material and Methods and stained for the times indicated. A darkening of the background staining over time can be seen.

Expression of the integrated area for an individual amount of protein as percentage value of that of the highest amount of proMMP-9 loaded (8000pg = 100%) showed that the increase in background staining over time did not alter the relative band intensities within a dilution series (Fig. 3.2 B). Both Fig. 3.2 A and Fig. 3.2 B indicated that a linear relationship existed between the amount of protein loaded and the band intensity in the range below 1000pg of proMMP-9. This range is shown in Fig. 3.2 C and indeed confirmed the linearity for all four time points of staining.

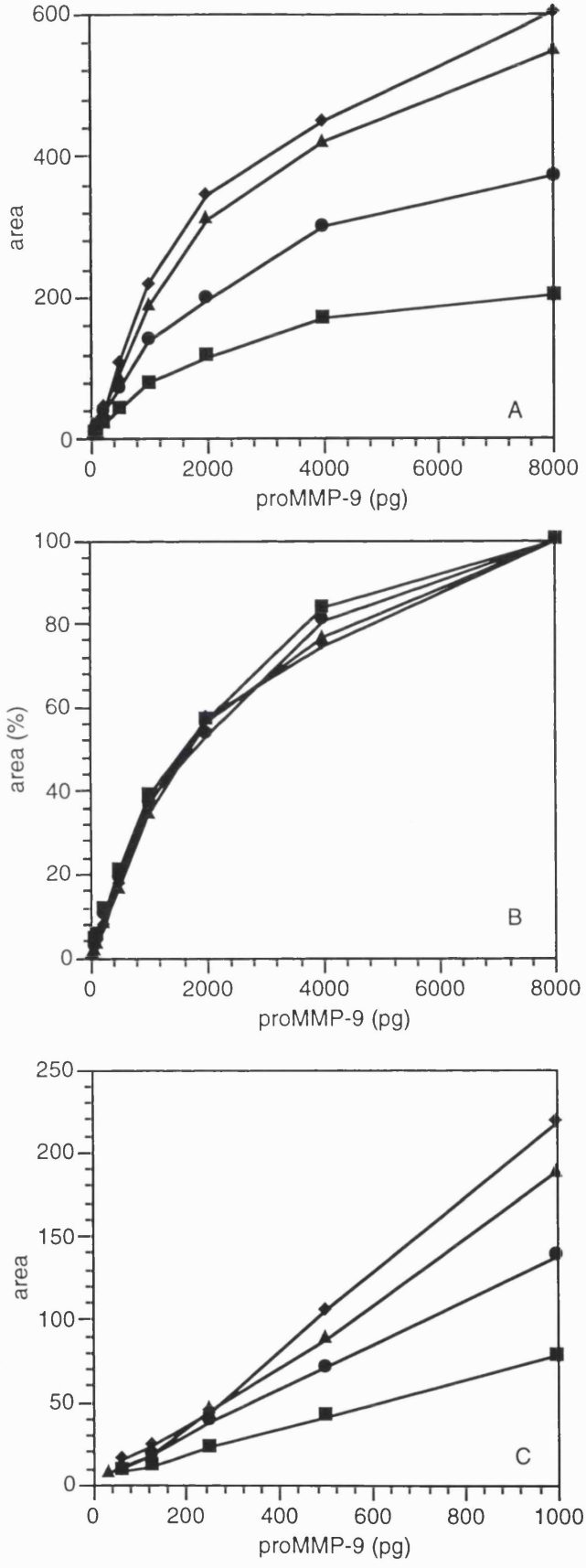


Fig. 3.2 Quantitation of the time course of zymogram staining

Fig. 3.2 A shows the area determined for the complete range of proMMP-9 loaded all four time points of staining (1(■), 2(●), 4.5(▲) and 6(◆) hours).

The value determined for a particular amount of protein increased with increasing staining time. This effect was diminished between time point 4.5 and 6 hours. In Fig. 3.2 B the individual values were expressed as a percentage of the value found for 8ng of proMMP-9. All periods of zymogram staining led to the same curve. A linear range between the area measured and the amount of proMMP-9 loaded existed in the range of 1ng proMMP-9 and below. This range is shown in Fig. 3.2 C. (Data shown are typical for three experiments performed)

3.3.2 *Intergel reproducibility of linear range and detection limit*

The time course experiment of zymogram staining was performed three times and the observations made were found to be consistent with those shown in Fig. 3.1 and 3.2 A-C. The areas measured for the range from 2000pg to 32pg proMMP-9 were expressed as percentage of the area found for 2000pg proMMP-9 (=100%) and plotted in Fig. 3.3. For all four time points there was a linear relationship between the amount of protein loaded and the area measured in the range of 1000pg proMMP-9 and below.

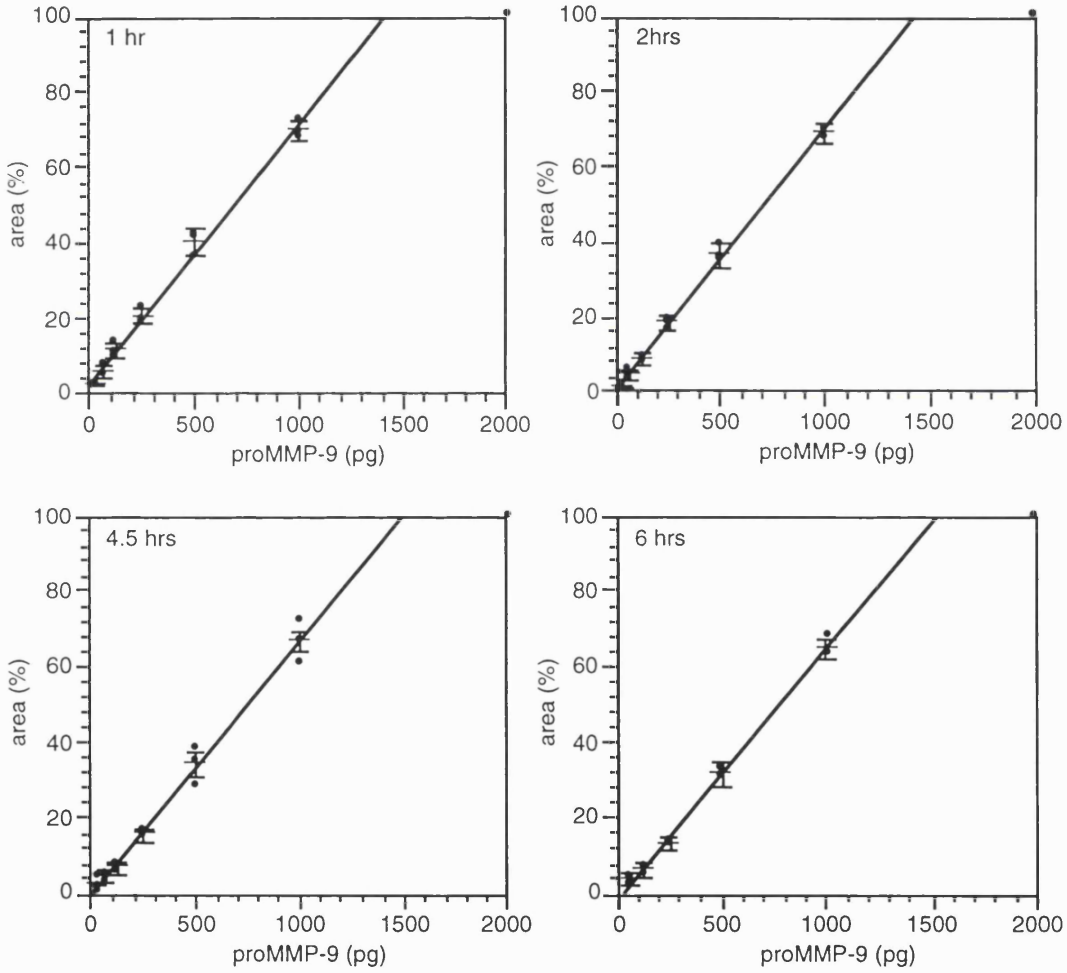


Fig. 3.3 Inter gel reproducibility of the linear range for proMMP-9
The values shown were calculated by expressing the integrated density of a particular amount of proMMP-9 as percentage value of that measured for 2ng of proMMP-9. For all four time points (1, 2, 4.5 and 6 hours) linearity between the amount of protein loaded and the area measured existed in the range of 1ng proMMP-9 and below. (● individual data; – average; error bars: standard deviation)

Detection of the lowest amount of MMP-9 loaded (32pg) was possible in 2 out of 3 experiments for an incubation period of 1 and 2 hours; in 3 out of 3 experiments for a staining period of 4.5 hours and failed in all three cases for a staining period of 6 hours. This indicated that the detection limit of the assay was approximately 32pg of proMMP-9.

3.3.3 Intragel variation of proMMP-9 quantitation

Four gels were poured from a single stock solution and on each gel the same amount of proMMP-9 was loaded eight times (1000, 500, 125 and 62.5pg). The gels were stained for 3 hours. The data and statistics are shown in Table 3.1. For all four amounts of protein, the coefficient of variation (CV) remained below 15% of the mean value, indicating an acceptable intra assay reproducibility.

proMMP-9	62.5pg	125pg	500pg	1000pg
n	8	8	8	8
mean (area)	6.43	15.96	53.38	133.36
stdev (area)	0.74	1.69	7.97	8.62
CV(%)	11.58	10.60	14.94	6.46

Table 3.1 Intra gel reproducibility of the linear range for proMMP-9
ProMMP-9 was diluted in doubling dilutions and each of the amounts were loaded eight times on a single gel. The zymograms were run as described in Material and Methods and the gels stained for 3 hours. For all four amounts of protein the coefficient of variation (CV) remained below 15% of the mean value.

3.4 Discussion

In this chapter an improved staining protocol merging the traditionally separated staining and destaining step of zymograms into a single procedure was established. This protocol overcomes the main disadvantages of the traditional method which were over-staining leading to loss of assay sensitivity and over-destaining which could "bleach" the bands and make them unsuitable for quantitation. Evidence was provided that the single step method led to reproducible staining of zymograms avoiding gel to gel judgements of the achieved background staining. On the basis of this protocol the linear range of the assay, the detection limit and the assay reproducibility for proMMP-9 was established. The findings indicated that proMMP-9 could be quantified reliably from 1000pg down to the detection limit, which was determined to be at 32pg. The range covered almost two log scales and was therefore comparable with ELISAs. The linear range was also wider than reported in a comparable study on proMMP-2 in which linearity was found to just exceed one log scale (10 to 120pg) (161); the higher assay sensitivity of the latter was probably due to a higher specific activity of proMMP-2 on zymograms and the higher protease concentration per volume unit gel due to the narrower combs in the mini-gel system used.

Further evidence was provided that over-staining and therefore loss of assay sensitivity was well controlled in this method and only appeared when the staining period was extended to 6 hours. Bleaching of bands could not be observed because of the combination of staining and destaining into a single step procedure. Linear quantitation of proMMP-9 was already possible after as little as 1 hour of staining. In practice, however, a longer staining period of 2-3 hours was found to be more convenient because it led to more intense background staining. Nevertheless, the procedure still remained faster than other protocols reported and required no additional handling of the gel.

In terms of reproducibility, satisfactory inter- and intra-gel reproducibility was demonstrated. The linear range was maintained over several independently prepared zymograms. Experiments performed to investigate the intragel reproducibility showed

that for the whole range of linearity, the coefficient of variation (CV) remained below 15% of the mean value.

This staining protocol was also useful for detection of MMP-2 on gelatin zymograms and MMP-7 on casein zymograms (data not shown). These findings were not unexpected since the staining procedure did not depend on the protease to be detected but only on the co-polymerised substrate. The method might therefore also be useful for detection of other proteases (uPA, tPA, other MMPs, etc.) which degrade a co-polymerised protein substrate that can be efficiently stained by Coomassie-Blue.

On the basis of the assay reproducibility and linearity, the use of a single point standard was suggested. Since all further data depended on the outcome of this single point standard we suggested using the mean of duplicate samples for quantitation. This allowed a reliable quantitation of proteolytic activity in 10 further samples on the same gel using a 12 well comb.

In this chapter a method for the detection and quantitation of MMP-9 was optimised and validated. This provided an essential tool for the analysis of MMP-9 release in a co-culture system as outlined in the following chapter.

4. Regulation of monocytic MMP-9 production in human ovarian cancer

4.1 Introduction

In chapter 2 of this thesis the MMP/TIMP gene expression profile in malignant and non malignant biopsies of human ovarian cancer was determined by RT-PCR. Gene expression of some MMPs/TIMPs (MMP-2, -11, MT-1-MMP, TIMP-1, -2, -3) was detected in all tissue biopsies, whilst others (MMP-1, -10, MT-2-MMP) were rarely detectable. Five MMPs (MMP-3, -7, -9, -13, MT-3-MMP) showed a variable pattern of expression. In the small number of biopsies analysed, expression of MMP-3, -7, -9 and -13 was at least twice as frequent in malignant samples (n=10) than in non-malignant ones (n=10). Of these four MMPs, MMP-9 gene expression and protein has been studied in biopsies of human ovarian cancer whereas to our knowledge no data are available for MMP-3, -7 and MMP-13. Analysis of tissue samples of human ovarian cancer by zymography revealed the presence of proMMP-9 protein (43). The pattern of MMP-9 gene expression in ovarian carcinomas as assessed by *in situ* hybridisation was discrete and seen in both tumour and stromal areas. Immunohistochemical studies with the macrophage marker CD68 showed a positive correlation with the pattern found for MMP-9 suggesting that tumour associated macrophages (TAMs) may be the source of MMP-9 (43). Naylor *et al.* also assessed expression of TNF-α, a potent stimulus of monocyte MMP-9 expression (168, 169), and TIMP-1 by *in situ* hybridisation in tissue sections of human ovarian cancer. Expression of TIMP-1 was found in stromal areas adjacent to tumour cells and, in some cases, in tumour cells (43). TNF-α expression was confined to epithelial tumour areas. Immunoreactive TNF-α protein was found in both tumour and stromal areas with a similar pattern to that of infiltrating macrophages (170). These results suggested that TAMs could be the source of MMP-9 and that TNF-α might play a role in its production (43, 170).

Co-culture of different cell types represents a relatively simple *in vitro* system to analyse tumour stroma interactions mediated by soluble proteins and/or cell-cell contact. MMP-9 production has been studied in co-cultures of fibroblasts and cancer cells.

Himelstein *et al.*, Kurogi *et al.* and Lengyel *et al.* suggested that a soluble, fibroblast derived factor can induce MMP-9 production from breast, osteosarcoma and squamous cancer cell lines, respectively (171-173). Others have shown that cell-cell contact between fibroblasts and colorectal or keratinocyte cancer cell lines was required for MMP-9 production (174, 175). Further Miyagi *et al.* (176) have suggested that ovarian cancer cells induced fibroblast MMP-9 production via a soluble factor and Shibata *et al.* (177) reported that mesothelial cells induced MMP-9 production in an ovarian cancer cell line. Both studies, however, were not in keeping with the *in vivo* findings by Naylor *et al.* showing that MMP-9 production is associated with tumour infiltrating macrophages rather than tumour or other stromal cells (43). Finally, while this work was in progress, Swallow *et al.* reported the induction of monocytic MMP-9 production by colorectal cancer cell lines (178). They provided evidence that a soluble factor derived from a metastatic colorectal cancer cells but not from non-metastatic colorectal cancer cells was able to induce MMP-9 production in the monocytic cell line THP-1.

In this chapter a co-culture system of an ovarian cancer and a monocytic cell line is described and the cellular interactions are analysed with respect to the production of MMP-9. A mechanism of monocytic proMMP-9, but not TIMP-1, production is proposed and evidence provided suggesting that a soluble tumour derived factor, tentatively named MMPSF (matrix metalloproteinase stimulating factor) synergises with autocrine or paracrine TNF-α to stimulate monocytic MMP-9 release.

4.2 Material and Methods

Cell culture techniques

The human ovarian cancer cell line PEO1 has been described in detail in the Materials and Methods of chapter 2. The human monocytic cell line THP-1 was obtained from ATCC (American type culture collection, Rockville, USA) and maintained at a cell concentration between 0.5 and $1x10^6$ cells/ml in RPMI containing 10% FCS and 50µM beta-mercaptoethanol (Sigma, Poole, UK). Cells were grown in Nunc tissue culture flasks (Nalge Nunc International, Denmark) and incubated in a humidified atmosphere at 37°C, 5% CO_2.

Experimental cell culture conditions and preparation of conditioned medium

PEO1 cells were grown to near confluency, detached from the tissue culture flask with trypsin/versene (Gibco, Paisley, UK), resuspended in culture medium, pelleted (210g, 5 min), washed up to three times in phosphate buffered saline (PBS) and resuspended in FCS-free Aim V medium (Gibco). Similarly, THP-1 cells were pelleted (210g, 5 min) and resuspended in FCS-free Aim V. Cells were counted using a modified Neubauer haemocytometer and, if not otherwise stated, the cell concentration adjusted to $1x10^6$ cells/ml. All experiments were set up in 24 or 96 well plates (Costar, Cambridge, MA, USA) and the cell culture supernatant was harvested after an incubation period of 48 hours, unless stated otherwise. The supernatant was cleared of cells and cell debris by centrifugation at 14000g in a microfuge prior to storage at -20°C or immediate use in zymography. For use as conditioned medium (CM) the supernatant was sterile filtered (Acrodisc 0.2µm, Gelman Sciences, Ann Arbor, MI, USA) prior to storage at -20°C or use.

Isolation of peripheral blood monocytes

50ml peripheral blood was taken from healthy volunteers by venupuncture, mixed with 5ml 3.8% sodium citrate and centrifuged (20 min, 300g) to obtain a cell pellet and platelet rich plasma (PRP). PRP was centrifuged twice (2000g, 10 min) to remove the platelets. The resulting platelet poor plasma (PPP) was stored on ice for later use. The cellular pellet was resuspended in 0.9% NaCl to 45ml and erythrocytes precipitated with 5ml

6% Dextran T-500 (Pharmacia, Uppsala, Sweden). The lymphocyte rich supernatant was transferred into a fresh tube, the cells pelleted by centrifugation (5 min, 200g), washed three times in wash buffer (0.9% NaCl, 10% PPP) and resuspended in 8ml PPP. Two ml of cell suspension were under layered with 2ml 42% Percoll (Pharmacia) prepared with PPP. After centrifugation (10 min, 300g) the monocyte rich interphase was harvested by aspiration, washed three times in wash buffer and resuspended in Aim V medium. To estimate the number of monocytes, a drop of cell suspension was transferred on a haemocytometer and the monocytes left to adhere for 30 min (37°C). Adherence changed their appearance sufficiently to determine the monocyte concentration by cell counting. The remaining cells were then seeded, incubated for 1 hour at 37°C, washed three times with Aim V to select the monocytes by adhesion and Aim V added to obtain a cell concentration of $1x10^6$ monocytes/ml. To assess the purity of the preparation, an aliquot of cells was left to adhere on a petri dish, washed in the same way as the cells used for the stimulation experiments, air dried, stained using the α-naphtyl acetate esterase method (179) and counter-stained with Meyers Hematoxylin. Monocytes appeared with multiple reddish-brown granules. The ratio of monocytes versus non monocytes was determined by phase contrast microscopy. In all experiments purity of monocytes exceeded 90%.

Endotoxin assessment

Solutions and buffers (RPMI, PBS, AIM V, water, CM) used in cell culture were checked for endotoxin content using a kinetic turbidity assay[2] (BioWhittaker, Reading, UK) or the endotoxin detection kit (0.50 EU/ml, detection limit 50pg/ml endotoxin from *E. coli* 055:B5) purchased from Associates of Cape Cod (Woods Hole, MA, USA). Levels were found to be below 100pg/ml. Dose response experiments of THP-1 cells to three types of endotoxin (*E. coli* 055:B5, 0111:B4 and *Salmonella minnesota*) showed that levels of 100pg/ml or less did not stimulate MMP-9 release in a detectable manner (Fig. 6.1). To achieve levels of MMP-9 comparable to those obtained in co-culture or CM experiments with THP-1 (> 5 fold background level) 1ng/ml or more of endotoxin was needed.

[2] The kinetic turbidity assay was kindly performed by Chris Selkirk (ICRF, Department of Biotherapeutics and Hybridoma Development).

Endotoxin levels present in FCS were assessed by Gibco and were found to be below 100pg/ml.

Zymogram analysis

Quantitative gelatinolytic zymography was performed as described in chapter 3 (180). For gel to gel comparison a standard of commercially available purified human proMMP-9 (TCS-Biologicals, Botolph Claydon, UK) was loaded on each gel in duplicate. All samples were assessed in the linear range of the assay and the individual MMP-9 activity expressed in ng(MMP-9) / µl(supernatant). All values of MMP-9 activity were based on at least three independent experiments. The error bars reflect the standard deviation.

Spin column experiment

Spin columns (SpinColumns-30 in distilled water, Clontech, Palo Alto, CA, USA), were pre-spun twice for 3 min (4°C, 1100g) to remove the equilibration buffer. Sample (50µl) was loaded onto the spin column and spun for 5 min (4°C, 1100g). The flow-through was harvested and the volume adjusted to 55µl with sterile PBS.

Immunoprecipitation of TNF-α

The monoclonal anti-TNF-α antibody 6H11 (kindly provided by Dr. N-B. Liabakk, Institute of Cancer Research and Center for Molecular Biology, Trondheim, Norway) was added to CM or control samples at a concentration of 5µg/ml. Samples were incubated for at least 1 hour at 4°C on a roller, then 40µl protein-G sepharose beads (Sigma) were added and samples incubated as described before. To pellet the beads, samples were spun for 5 min at 14000rpm in a microfuge and the supernatant carefully removed. To assess the MMP-9 inducing activity, sample was added to THP-1 cells at a 1:10 dilution followed by quantitative zymography. To check the efficiency and specificity of TNF-α precipitation, samples spiked with recombinant human TNF-α at a concentration of 10ng/ml were prepared. Precipitation of TNF-α and removal of the anti TNF-α antibody was complete and specific. The experiments have been repeated with a commercially available monoclonal anti-TNF-α antibody (R&D Systems, Abingdon,

UK) and an unrelated antibody of the same antibody isotype (IgG_1) as a negative control.

Detection of TNF-α protein

TNF-α protein was detected using a WEHI-164 based bioassay[3] (181) or a commercially available ELISA-assay (R&D Systems). A standard curve using recombinant human TNF-α was set up and validation of the assay showed that accurate results could be obtained in the range between 20 and 500pg/ml TNF-α for the bioassay and 15-1000pg/ml for the ELISA.

RNA preparation and northern blotting:

Total RNA was isolated using Tri-Reagent (Molecular Research Center, Cincinnati, Ohio, USA) according to the manufacturers instructions. Total RNA (10μg) was heated to 65°C for 5 min, resolved on a 1% (w/v) denaturing formaldehyde-agarose gel and blotted onto a Hybond N^+ membrane (Amersham, Slough, UK) by capillary transfer (182). RNA was UV crosslinked (1200mJ) to the nylon membrane using a Stratalinker (Stratagene, Cambridge, UK) and blots pre-hybridised and hybridised as described (182). cDNA probes for TNF-α, MMP-9 and β-actin were radioactively labelled (^{32}P-dCTP, Amersham) by random priming using the Prime-It or the RmT-Prime-It labelling kit (Stratagene) according to the manufacturers instructions. After hybridisation, blots were washed twice for 10 min at room temperature in 2xSSC (20xSSC: 3M NaCl, 0.3M sodium citrate, pH 7.0), 0.1% (w/v) SDS, then incubated twice for 15 min in 0.1xSSC, 0.1% (w/v) SDS at 65°C and finally washed for 10 min in 2xSSC at room temperature. For detection blots were wrapped in Saranwrap (Dow Chemicals) and exposed at -70°C to BioMax X-ray film (Kodak, Rochester, NY, USA) for 4 to 56 hours with intensifying screens.

Western blotting

Supernatants of THP-1/PEO1 co-cultures and purified proMMP-9 were resolved by 10% SDS-PAGE under reducing conditions and electroblotted onto a nitrocellulose

[3] The WEHI-bioassay was kindly performed by P. Thavasu (ICRF, Biol. Therap. Laboratory).

membrane (Amersham) for 2 hours at 60 volts. The membranes were blocked for 16 hours at 4°C with phosphate buffered saline (PBS) containing 10% non-fat dry milk and 0.1% (v/v) Tween-20 (Sigma), briefly washed with PBS/0.1% (v/v) Tween-20 and incubated for at least 1 hour with the monoclonal anti MMP-9 antibody Ab-2 (Oncogene Science, Cambridge, MA, USA) or CA-209 (kindly provided by Dr. Raphael Fridman, Wayne State University, Detroit, USA) diluted in PBS/0.1% (v/v) Tween-20 1:5000 and 1:10000, respectively. The blots were washed three times for 10 min in PBS/0.1% (v/v) Tween-20 and incubated with the horse radish peroxidase labelled second layer antibody (Sigma) diluted 1:10000 in PBS/0.1% (v/v) Tween-20. Immunodetection was performed after extensive washing (10 times 15 min in PBS/0.1% (v/v) Tween-20) using the enhanced luminescence kit ECL (Amersham) or with SuperSignal ULTRA (Pierce, Chester, UK) according to the manufacturers instructions.

Statistical analysis

Data were analysed statistically with the Student's T-test using data from at least three independent experiments. P-values were determined with StatView 4.5 (Abacus Concepts, Berkeley, CA, USA) and differences considered significant for $p<0.05$.

Inhibitory constants representing a 50% inhibition (IC_{50}) were calculated using the curve fitting tool within DeltaGraph (DeltaPoint, Monterey, CA, USA). The formula was y=(a-d)/(1+(x/c)^b)+d. The parameter 'a' represents the maximum, 'b' the slope, 'c' the point of inflection and hence the IC_{50} value and 'd' the minimum of the sigmoid curve. Parameter 'a' was set to 1000, 'b' and 'c' to 1 and 'd' to 0 and 50 iteration steps calculated.

Other materials

Recombinant TNF-α, kindly provided by Knoll AG, Friedrichshafen, Germany, was prepared as a 15µg/ml stock in sterile filtered PBS, 0.3% (w/v) bovine serum albumin (Sigma) and stored at -20°C. The synthetic MMP inhibitor BB-2116 (gift of British Biotech Pharmaceuticals Ltd., Oxford, UK) was prepared as a 100mM stock solution in dimethyl sulfoxide (DMSO, Sigma) and diluted in PBS to, if not stated differently, a concentration of 30µM. Microbial lipopolysaccharide (LPS, *Salmonella minnesota*, Sigma) was prepared as a stock solution of 1mg/ml in PBS and stored at -20°C. Once thawed

the stock solution was kept at 4°C. LPS stock solution was vortexed to solubilise precipitated material prior to use. The polyclonal agonistic and the monoclonal blocking antibody to the p55 TNF-α receptor were purchased lyophilised form R&D-Systems. The antibodies were reconstituted in endotoxin free PBS and stored at 4°C. The monoclonal mouse anti human TIMP-1 antibody was purchased from Oncogene Science (Cambridge, UK) and the polyclonal rabbit anti human TIMP-1 antibody from TCS Biologicals (Buckingham, UK).

4.3 Results

4.3.1 Analysis of proMMP-9 production in co-cultures of ovarian cancer cells and monocytic cells

The co-culture system consisted of the ovarian cancer cell line PEO1 and the monocytic cell line THP-1. As shown in Fig. 4.1, supernatants generated when these cells were cultured separately contained low levels of the 92kDa form of MMP-9 as measured by quantitative zymography.

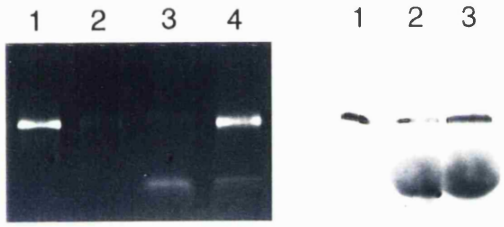


Fig. 4.1 Co-culture of the monocytic THP-1 cell line and the ovarian cancer cell line PEO1
ProMMP-9 production was increased in co-cultures of the monocytic THP-1 cell line and the ovarian cancer cell line PEO1 (1:1 ratio). The left panel shows a typical zymogram of cell culture supernatants, the right panel a western blot for MMP-9. Only proMMP-9 (92kDa) can be detected. Left panel: 1: purified proMMP-9; 2: THP-1 supernatant; 3: PEO1 supernatant; 4: THP-1/PEO1 co-culture supernatant; Right panel: 1: purified MMP-9; 2: THP-1 stimulated with CM derived from PEO1 cells; 3: THP-1/PEO1 co-culture supernatant.

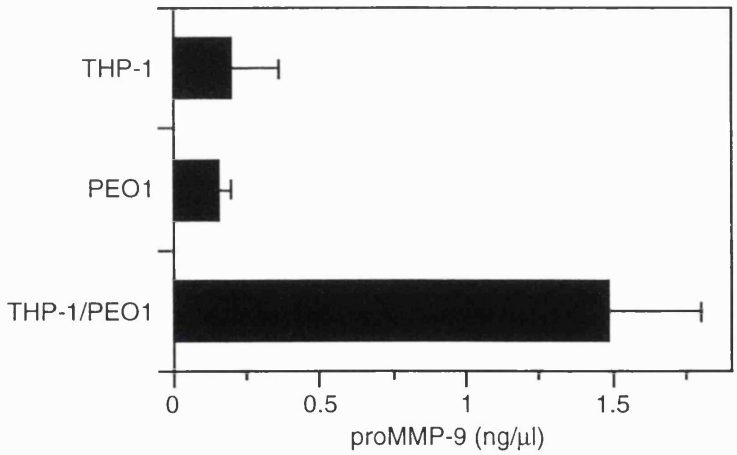


Fig. 4.2 Quantitation of proMMP-9 release in co-cultures of THP-1 and PEO1
The cell lines THP-1 and PEO1 were cultured separately or in co-culture for 48 hours and the cell culture supernatant was analysed by quantitative zymography. In co-culture proMMP-9 stimulation exceeded background levels 4-6 fold. The graph shows a typical result of four experiments. Statistical analysis using Students t-test: THP-1 vs. THP-1/PEO1: p=0.039; PEO1 vs. THP-1/PEO1: p=0.034

However, co-culture at a 1:1 ratio of tumour cells to monocytes resulted in a strong release of proMMP-9 in supernatants. The identity of MMP-9 was confirmed by western blotting (Fig. 4.1, right panel). A quantitative analysis of the proMMP-9 activity detected on zymograms is shown in Fig. 4.2. The proMMP-9 levels in co-cultures were 4-6 fold higher than in the supernatant of the individual cell lines.

4.3.2 Cell ratio experiments of the ovarian cancer cell line PEO1 and the monocytic cell line THP-1.

Co-culture experiments showed that a cell ratio of 1:1 was optimal (Fig. 4.3). For a fixed number of PEO1 cells, proMMP-9 release was strongly enhanced with an increasing number of THP-1 cells. For a fixed number of THP-1 cells, proMMP-9 release was slightly enhanced with an increasing number of PEO1 cells.

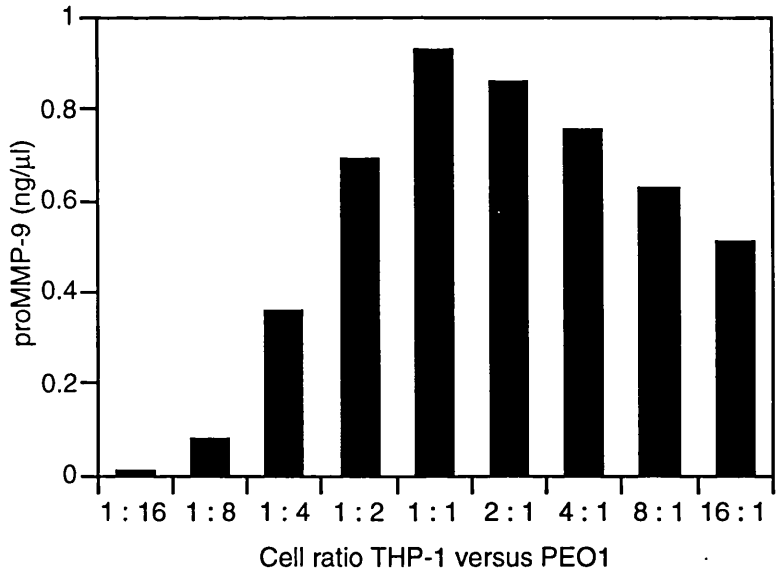


Fig. 4.3 Effect of the PEO1/THP-1 cell ratio on the proMMP-9 production
Cells were diluted in doubling dilutions starting from $1x10^6$ cells/ml and added to $1x10^6$ cells/ml of the other cell line. After 48 hours, the cell culture supernatant was assessed by quantitative zymography. Maximal proMMP-9 release was obtained with a 1:1 ratio of tumour cells and monocytes (The graph shows a typical experiment out of four experiments performed).

4.3.3 *Co-culture experiments without direct cell-cell contact*

Cell culture inserts (Millicell-CM, Millipore, Molsheim, France) were used to physically separate the two cell lines without preventing the exchange of soluble factors. As shown in Fig. 4.4 similar amounts of proMMP-9 were detected with and without cell culture inserts.

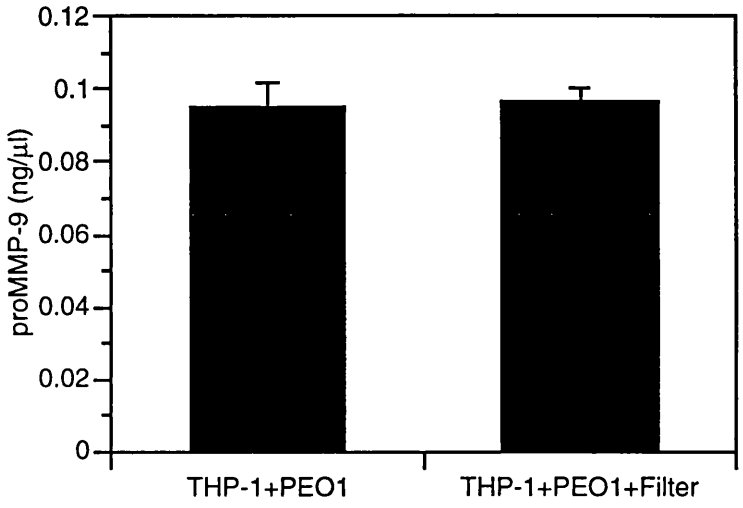


Fig. 4.4 Role of cell cell contact on proMMP-9 production in co-cultures
Co-cultures of the ovarian cancer cell line PEO1 and the monocytic cell line THP-1 were set up at a 1:1 ratio. Cells were either mixed to allow direct cell cell contact or separated by a tissue culture insert which prevented direct cell cell contact without blocking the exchange of soluble factors. The cell culture supernatant was analysed after an incubation period of 48 hours. Similar levels of MMP-9 production were achieved with or without direct cell cell contact. The data shown are representative for three experiments.

4.3.4 *Tumour cell derived CM induced proMMP-9 production by the monocytic cell line THP-1*

The results obtained so far suggested that cell-cell contact was not required and that the monocytic cells were the source of proMMP-9. To test this, conditioned medium (CM) from both PEO1 and THP-1 cells was prepared. CM from THP-1 cells did not enhance MMP-9 release from PEO1 (data not shown) whereas PEO1 derived CM induced proMMP-9 production by THP-1 (Fig. 4.5). This induction was dose dependent and even at a 1:60 dilution PEO1 derived CM had a clear proMMP-9 inducing effect on THP-1 cells. The identity of the protein induced by CM was confirmed as MMP-9 by western blotting (Fig. 4.1, right panel). Cell counting experiments showed that the cell numbers in CM stimulated and control cultures were not significantly different (data not shown).

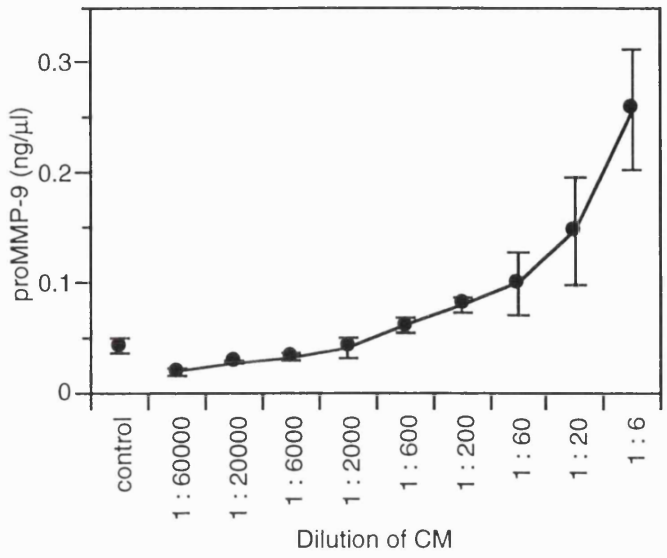


Fig. 4.5 Dose dependent production of proMMP-9 by CM stimulated THP-1 cells
THP-1 cells were seeded and stimulated with different amounts of PEO1 derived conditioned medium (CM). The cell culture supernatant was analysed by quantitative zymography after an incubation period of 48 hours. CM induced THP-1 cells to produce MMP-9 in a dose dependent manner. For all further stimulation experiments a 1:10 dilution of CM was used.

4.3.5 Tumour cell derived CM induced proMMP-9 release from isolated peripheral blood monocytes

Similar to the monocytic cell line THP-1, isolated peripheral blood monocytes released increased amounts of proMMP-9 in response to CM. However, to achieve the level of induction shown (Fig. 4.6), CM had to be concentrated 10 fold by ultrafiltration (NanoSpin Plus, MWCO 10000, Gelman Sciences, Ann Arbor, MI, USA)

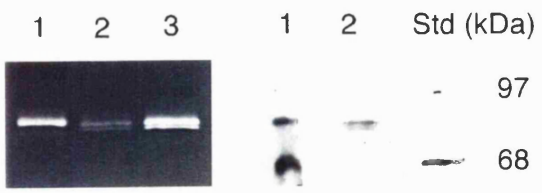


Fig. 4.6 CM stimulation of isolated peripheral blood monocytes
Isolated peripheral blood monocytes (PBMs) were stimulated with 10x concentrated CM. The cell culture supernatant was analysed by zymography and western blotting after an incubation period of 48 hours. The zymogram (left panel) shows two closely together migrating activities which were both enhanced by CM stimulation of the monocytes. Both proteins were identified as MMP-9 by western blotting using the monoclonal anti MMP-9 antibody CA-209 (right panel). In other experiments only the upper band was detectable. This is a typical result of three independent experiments. (left panel: lane 1: proMMP-9, lane 2: unstimulated PBMs, lane 3: CM stimulated PBMs; right panel: lane 1: proMMP-9, lane 2: CM stimulated PBMs)

4.3.6 Analysis of MMP-9 and TIMP-1 gene expression and protein

MMP-9 and TIMP-1 gene expression and protein production was studied in CM stimulated and unstimulated THP-1 cells over 48 hours of incubation. Using northern analysis, MMP-9 mRNA was detected in both CM stimulated and unstimulated THP-1 cells at 6 hours. In unstimulated cells the signal remained weak and decreased to below the detection limit after 24 hours of incubation (Fig. 4.7). In CM stimulated cells, however, MMP-9 expression was strongly increased, peaked at 14 hours and remained detectable until 48 hours (Fig. 4.7). Reprobing of the same blot for TIMP-1 mRNA showed no difference in TIMP-1 expression between unstimulated and CM stimulated THP-1 cells (Fig. 4.7). Reprobing of the blot with β-actin showed that loading was even (Fig. 4.7).

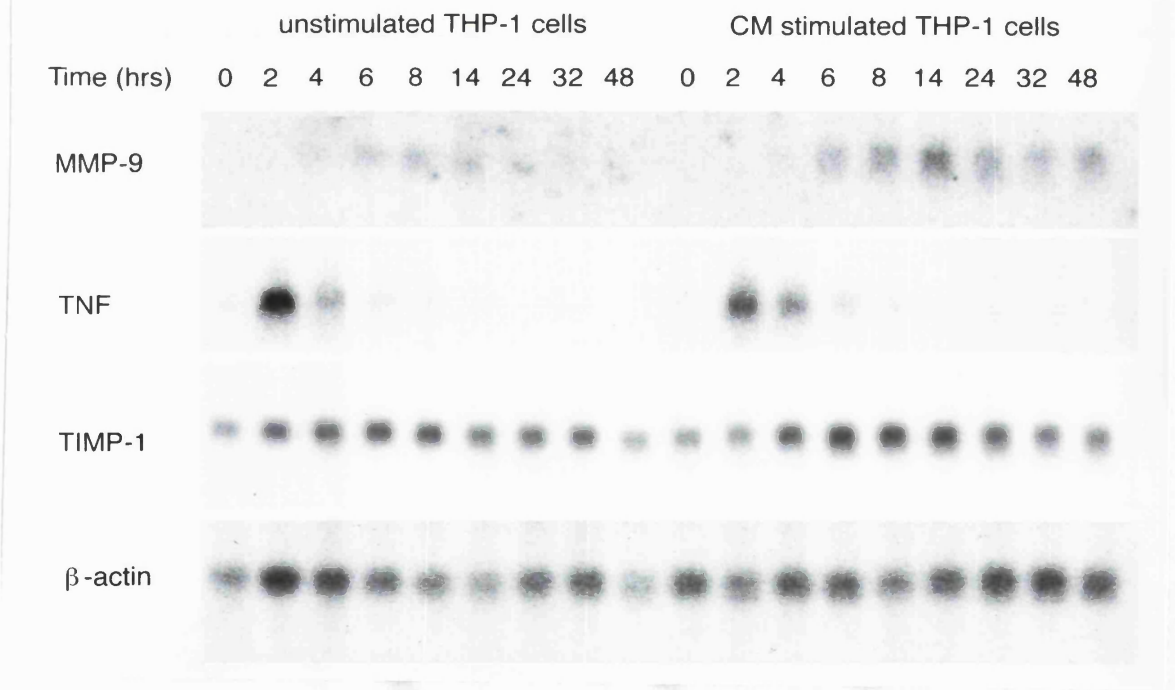


Fig. 4.7 Kinetics of proMMP-9, TNF-α and TIMP-1 gene expression in unstimulated and CM stimulated THP-1 cells
MMP-9 gene expression is increased in CM stimulated THP-1 cells whereas TIMP-1 expression remained unaffected. TNF-α gene expression was increased in both CM stimulated and unstimulated THP-1 cells. Probing for β-actin showed even loading of the samples. This is a typical result of four experiments performed.

MMP-9 protein in the cell culture supernatant was analysed by zymography. First proMMP-9 proteolytic activity was detected in the supernatant after 12 hours and levels seemed to increase steadily thereafter (Fig. 4.8). In CM stimulated cells, however, the amount of MMP-9 measured was higher then in unstimulated control cells. Western blotting for TIMP-1 using a commercially available monoclonal (Oncogene Science) or polyclonal (TCS-Biologicals) antibody did not lead to the detection of a specific band (28kDa). Non specific binding to proteins with higher molecular weights (>60kDa) was, however, observed. Similar results were obtained in three further experiments.

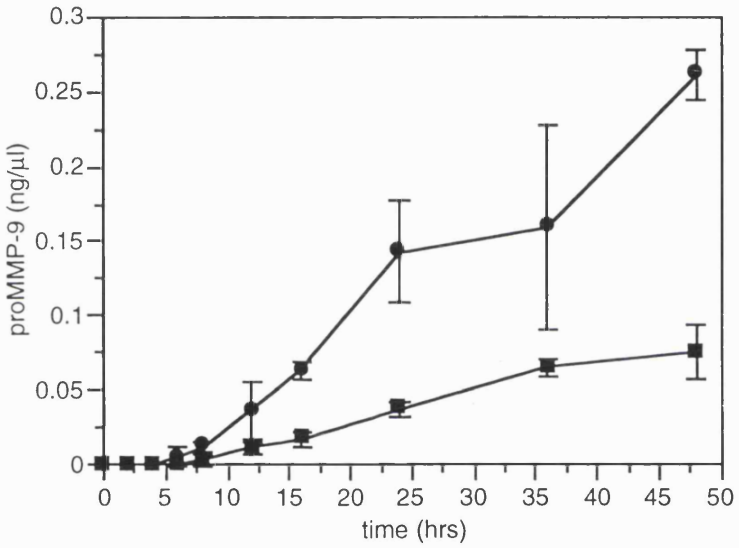


Fig. 4.8 Time course of MMP-9 production in unstimulated and CM stimulated THP-1 cells
THP-1 cells were stimulated with CM at a 1:10 dilution, the cell culture supernatant was harvested at the time points indicated and MMP-9 content analysed by quantitative zymography. ProMMP-9 was detectable at 12 hours. CM stimulated THP-1 cells produced more proMMP-9 then unstimulated cells over the period of time analysed. (■ unstimulated THP-1 control, ● CM stimulated THP-1 cells). This is a typical experiment of four performed.

Several experiments suggested a role for TNF-α in the synthesis of monocytic proMMP-9.

4.3.7 TNF-α stimulated proMMP-9 production by THP-1 cells in a dose dependent manner

Recombinant TNF-α induced proMMP-9 production by THP-1 cells in a dose dependent manner. ProMMP-9 release was first detected at 0.3ng/ml TNF-α and reached its maximum at 10ng/ml TNF-α (Fig. 4.9). This finding is consistent with published results (78, 168, 169, 183). TNF-α had, however, a much smaller effect on proMMP-9 release from PEO1 cells. At 10ng/ml TNF-α a maximal 2.8 fold increase of MMP-9 production was observed (control: 0.12ng/µl +/-0.02 proMMP-9, TNF-α: 0.33ng/µl +/-0.06 proMMP-9) whereas THP-1 cells increased their proMMP-9 production more then 6 fold.

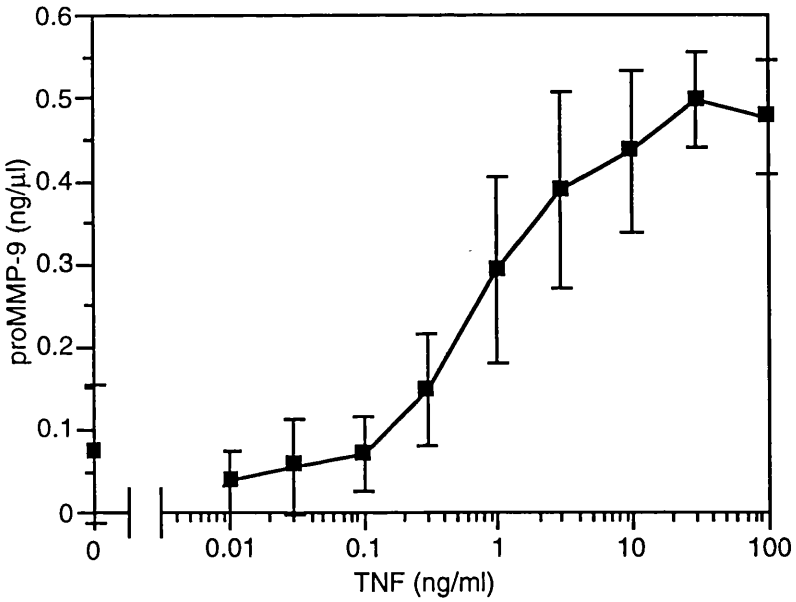


Fig. 4.9 Dose dependent production of proMMP-9 by TNF-α stimulated THP-1 cells
THP-1 cells were stimulated with recombinant TNF-α as indicated. The cell culture supernatant was analysed for MMP-9 by quantitative zymography after an incubation period of 48 hours. First clear production of MMP-9 protein was obtained with 0.3ng/ml and maximal MMP-9 production was obtained at 10ng/ml TNF-α.

4.3.8 *Antibodies to TNF-α blocked proMMP-9 production in both co-cultures and CM stimulated THP-1 cells*

A monoclonal antibody that neutralised TNF-α bioactivity inhibited proMMP-9 release in co-cultures and also in CM stimulated THP-1 cultures (Fig. 4.10). These experiments suggested that the soluble factor present in PEO1 derived CM could be TNF-α. However, further experiments argued against this hypothesis.

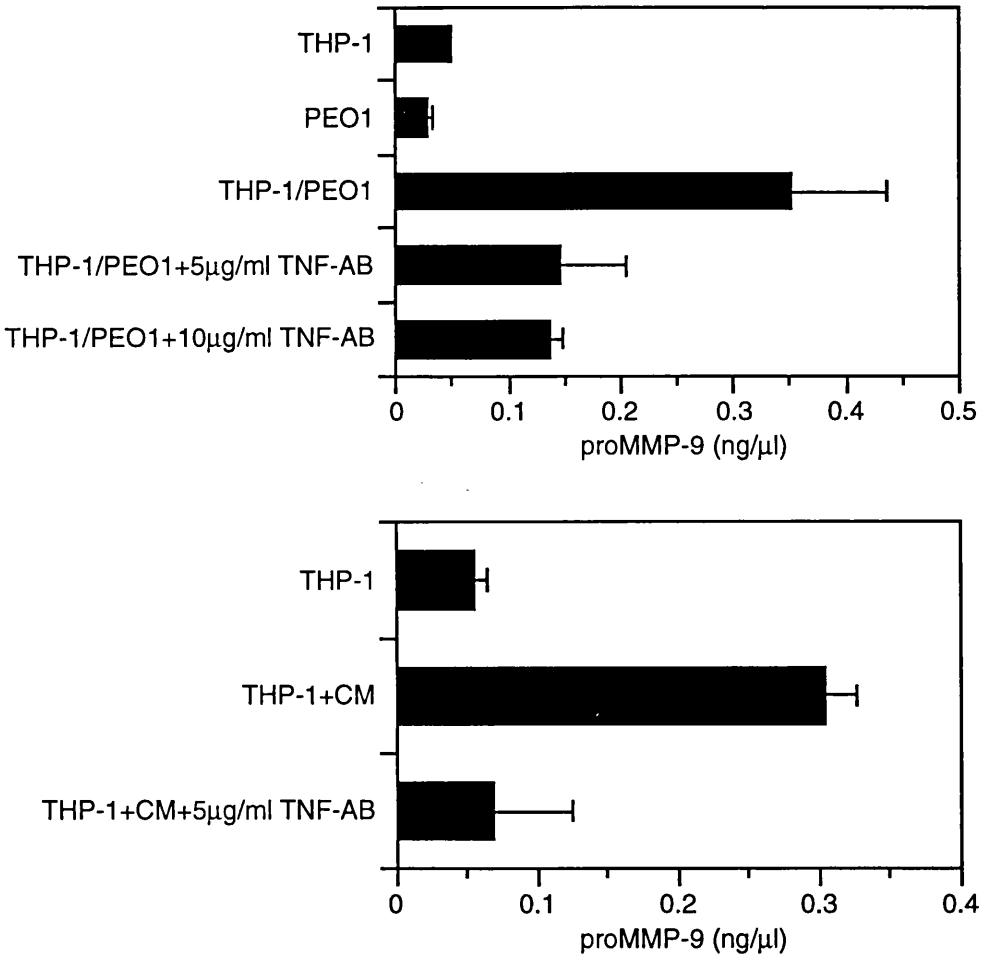


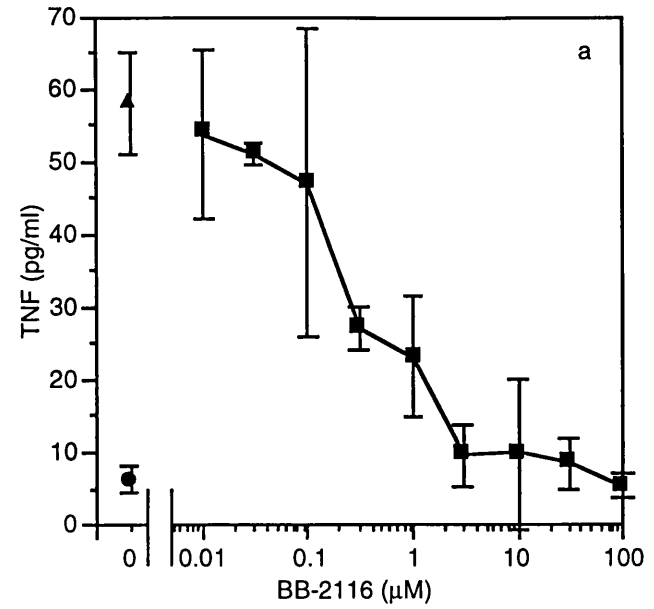
Fig. 4.10 Effect of an anti TNF-α antibody on proMMP-9 release
The monoclonal anti TNF-α antibody 6H11 was added to CM stimulated THP-1 cells and co-cultures of PEO1 and THP-1. The cell culture supernatant was analysed by quantitative zymography after an incubation period of 48 hours. The anti TNF-α antibody blocked MMP-9 production in both THP-1/PEO1 co-cultures (upper graph) and from CM stimulated THP-1 cells (lower graph).
(Statistical analysis using paired Students T-test. Upper graph: THP-1/PEO1 vs. THP-1/PEO1 in presence of 5µg/ml TNF-α antibody: $p=0.028$; THP-1/PEO1 vs. THP-1/PEO1 in presence of 10µg/ml TNF-α antibody $p=0.044$. Lower graph: THP-1 vs. THP-1+CM: $p<0.001$; THP-1 vs THP-1+CM+ TNF-AB: $p=0.003$)

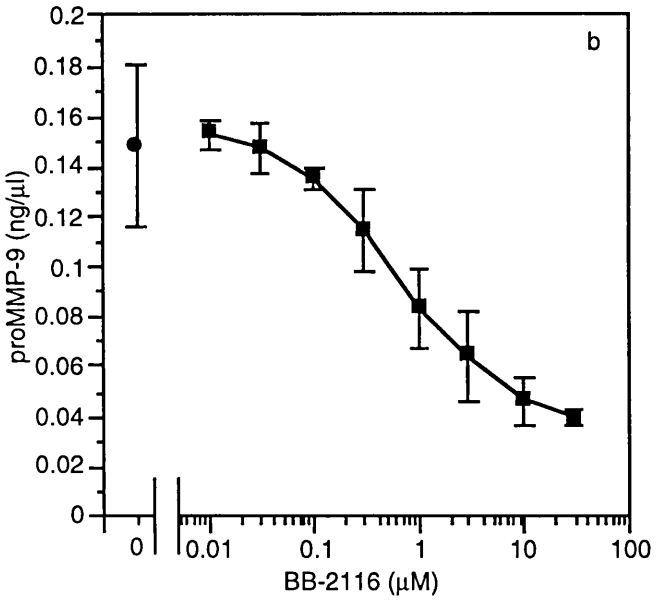
4.3.9 The compound BB-2116 blocked TNF-α release and proMMP-9 production in both co-cultures and CM stimulated THP-1 cells

In accordance with published results (105, 184) the matrix metalloprotease inhibitor (MMPI) BB-2116 blocked shedding of TNF-α from its membrane spanning precursor after LPS (100ng/ml) stimulation of THP-1 cells (Fig. 4.11a, IC_{50} 300nM). This MMPI also inhibited proMMP-9 production with a similar IC_{50} in co-culture and in CM experiments (Fig 4.11b and 4.11c, IC_{50}: 590nM and 860nM). At the concentrations used BB-2116 had no cytotoxic or cytostatic effects on THP-1 cells as assessed by cell counting and phase contrast microscopy (data not shown). Further, BB-2116 did not interfere with zymogram analysis of proMMP-9 in the assessed range (< 300mM, data not shown). The observation that an inhibitor of TNF-α release also inhibited proMMP-9 release from monocytic cells when stimulated with CM, suggested that TNF-α production by the monocytic cell line THP-1 was involved in the CM stimulated production of proMMP-9.

4.3.10 Analysis of TNF-α gene expression and protein in THP-1 cells

Northern blotting and probing for TNF-α mRNA showed gene expression in both CM stimulated and unstimulated THP-1 cells with similar kinetic and level of expression (Fig. 4.7). TNF-α mRNA was not detectable at the beginning of culture, peaked after 2 hours and declined thereafter. TNF-α protein, as detected by bioassay or ELISA in three independent experiments, followed the pattern observed for mRNA. Maximal TNF-α protein was detected 5 to 8 hours after stimulation (60pg/ml and 21pg/ml as determined by bioassay and ELISA, respectively). No TNF-α was detected at the beginning of the incubation period and the amounts decreased to detection limit (ELISA, 15pg/ml) or below (bioassay, 19pg/ml; data not shown) by 16 hours.





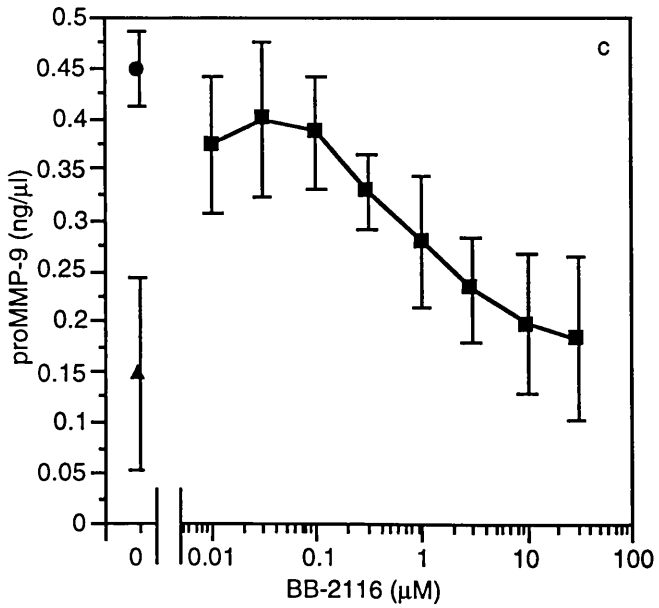


Fig. 4.11 Effect of BB-2116 on release of TNF-α and proMMP-9
BB-2116 was added to LPS stimulated THP-1 (4.11a), THP-1/ PEO1 co-cultures (4.11b) and CM stimulated THP-1 cells (4.11c). After an incubation period of 48 hours the cell culture supernatant was assayed for TNF-α (4.11a) and MMP-9 (4.11b and c). The MMP inhibitor BB-2116 inhibited TNF-α release from LPS stimulated THP-1 cells in a dose dependent manner (Fig. 4.11a, ■ LPS + BB-2116 ▲ LPS, ● unstimulated). The IC_{50} was 300nM as determined by computer assisted curve fitting. Further, BB-2116 inhibited proMMP-9 release in a dose dependent manner in both co-cultures (Fig. 4.11b, IC_{50} 590nM, ■ BB-2116, ● control) and CM stimulated THP-1 cells (Fig. 4.11c, IC_{50} 860nM, ■ CM + BB-2116, ● CM, ▲ unstimulated).

4.3.11 TNF-α acts in a synergistic fashion with PEO1 derived CM

The data presented so far suggested that synergistic action of autocrine TNF-α and CM, led to monocytic proMMP-9 production. To test this hypothesis we used the observation that TNF-α gene expression was only detectable shortly after seeding of THP-1 cells (Fig 4.7) and that TNF-α protein detectable in the culture medium decreased to below detection level by 12 hours post seeding of the cells (data not shown). Thus, seeding of THP-1 cells followed by incubation for 16 hours prior to stimulation resulted in culture medium containing very little residual bioactive TNF-α. As shown in Fig. 4.12, under these conditions CM stimulation did not result in proMMP-9 production as detected by zymography after a further incubation period of 48 hours. A polyclonal agonistic antibody to the p55 TNF-α receptor induced a small amount of proMMP-9. Presence of both CM and the agonistic antibody resulted in a stronger induction of proMMP-9 then addition of the individual effects.

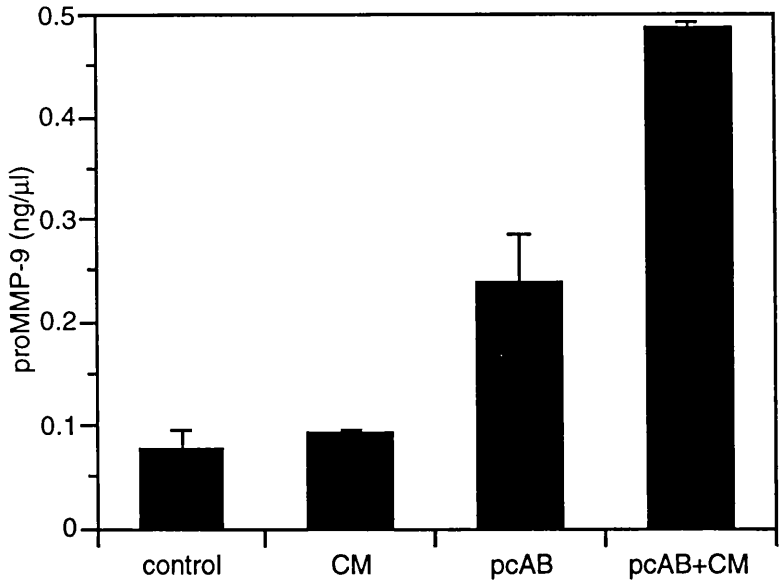


Fig. 4.12 Synergistic action of CM with an agonistic polyclonal antibody specific for the p55 TNF-α receptor
THP-1 cells were incubated for 16 hours to allow for very low levels of residual TNF-α in the cell culture supernatant. Cells were stimulated with CM (1:10 dilution) or 5µg/ml of an agonistic polyclonal antibody specific for the p55 TNF-α receptor (pcAB). Under these conditions CM did not and pcAB induced some proMMP-9 production. Together, however, proMMP-9 production exceeded the added effects of CM and pcAB. The graph shows a typical experiment. Each condition was set up in duplicate. The error bars reflect the standard deviation of the two values. Statistics were performed using a paired T-test of four independent experiments: control vs. CM: $p>0.05$; control vs. pcAB $p>0.05$; control vs. CM+pcAB $p=0.0063$; CM vs. pcAB $p>0.05$; pcAB vs. CM+pcAB $p=0.0042$.

4.3.12 Immunoprecipitation of TNF-α from CM did not alter CM's ability to induce proMMP-9 from THP-1 cells

The results shown above suggested a synergistic interaction between autocrine TNF-α and a soluble factor present in CM. TNF-α was a likely candidate for this factor due to its stimulatory activity on proMMP-9 production (Fig. 4.9). Several experiments, however, suggested that the factor was not TNF-α. Immunoprecipitation of potential TNF-α present in CM had only a minor effect on its MMP-9 stimulating activity (Fig. 4.13). Controls showed that recombinant TNF-α induced proMMP-9 production and that the monoclonal anti TNF-α antibody 6H11 blocked this induction. Further controls showed that immunoprecipitation of recombinant TNF-α was efficient and that the TNF-α antibody 6H11 was fully removed by protein-G sepharose precipitation. Finally, neither protein-G sepharose on its own or in combination with an unrelated monoclonal antibody of the same IgG type as 6H11 was able to bind and remove recombinant TNF-α.

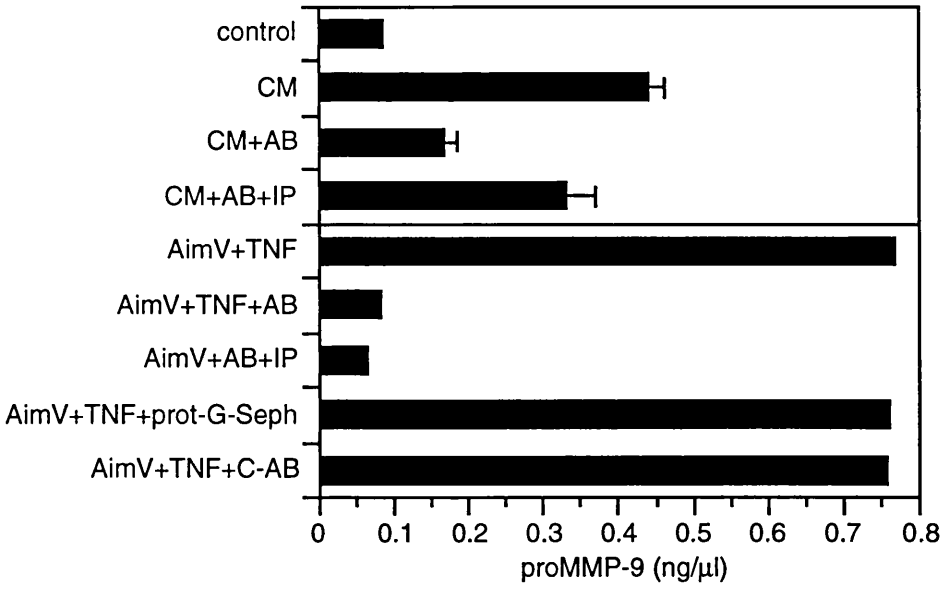


Fig. 4.13 The proMMP-9 release stimulating activity in CM can not be precipitated with an anti TNF-α antibody
Immunoprecipitation of TNF-α potentially present in PEO1 derived CM had little effect on its capacity to induce MMP-9 release from THP-1 cells. Controls showed that recombinant TNF-α could be precipitated efficiently and the TNF-α antibody 6H11 was fully removed by protein-G sepharose precipitation. This result is typical for three experiments performed. Statistical analysis using Student's T-test: CM vs. CM+AB: $p<0.0001$; CM vs. CM+AB+IP; $p=0.011$; CM + AB vs. CM+AB+IP: $p=0.0023$. (control: unstimulated THP-1 cells, AB: TNF-α antibody 6H11, IP: immunoprecipitation, Aim V: culture medium, prot-G Seph: protein-G Sepharose, C-AB: control antibody of same IgG-isotype as 6H11 (IgG_1))

4.3.13 Production of CM in presence of BB-2116 did not alter CM's capacity to induce proMMP-9 from THP-1 cells.

As shown before (Fig. 4.11) BB-2116 at a concentration of 30μM blocked release of TNF-α completely (IC_{50}=300nM). Fig. 4.14 shows proMMP-9 production by THP-1 cells stimulated with CM produced in presence of 30μM BB-2116. ProMMP-9 production was partly inhibited when THP-1 cells were stimulated with this CM in a 1:10 dilution. However, when BB-2116 was removed by gel filtration, CM still stimulated proMMP-9 release by the monocytic cells. Taken together, the results shown in Fig. 4.13 and Fig. 4.14 suggested that tumour cell derived TNF-α did not play a major role in stimulation of monocytic MMP-9 production by CM. This was further supported by the observation that the amount of TNF-α detected in CM was variable (0-160pg/ml, measured by WEHI-164 bioassay), without affecting its capacity to induce monocytic proMMP-9 (data not shown).

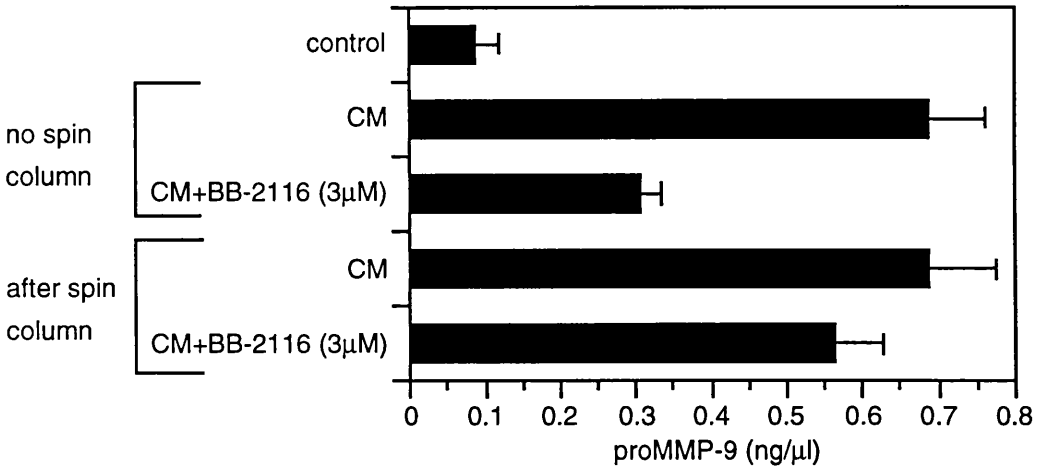


Fig. 4.14 Release of the proMMP-9 inducing activity present in CM is not inhibited by BB-2116
Conditioned medium from PEO1 cells was prepared in presence of BB-2116 (30μM). Addition of this CM to THP-1 cells in a 1:10 dilution resulted in a partial reduction of MMP-9 release from THP-1 cells. When BB-2116 was removed from CM by gel filtration, the inhibitory effect of BB-2116 disappeared and full MMP-9 stimulation was achieved. (Statistical analysis using Student's T-test: control vs. CM: p=0.009; no spin column: CM vs. CM+BB-2116 (3μM): p=0.022; after spin column: CM vs. CM+BB-2116 (3μM): p=0.251)

4.3.14 Heating of CM reduced its proMMP-9 inducing activity

Heating of CM showed that the factor(s) present in CM, tentatively named Matrix Metalloproteinase Stimulating Factor (MMPSF), was temperature sensitive. The ability of MMPSF to induce MMP-9 from THP-1 cells was slightly reduced after an incubation at 45°C and completely abrogated after an incubation at 85°C for 20 min (Fig 4.15).

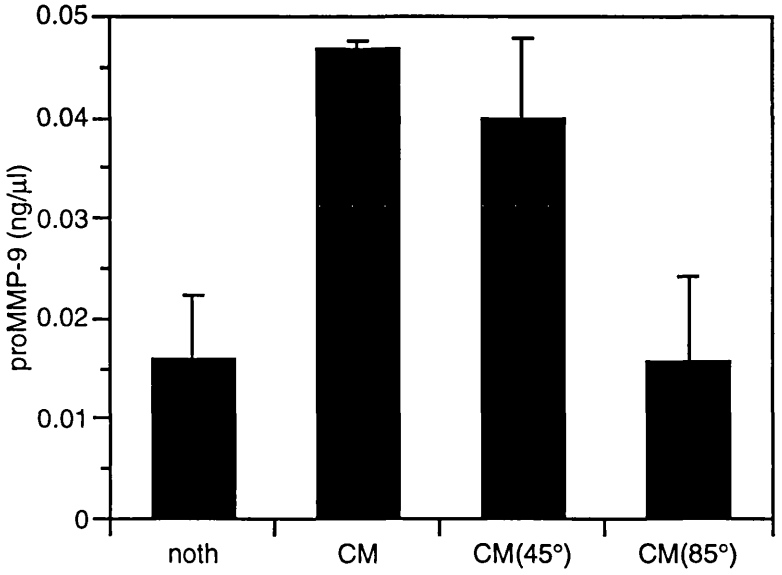


Fig. 4.15 Warming of CM abrogates proMMP-9 induction from THP-1 cells
CM was heated for 20 min to the temperatures indicated and then added to THP-1 cells in a 1:10 dilution. There is a slight reduction in proMMP-9 release after heating to 45°C as compared to control CM. Heating to 85°C completely abrogates induction of proMMP-9. The data shown are typical for two experiments performed.

4.4 Discussion

In this chapter the interaction of ovarian cancer cells and monocytic cells with respect to MMP-9 production was investigated and the role of TNF-α in this process determined. The results showed that co-cultures of the ovarian cancer cell line PEO1 and the monocytic cell line THP-1 led to increased proMMP-9 in the cell culture supernatant. Further experiments indicated that the monocytic cells were the source of proMMP-9 and that a soluble factor, tentatively named MMPSF, present in tumour derived conditioned medium (CM), was sufficient to induce proMMP-9 production in both the monocytic cell line THP-1 and peripheral blood monocytes. Experiments with neutralising antibodies to TNF-α, the inhibitor BB-2116 which blocked shedding of TNF-α from its membrane spanning precursor, and northern analysis, revealed that autocrine TNF-α production by the monocytic cells was also involved in monocytic proMMP-9 synthesis. These experiments also showed that, in contrast to MMP-9 production, TIMP-1 gene expression remained unchanged. Experiments involving agonistic and neutralising antibodies to the p55 TNF-α receptor suggested that TNF-α was signalling through this receptor (data not shown) and northern analysis revealed that CM stimulation of THP-1 cells led to an increase in p55 TNF-α receptor gene expression (data not shown). It remained to establish whether the increased gene expression of the p55 TNF-α receptor and MMP-9 gene were due to the same or different factors. Immunoprecipitation experiments and production of CM in presence of BB-2116 showed that MMPSF was distinct from TNF-α. Thus, synergistic action of both MMPSF and TNF-α was required for monocytic proMMP-9 production and experiments performed confirmed this hypothesis. These results were consistent with the model shown in Fig. 4.16.

This study was based on observations made in biopsies of human ovarian cancer. *In situ* hybridisation and immunohistochemical studies have revealed similarities in the expression pattern of MMP-9 mRNA and the distribution of tumour associated macrophages (TAMs) (43). Further, TNF-α expression has been localised by *in situ* hybridisation to tumour cells (43) whereas immunohistochemical evidence has suggested localisation of TNF-α protein and TAMs (170). To investigate the relationship between

monocytes, tumour cells, TNF-α and MMP-9, a co-culture system consisting of the ovarian cancer cell line PEO1 and the monocytic cell line THP-1 or isolated peripheral blood monocytes was established. The findings that MMP-9 was released by the monocytes and that this induction was due to a soluble tumour derived factor, MMPSF, were consistent with the *in vivo* observations (43, 170).

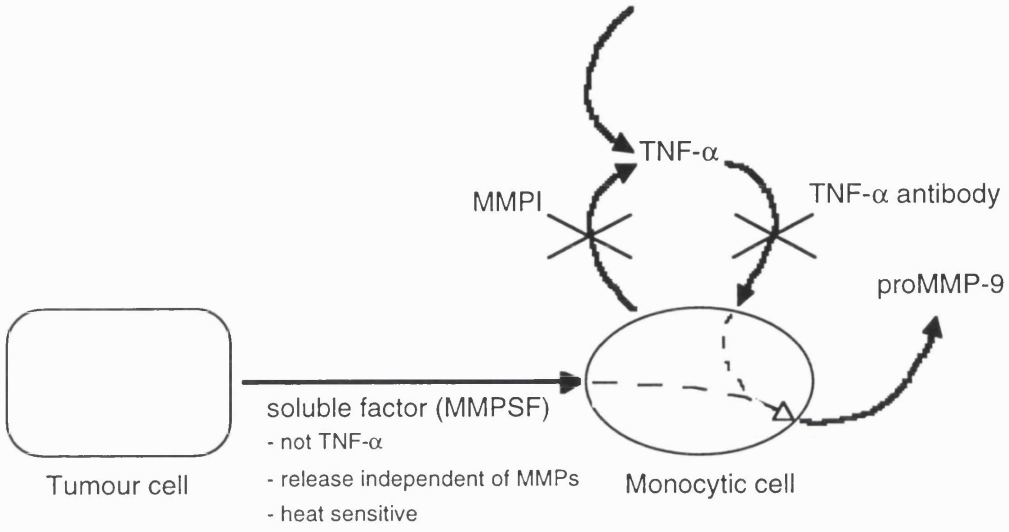


Fig. 4.16 Model of monocytic proMMP-9 production
A soluble factor (MMPSF) released from tumour cells acts synergistically with autocrine or paracrine TNF-α to stimulate monocytic proMMP-9 production.

Detection of TNF-α mRNA and protein in the *in vitro* system showed that the monocytic cell line THP-1 produced TNF-α between 2-4 hours after seeding. This TNF-α production was not influenced by stimulating the cells with CM. *In vivo* observations, however, have localised TNF-α gene expression to the tumour area (43) but TNF-α protein to TAMs. Therefore, *in vivo*, cells other than the monocytes might also be the source of TNF-α protein. In summary, the results suggested that in ovarian cancer TNF-α gene expression in epithelial tumour areas led to low level of tissue TNF-α which promoted, together with tumour derived MMPSF, monocytic proMMP-9 production.

This MMP-9 induction, however, did not affect TIMP-1 gene expression suggesting a net production of potential proteolytic activity. An interesting observation was that signalling of TNF-α through the p55 TNF-α receptor I was sufficient for proMMP-9 production and that its gene expression was upregulated upon stimulation of THP-1

cells with CM (data not shown). This upregulation was due to a component of PEO1 derived CM and it remained to be determined whether the same factors were responsible for both the upregulation of MMP-9 and p55 TNF-α receptor I gene expression.

Regulation of monocytic proMMP-9 production has been the focus of several recent papers. T-cell derived gp-39 (185), TNF-α and IL-1β (169) have been shown to be potent inducers of monocytic proMMP-9 production. These proteins, particularly TNF-α since it had been detected in tissue biopsies of human ovarian cancer (43), were potential candidates for the soluble, tumour cell derived factor MMPSF. Experiments showed, however, that MMPSF was distinct from TNF-α. MMPSF could not be precipitated with a monoclonal anti TNF-α antibody and BB-2116 did not inhibit its release. This eliminated a series of proteins for which such an inhibition has been reported (e.g. TGF-α, M-CSF, FasL etc.) (107, 108, 186-191). The characterisation of MMPSF will be the subject of chapter 5.

Our results further suggested a new mechanism of action of synthetic MMPIs in cancer. MMPIs have been designed originally to reduce excessive MMP activity due to an imbalance of MMPs and their natural inhibitors (TIMPs). However, synthetic MMPIs also inhibited shedding of TNF-α from its membrane spanning precursor (105, 184) and of other cytokines: TGF-α, FasL, IL-6 receptor, stem cell factor, M-CSF, TNF-α receptors, L-selectin and Thyrotropin receptor ectodomain (107, 108, 186-191). Therefore, inhibition of the release of cytokines which might play a role in tumour development represented an alternative or additional mechanism of action of MMPIs. This option might be of particular importance if the cytokine has stimulatory activity on MMP gene expression as it has been shown to be the case for TNF-α (168, 169). However, the IC_{50} values of synthetic MMPIs for MMPs were generally 10-100 fold lower than those that influence cytokine release (192). Hence, the effect might only be a secondary one to inhibition of MMP activity. However, because of the broad activities of cytokines and their potency, even small changes might contribute to the observations made in animal models of human cancer (22, 28) or in current clinical trials.

In this chapter a simple *in vitro* system was established to analyse mechanisms leading to monocytic MMP-9 production. The *in vitro* findings were in keeping with the observations made on biopsy material of human ovarian cancer. Further studies were required to characterise MMPSF (chapter 5) and to determine the biological role of the 92kDa pro-form of MMP-9 (chapter 6).

5. Characterisation of MMPSF

5.1 Introduction

In chapter 4 of this thesis a co-culture system of the ovarian cancer cell line PEO1 and the monocytic cell line THP-1 was established and interactions leading to production of proMMP-9 were analysed. The results suggested that a soluble, tumour cell derived factor (tentatively named Matrix Metalloproteinase Stimulating Factor, MMPSF) induced production of monocytic proMMP-9 in synergistic fashion with TNF-α. Several soluble proteins have been reported to stimulate MMP-9 gene expression: TNF-α (168, 169), IL-1α and β (193-196), epidermal growth factor (EGF) and amphiregulin (197, 198), thrombin (195), transforming growth factor-beta (TGF-β) (199, 200), fibroblast growth factor-2 (FGF-2) (201), platelet activating factor (PAF) (202) and TGF-α (198). Others, such as TNF-β, IL-4 and interferon-γ (IFN-γ) (78, 203) down regulate MMP-9 gene expression.

Results from chapter 4 demonstrated that MMPSF was distinct from TNF-α and that its release from the tumour cells could not be blocked with a synthetic MMP inhibitor. Thus proteins like TNF-α (105, 184), TGF-α (107), Fas Ligand (107, 108), IL-6 receptor (189), p55 TNF-α-receptor (189), p75 TNF-α-receptor (204), stem cell factor, M-CSF (107), L-selectin (186, 205) and the thyrotropin receptor ectodomain (191) were unlikely candidates for MMPSF since their release is inhibited by compounds homologous to BB-2116. Furthermore, as shown in chapter 4, MMPSF required synergistic action of TNF-α to induce MMP-9 gene expression. This observation has to our knowledge not been reported yet for any of the potential candidates named. It was therefore the aim of this chapter to develop a protocol for the purification of MMPSF from PEO1 derived conditioned medium.

5.2 Materials and Methods

Preparation of conditioned medium

Conditioned medium (CM, 8ltr) was prepared essentially as described in chapter 4. Briefly, PEO1 cells were grown in 175cm^2 flasks, detached with trypsin/versene, pelleted, resuspended in FCS-free Aim V medium and seeded at a concentration of 1x10^6 cells/ml, 30ml per flask. After an incubation period of 48 hours at 37°C, 5%CO_2, the cell culture supernatant was harvested. Cell debris was pelleted by centrifugation (3000g, 10 min), the supernatant was filter sterilised (Nalgene, 0.2µm) and stored at -20°C.

Bioassay for MMPSF and zymography

The bioassay for MMPSF was based on experiments described in chapter 4. Briefly, THP-1 cells were pelleted, resuspended in FCS-free Aim V medium, the cell concentration was adjusted to 1x10^6 cells/ml and the cells were seeded in 100-200µl cultures in 96 well plates. Test samples were added to THP-1 cultures at the dilution indicated (1:6 to 1:200), the cells incubated for 48 hours and the supernatant analysed for proMMP-9 activity by zymography as described in chapter 3. Test samples were prepared under endotoxin free conditions and regularly tested for endotoxin content. They were used if endotoxin content was below 0.5EU/ml (50pg/ml endotoxin from *E. coli* 055:B5) as determined with a Limulus amoebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA, USA)

Ion exchange chromatography by FPLC/HPLC

Ion exchange chromatography (MonoQ 1.6/50, Pharmacia, Uppsala, Sweden) was performed on a FPLC/HPLC system (Smart System, Pharmacia) equipped with a µ-peak UV detector (280nm), a conductivity meter and a fractionator (48 samples). An external cooling unit was installed to maintain a temperature of 4-8°C. Prior to use, the entire system including pumps, sample loop and column was washed sequentially with 2M NaOH, endotoxin free water (Baxter, Thetford, UK), 1M HCl and endotoxin free water (Baxter) to obtain endotoxin levels in the effluent below 0.5EU/ml (50pg/ml endotoxin from *E.coli* 055:B5, Associates of Cape Cod). The 50ml SuperLoop

(Pharmacia) used in some experiments was newly purchased and found to be endotoxin free. Finally, the system was equilibrated with 20mM triethanolamine (TEA, pH 7.5) prepared with endotoxin free water (Baxter). Pump A was filled with equilibration buffer, pump B with the same buffer containing 1M NaCl. All buffers and solutions were prepared under sterile and endotoxin free conditions, passed through a 0.2μm filter and degassed prior to use on the Smart System.

Gel filtration

Gel filtration was performed on the Smart System using a Superdex PC 75 3.2/30 (exclusion size: 100kDa, optimal separation range: 3-70kDa, Pharmacia) or a Superdex PC 200 3.2/30 (exclusion size: 1300kDa, optimal separation range: 10-600kDa, Pharmacia) column. Molecular weight markers (Pharmacia) were used according to the manufacturers instructions. Prior to use, the chromatography system and columns were washed with 5ml 2M NaOH followed by endotoxin free water and equilibrated with endotoxin free PBS (Gibco, Paisley, UK). Effluents were tested for endotoxin content and washing continued until endotoxin levels were below 0.5EU/ml (50pg/ml endotoxin from *E.coli* 055:B5, Associates of Cape Cod).

SDS-PAGE

8% or 11% SDS gels were poured using the reagents described in chapter 3. Samples were prepared in a reducing loading buffer (100mM TrisHCl (pH 8.5), 50mM DTT, 1% (w/v) SDS, 10% (v/v) glycerol, 0.01% (w/v) Bromophenol Blue), heated to 65°C for 10 min, briefly centrifuged and loaded. DTT was prepared as a 1M stock solution as described elsewhere (150), aliquoted and stored at -20°C until use. Gels were run at 160 volts until the loading dye reached the bottom of the gel. Rainbow (Amersham), Mark 12 (Novex, San Diego, CA, USA) or SeeBlue (Novex) molecular weight markers were loaded at 10 or 5μl per lane.

Lectin blot

Samples were resolved by SDS-PAGE under reducing conditions and blotted onto a PVDF membrane (Immobilon-P, Millipore). After transfer the blot was rinsed with methanol and air dried for 1 hour. The blot was incubated with biotinylated WGA

(1μg/ml, Pierce) in PBS containing 1% (w/v) bovine serum albumin (BSA, Sigma) and 0.01% (v/v) Tween-20 (Sigma) for 1 hour at room temperature on a shaker, followed by several washes in PBS/0.01% (v/v) Tween-20 (Sigma). Then the blot was incubated with strepavidin labelled horseradish peroxidase (Sigma, 1μg/ml) in PBS/1% (w/v) BSA/0.01% Tween-20 for 1 hour at room temperature on a shaker, washed several times in PBS/0.01% (v/v) Tween-20 and proteins detected with the ECL system (Amersham).

Silver staining

Silver staining was performed according to a modified protocol published elsewhere (206). Briefly, after electrophoresis the gel was incubated for 10 min in methanol/acetic acid/water (v/v/v=5/1/4), 5 min in acetone/ethanol/water (v/v/v = 1/1/1), washed 3 times for 2 min in distilled water, incubated for 5 min in 3.4mM potassium dichromate, 3.2mM HNO_3, rinsed in water (3 times, 1 min), incubated in 12mM $AgNO_3$ for 30 min, rinsed in water and developed in 29.7g/l Na_2CO_3, 0.5ml/l 37% (v/v) formaldehyde. Colour development was stopped by replacing the developing solution with water containing 1% (v/v) acetic acid. All steps were performed at room temperature on a shaker.

Chromatography resins

DEAE-, CM-, Q-, S-, Blue-, Heparin-, Phenyl- and Octyl-sepharose were purchased from Pharmacia. Wheat-germ agglutinin-sepharose 6MB (WGA) was from Sigma. Prior to use all resins were washed extensively in endotoxin free PBS.

5.3 Results

5.3.1 Binding studies

Binding studies were performed to determine a chromatographic matrix for a first purification step of MMPSF from PEO1 derived conditioned medium (CM). Resins for ion exchange-, affinity- or hydrophic interaction-chromatography were tested (DEAE-, CM-, Q-, S-, wheat germ agglutinin-, blue-, heparin-, phenyl- and octyl-sepharose) and the flow through was analysed for residual MMPSF at a 1:10 dilution. Once binding was observed, protein was eluted from the resin and also tested by bioassay.

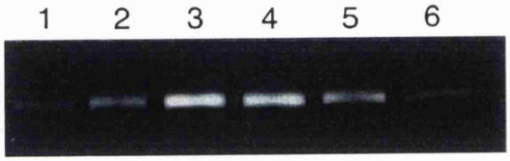


Fig. 5.1 Binding of MMPSF to wheat germ agglutinin (WGA) beads
WGA-sepharose beads (50µl) were added to 50ml CM and the suspension incubated on a roller (20 min, 4°C). Beads were left to sediment (15 min, 4°C), washed with PBS (Gibco) and protein eluted in batches of one bed volume with 0.1M N-acetyl glucosamine (Sigma). Flow through and eluates were tested for proMMP-9 inducing activity by bioassay (1:6 dilution) and zymography. MMPSF was enriched with WGA (WGA-MMPSF) and the activity eluted in fractions E1, E2 and E3. Binding of MMPSF was incomplete. In all experiments some activity remained in the flow through. Typical results are shown. Lane 1: control (unstimulated); 2: flow through, 3: E1 (eluate-1); 4: E2; 5: E3; 6: control (0.1M N-acetyl glucosamine).

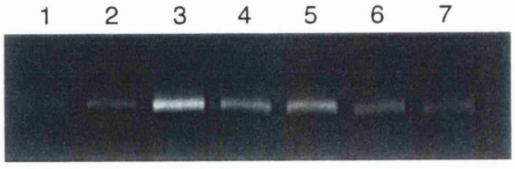


Fig. 5.2 Binding of MMPSF with Q-Sepharose
CM (1ml) was diluted 1:10 in 10mM TEA (pH 7.5) and 30µl Q-Sepharose beads were added. The suspension was incubated on a roller for 30 min at 4°C, the beads pelleted by centrifugation (5 min, 100g) and the flow through removed. Activity was eluted in batches of 2 bed volumes with 400mM NaCl in 10mM TEA, pH 7.5. Eluates were tested by bioassay (1:10 dilution) and zymography. Strong proMMP-9 inducing activity was present in eluates E1, E2 and E3. No activity was detected in the flow through (data not shown). Lane 1: control (unstimulated); 2: control (CM stimulated); 3: E1 (eluate 1); 4: E2; 5: E3; 6: E4; 7: E5

Blue sepharose and both phenyl and octyl-sepharose showed binding of MMPSF in the presence of 1M NaCl but recovery of activity was poor (data not shown). Satisfactory binding and recovery of activity was obtained with wheat germ agglutinin

(WGA-MMPSF, Fig. 5.1) and Q-sepharose (Q-MMPSF) if CM was diluted 1:10 in 20mM triethanolamine (TEA, pH 7.5) to reduce the salt concentration (Fig. 5.2).

5.3.2 Partial characterisation of WGA binding proMMP-9 inducing activity (WGA-MMPSF)

As shown in Fig. 5.1, binding of proMMP-9 inducing activity could be achieved with the lectin binding resin wheat germ agglutinin (WGA-MMPSF). This binding was found to be partial, leaving considerable proMMP-9 activity in the flow through (Fig. 5.1). Attempts to increase the efficiency of binding through the addition of bivalent ions such as Mn^{2+} and Ca^{2+} suggested elsewhere (207) were unsuccessful (data not shown).

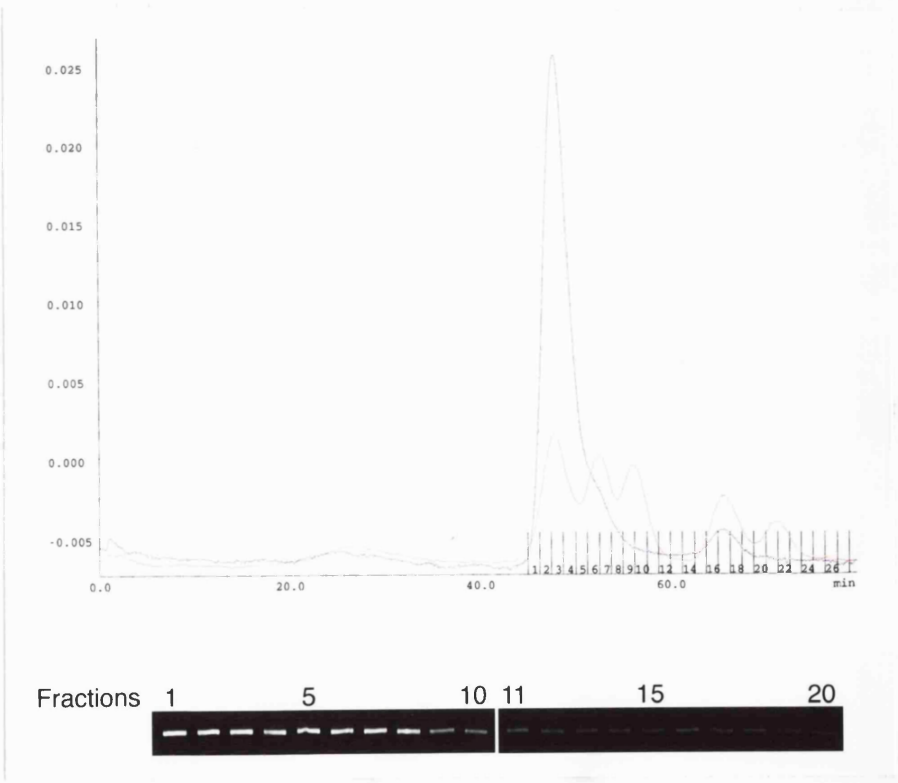


Fig. 5.3 Gel filtration of WGA binding proMMP-9 inducing activity (WGA-MMPSF)
WGA-MMPSF present in 600ml CM was bound to 80µl WGA-sepharose, the beads left to sediment (15 min, 4°C), washed in ice cold PBS (Gibco) and protein eluted in batches of 1 bed volume with 0.3M N-acetyl glucosamine (Sigma). Eluates containing proMMP-9 inducing activity were pooled and 50µl resolved by gel filtration on a Superdex PC 75 column at a flow rate of 40µl/min. Fractions of 25µl were harvested in the range shown and tested for proMMP-9 inducing activity by bioassay (1:12 dilution) and zymography. A strong peak of protein co-migrated with dextran blue in the void volume of the column. ProMMP-9 inducing activity was associated with this protein peak (fractions 1-8).
pink: sample, orange: molecular weight marker (dextran blue: 2000kDa, albumin: 67kDa, ovalbumin: 43kDa, RNAse-A: 25kDa, chymotrypsinogen-A: 13.7kDa)

For further analysis, WGA-MMPSF was purified from 600ml CM and a sample resolved by gel filtration on a Superdex 75 PC column. This column offered best resolution in the range from 70 to 3kDa and was used because preliminary studies based on ultrafiltration of CM suggested that MMPSF has a molecular weight below 100kDa (data not shown). Eluates were harvested and analysed for proMMP-9 inducing activity by bioassay and zymography (Fig. 5.3). Strong proMMP-9 inducing activity was present in fractions 1-8. This comprised a molecular weight range from the exclusion size of 100kDa to approximately 50kDa. These fractions were analysed by lectin blotting. As shown in Fig. 5.4 several proteins were detectable in fractions 2-8. Two bands showed a molecular weight close to 200kDa, another two bands were detectable at approximately 100kDa. No bands were detected in fractions 9-20 (data not shown).

Taken together these data suggested that WGA was a suitable resin for enrichment of proMMP-9 inducing activity (WGA-MMPSF) from PEO1 derived CM. WGA-MMPSF has a molecular weight of more then 100kDa as determined by gel filtration and might be a multimer of proteins with a molecular weight of about 100 and 200kDa (Fig. 5.4).

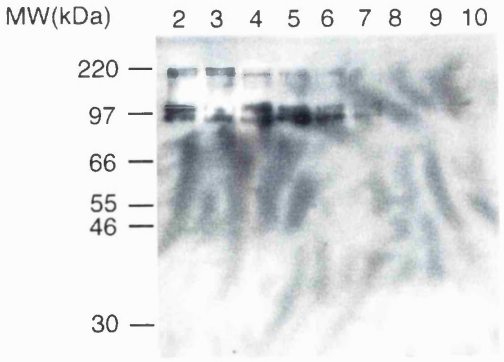


Fig. 5.4 Lectin blot of samples obtained by gel filtration
Samples 2-20 obtained by gel filtration (Superdex PC 75) were resolved by 11% SDS-PAGE under reducing conditions and blotted onto a PVDF membrane. Lectins were detected with biotinylated WGA and strepavidin labelled horseradish peroxidase. Several bands with a molecular weight of approximately 200 and 100kDa were detectable. The data show fractions 2-10. No bands were seen in fractions 11-20 (data not shown).

5.3.3 *Partial characterisation of Q-Sepharose binding proMMP-9 inducing activity (Q-MMPSF)*

As shown in Fig. 5.2, binding and elution of proMMP-9 inducing activity could also be achieved with Q-Sepharose (Q-MMPSF). Eluate 1 (E1) contained between 5-20 fold the activity measured in CM (data not shown). Further experiments were performed to optimise the salt concentration required for the elution of Q-MMPSF (Fig. 5.5). No activity was eluted at 60mM and little at 100mM NaCl. Most Q-MMPSF was detectable in the fraction eluted with 200mM NaCl (Fig. 5.5). This or slightly higher salt concentrations (250mM NaCl) were used in all further experiments.

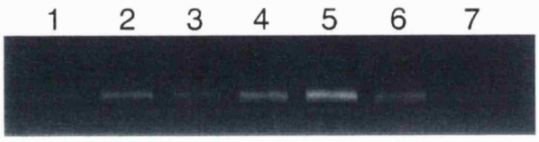


Fig. 5.5 Optimisation of the salt concentration required for the elution of Q-MMPSF
CM (1ml) was diluted 1:10 in 10mM TEA (pH 7.5) and 30µl Q-Sepharose beads were added. The suspension was incubated on a roller for 30 min at 4°C, the beads pelleted by centrifugation (5 min, 100g) and the flow through removed. Activity was eluted in batches of 2 bed volumes with increasing salt concentration (60mM, 100mM, 200mM, 300mM, 400mM NaCl). Eluates were tested by bioassay (1:6 dilution) and zymography. Some Q-MMPSF was eluted at 60 and 100mM NaCl. Most Q-MMPSF was eluted at 200mM NaCl.
Lane 1: control (unstimulated); 2: control (CM stimulated); 3: elution with 60mM NaCl; 4: 100mM NaCl; 5: 200mM NaCl; 6: 300mM NaCl; 7: 400mM NaCl

Finally, the amount of Q-Sepharose required for binding was optimised to obtain maximal concentration of Q-MMPSF (Fig. 5.6). CM was diluted 10 fold and 15 or 30µl Q-Sepharose added. Q-MMPSF was eluted in batches of 2 bed volumes and tested by bioassay (Fig. 5.6). Using 30µl Q-Sepharose, MMPSF was eluted in fractions E1 to E3 with maximal activity in E1. With 15µl Q-Sepharose, maximal activity was seen in E1 and E2. No activity was detectable in the wash buffer and in the flow through (data not shown).

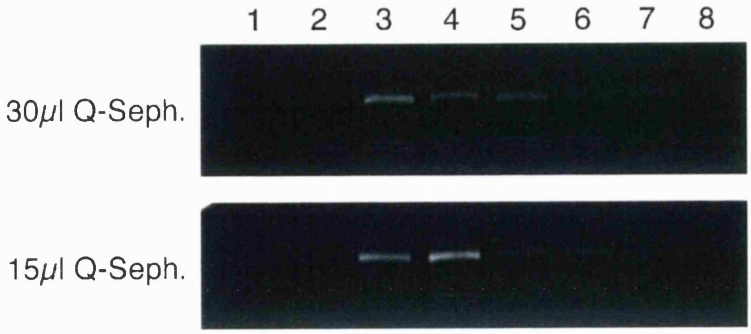


Fig. 5.6 Optimisation of the amount of Q-Sepharose required for maximal concentration of Q-MMPSF
CM (1ml) was diluted 1:10 in 10mM TEA (pH 7.5) and 15 or 30µl Q-Sepharose beads were added. The suspension was incubated on a roller (30 min, 4°C), the beads pelleted by centrifugation (10 min, 4°C, 100g), the flow through removed and the beads washed in TEA (pH 7.5), 30mM NaCl. Activity was eluted in batches of 2 bed volumes with TEA (pH 7.5), 200mM NaCl. Eluates and flow through were tested by bioassay (1:10 dilution) and zymography. The stronger concentration of Q-MMPSF activity was seen with 15µl Q-Sepharose per ml undiluted CM.
Lane 1: control (unstimulated); 2: wash ; 3: E1 (eluate 1); 4: E2; 5: E3; 6: E4; 7: E5, 8: E6.

To analyse the protein bound and eluted from Q-Sepharose, E2 was concentrated 50 fold by ultrafiltration (NanoSpin Plus, MWCO 10.000, Gelman Science, Ann Arbor, MI, USA), resolved by SDS-PAGE and proteins stained with silver nitrate. As shown in Fig. 5.7 large amounts of protein were detectable in the concentrate and very little in the flow through. The different amounts loaded per lane suggested that a single protein might be responsible for the pattern seen (Fig. 5.7).

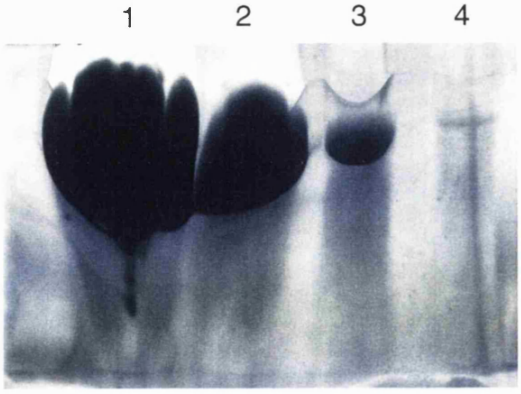


Fig. 5.7 Analysis of Q-Sepharose eluate by SDS-PAGE and silver staining
E2 (2ml) obtained from Q-Sepharose was concentrated by ultrafiltration, different amounts of concentrate and flow through were resolved by 10% SDS-PAGE and proteins stained with silver nitrate. Strong bands of protein were detectable. The band pattern observed when loading different amounts per lane suggested that this might be due to a single protein. The molecular weight marker was not detectable because the development of the gel had to be stopped before they became visible. Lanes 1-3: 30µl, 10µl, 1µl of concentrate; lane 4: 30µl flow through.

In view of the results obtained in Fig. 5.7 experiments were performed to investigate whether extended washing of the Q-Sepharose beads prior to elution would reduce the amount of contaminating protein eluted. Q-MMPSF was bound to Q-Sepharose, beads extensively washed and proteins eluted. Samples from washes and eluates were analysed by SDS-PAGE and Coomassie Blue staining of the gel. The results (Fig. 5.8) showed that extended washing did not lead to a reduction in the amount of protein eluted from Q-Sepharose. Most of the contaminating protein migrated in a single band at a molecular weight of approximately 70kDa. In addition, several experiment were performed to bind this protein to cholic-acid-, S-, CM-, octyl- and phenyl-sepharose. However, no satisfactory removal of this protein from CM could be achieved (data not shown).

Fig. 5.8 Extensive washing of Q-Sepharose beads did not reduce the amount of contaminating protein co-eluted with Q-MMPSF
CM (1ml) was diluted 1:10 in 10mM TEA (pH 7.5) and 20µl Q-Sepharose beads were added. The suspension was incubated on a roller for 30 min at 4°C, the beads pelleted by centrifugation (5 min, 100g) and the flow through removed. Beads were washed 6 times with 40µl 30mM NaCl, TEA (pH 7.5) and proteins eluted with 40µl 200mM NaCl, TEA (pH 7.5). Samples were resolved under reducing conditions by 10% SDS-PAGE and the gel stained with Coomassie Blue. Little unbound protein was detectable in the flow through after Q-Sepharose and no protein was detectable in the washes. One strong protein band (approx. 70kDa) was eluted in eluates 1-3. Lane 1: flow through; lane 2-7: washes 1-6; lane 8-10: eluate 1-3.

Taken together, these results suggested that Q-MMPSF could be efficiently concentrated from 1:10 diluted CM with 1.5µl Q-Sepharose per ml diluted CM. Elution was effective with 200mM NaCl in TEA (pH 7.5) but a large amount of a contaminating protein was co-eluted.

The protocol for the enrichment of Q-MMPSF from CM was then scaled up to initially 5ml (data not shown) and then to 500ml CM (Fig. 5.9). No activity could be detected in the

washes whereas activity in eluates 1 to 3 exceeded proMMP-9 stimulating activity observed in CM stimulated THP-1 cells approximately 5 fold (Fig. 5.9). Little activity was detectable in the eluates of a second round of purification using the flow through from the first one as starting material (data not shown). All activity containing fractions were pooled, centrifuged (1000g, 4°C) to pellet all remaining Q-Sepharose beads, sterile filtered (0.2µm, Nalgene) and stored at -20°C.

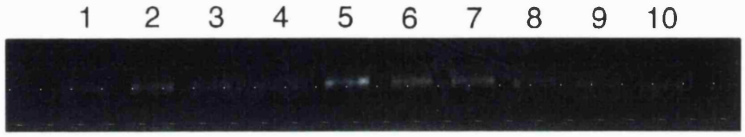


Fig. 5.9 Large scale enrichment of Q-MMPSF from CM with Q-Sepharose
CM (500ml) was diluted 1:10 in 10mM TEA (pH 7.5) and 7.5ml Q-Sepharose beads were added. The suspension was incubated on a roller (30 min, 4°C), the beads pelleted by centrifugation (10 min, 4°C, 100g), the flow through removed and the beads washed in TEA (pH 7.5), 30mM NaCl. Activity was eluted in batches of 1 bed volume with TEA (pH 7.5), 250mM NaCl. CM and eluates were tested by bioassay (1:10 dilution) and zymography (sample loading: 10µl). As in the small scale Q-MMPSF was eluted in fractions 1 to 3 (lanes 5-7). The degree of concentration was approximately 5 fold in comparison to the stimulation seen with CM. The data are representative of 6 experiments performed. Lane 1: control (unstimulated); 2: control (CM stimulated); 3: wash-1; 4: wash-2; 5: E1 (eluate 1); 6: E2; 7: E3; 8:E4; 9: E5; 10: E6.

5.3.4 Binding and elution of MMPSF to MonoQ

The purification described so far provided approximately 5 fold concentrated Q-MMPSF in a buffer consisting of TEA (pH 7.5), 250mM NaCl. These fractions were loaded onto a ion exchange column (MonoQ PC 1.6/5, Pharmacia) and Q-MMPSF was eluted by a salt gradient. A typical elution profile and a zymogram indicating the fractions containing proMMP-9 stimulating activity is shown in Fig. 5.10. The majority of Q-MMPSF was eluted in a step from 0 to 150mM NaCl but some activity was also eluted in a step from 150 to 240mM NaCl. Contaminating protein which eluted at salt concentrations higher then 240mM NaCl was efficiently removed. Alternative elution profiles including gradients, alternative steps and elution at pH 8.5 were also tried. Better resolution and concentration of MMPSF, however, could not be achieved (data not shown). Q-MMPSF containing samples were resolved by SDS-PAGE and proteins stained with silver nitrate. There were large amounts of protein detectable similar to Fig. 5.7 (data not shown).

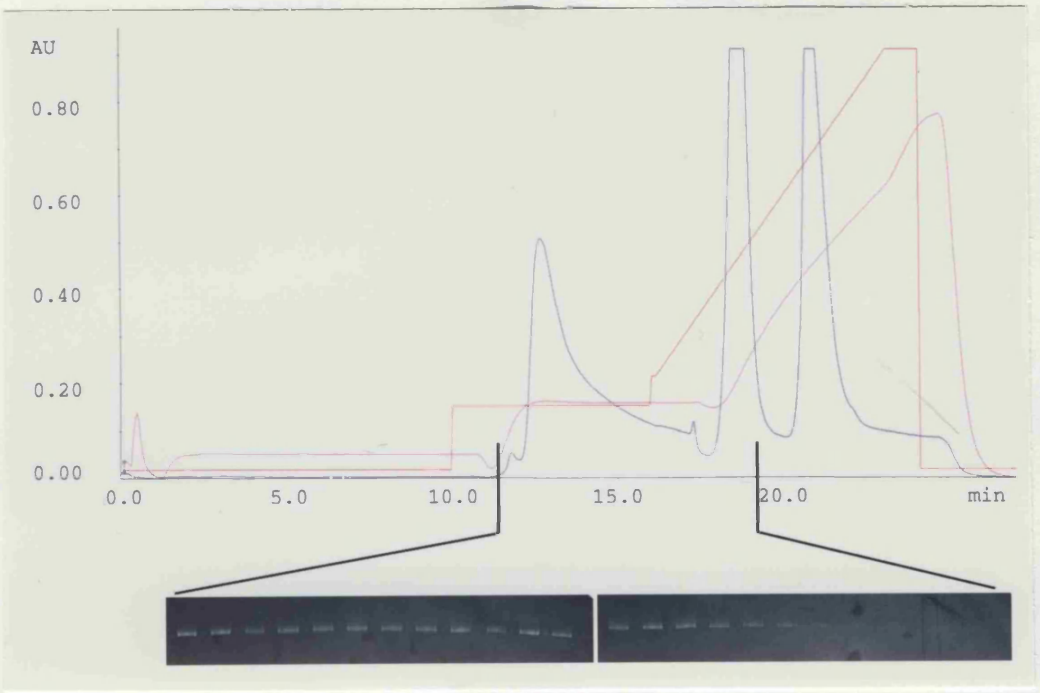


Fig. 5.10 Binding Q-MMPSF to MonoQ at pH 7.5
Eluates obtained from Q-Sepharose were diluted 1:5 in TEA (pH 7.5) and loaded on a MonoQ 1.6/5 column in batches of 2ml from a 50ml SuperLoop at a flow rate of 200µl/min. Proteins were eluted at a flow rate of 100µl/min in two steps from 0 to 150mM NaCl and 150 to 240mM NaCl followed by a linear gradient to 1M NaCl. Fractions (25µl) were collected during elution and tested by bioassay (dilution 1:15) and zymography (sample loading: 2µl). A typical zymogram is shown. MMPSF could be detected over a number of fractions. Most activity was eluted after the first stepwise increase in salt concentration but some was also detectable after the second step. (blue: sample, pink: conductivity, red: planned elution profile).

5.3.5 Gel filtration of Q-MMPSF obtained from MonoQ at pH 7.5

Eluates containing proMMP-9 inducing activity were pooled and a sample submitted to gel filtration (Fig. 5.11). Fractions in the range between the exclusion size (1300kDa) and 17.3kDa were taken. Presence of Q-MMPSF was analysed by bioassay and zymography (Fig. 5.11). Two peaks of activity were detected. One (fraction 1-3) showed a molecular weight close to the exclusion volume of the column. The second activity was detectable in fraction 24-28. To size this activity more accurately, further zymograms were run and fractions 25 and 26 were found to contain most proMMP-9. This suggested a molecular weight of 92±10kDa as compared to molecular weight marker (Fig. 5.12).

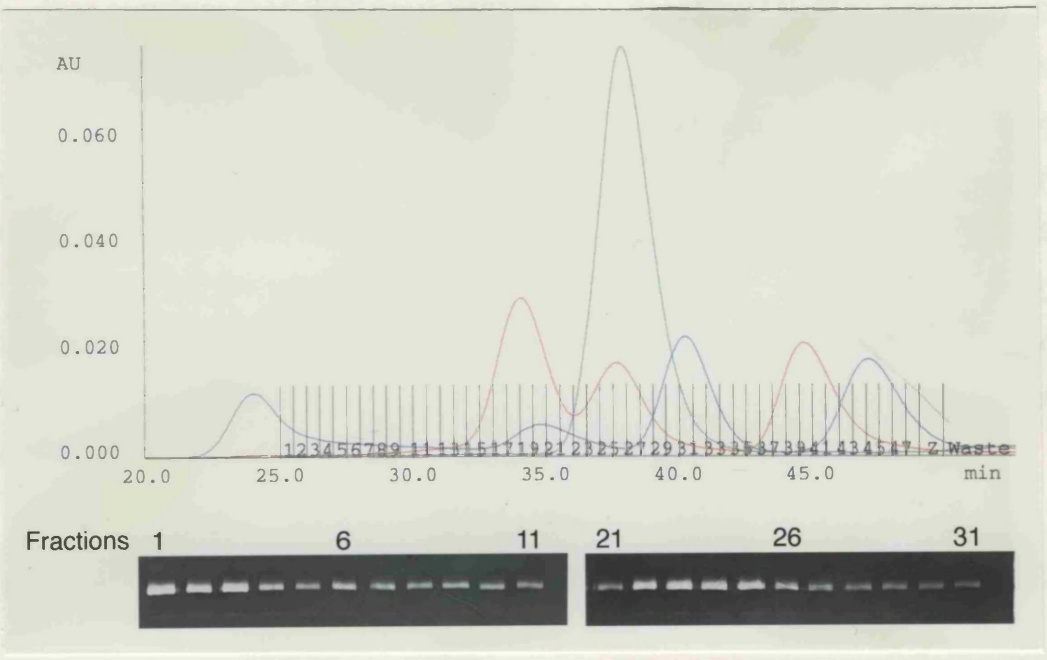


Fig. 5.11 Gel filtration of eluate obtained from MonoQ
Fractions containing proMMP-9 inducing activity obtained from the MonoQ column were pooled and 50µl were resolved on a Superdex 200 PC column (Pharmacia) at a flow rate of 40µl/min. Fractions (20µl) were taken in the range indicated and analysed by bioassay (1:10 dilution) and zymography. Two peaks of proMMP-9 inducing activity were observed. One (fraction 1-3) showed a molecular weight close to the exclusion volume (1300kDa) of the column. The second activity was observed in fractions 24-28. The data shown are representative for two experiments performed.
green: sample; blue: molecular weight marker I: dextran blue (2000kDa), aldolase (158kDa), ovalbumin (43kDa), RNAse-A (25kDa); red: molecular weight marker II: catalase (232kDa); albumin (67kDa); chymotrypsinogen A (13.7kDa)

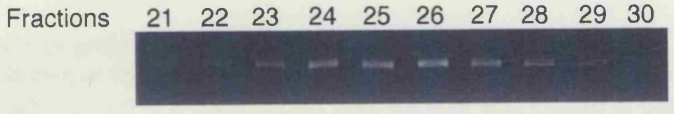


Fig. 5.12 Sizing of proMMP-9 inducing activity present in fractions 24-28
Fractions 21-30 were analysed by zymography and fractions 25 (t/to=1.544) and 26 (t/to=1.564) found to contain maximal Q-MMPSF. This corresponded a molecular weight of 92±10kDa as measured against a standard curve consisting of dextran blue (2000kDa, to=23.97), catalase (232kDa, t=34.08), aldolase (158kDa, t=34.96), albumin (67kDa, t=37.6), ovalbumin (43kDa, t=40.27), RNAse-A (25kDa, t=44.69); chymotrypsinogen A (13.7kDa, t=47.2). (t: elution time; to: elution time of dextran blue).

5.3.6 *Re-binding of MMPSF to MonoQ and elution at pH 6.5*

Fractions containing Q-MMPSF which had been eluted at 150mM NaCl from the Mono Q column (Fig. 5.10) were pooled, diluted 1:5 in TEA (pH 7.5) equilibration buffer and re-loaded onto the MonoQ column. The column was then equilibrated with a 20mM histidine buffer, pH 6.5 and protein eluted by increasing the NaCl concentration. A typical elution profile and a zymogram are shown in Fig. 5.13. A small peak containing proMMP-9 inducing activity was separated from the majority of protein which retained strong proMMP-9 inducing activity. The peak was obtained from 60mM to 90mM NaCl and 80mM NaCl was found to be optimal. A salt concentration of 100mM resulted in a reduced separation of the peak from the majority of protein (data not shown).

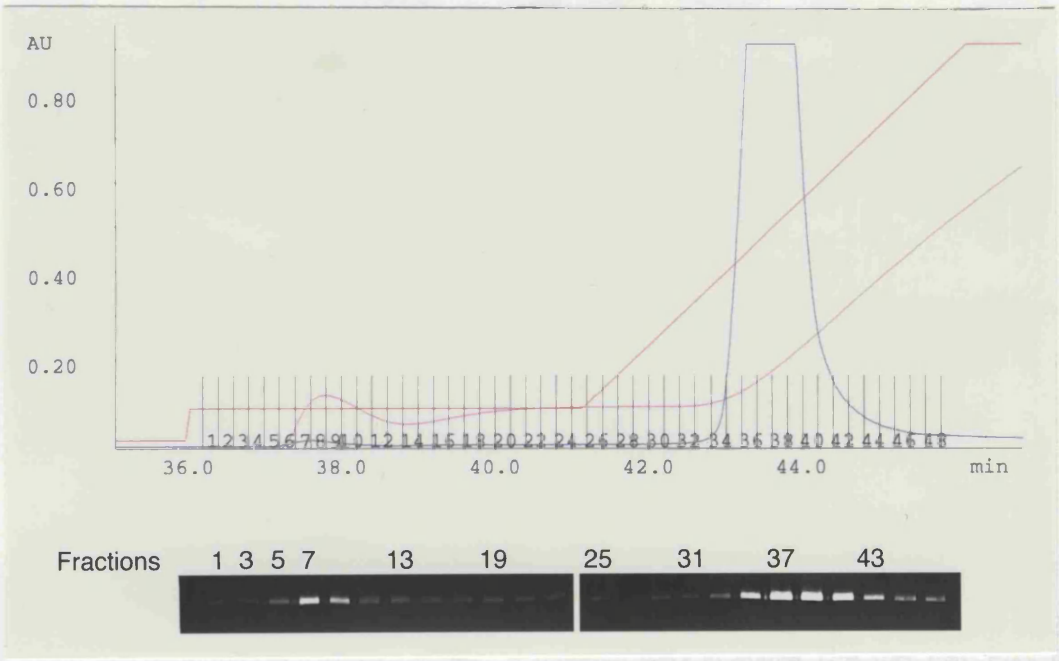


Fig. 5.13 Elution of proMMP-9 inducing activity from MonoQ at pH 6.5
Q-MMPSF obtained at 150mM NaCl from MonoQ at pH 7.5 was pooled, diluted 1:5 with 20mM TEA (pH 7.5) and loaded in a 50ml SuperLoop. Sample was loaded in batches of 1.5 to 2ml on the MonoQ column, the column was equilibrated with 20mM histidine, pH 6.5 (pump A) and proteins eluted in a step to 80mM NaCl followed by a linear gradient to 1M NaCl (pump B: 20mM histidine, pH 6.5, 1M NaCl). Fractions (20µl) were collected and tested for proMMP-9 inducing activity by bioassay (1:10 dilution) and zymography (loading: 5µl). Two peaks of proMMP-9 inducing activity were detected. The one present in fraction 5-11 was well separated from the majority of protein which eluted in a single peak and retained strong activity. (blue: sample; pink: conductivity; red: planned elution profile)

5.3.7 Concentration of Q-MMPSF obtained at pH 6.5 in fractions 4-11

Fractions 4 to 11 eluted at pH 6.5 were diluted 1:2 in 50mM TEA (pH 7.5), bound to the MonoQ column and eluted in a single step form 0-1000mM NaCl, 20mM TEA (pH 7.5) (Fig. 5.14). Bioassay and zymogram analysis showed very strong proMMP-9 inducing activity. Two protein peaks were detectable. Dilution assays to quantitate the concentration of the activity failed probably due to loss of protein through non-specific adherence to plastic tubes and tips. A sample (fraction 6) was resolved by 10% SDS-PAGE followed by silver staining. (Fig. 5.15). Several bands were detectable ranging from more then 200kDa to approximately 40kDa. A strong band co-migrates with BSA in the protein size marker (67kDa, Fig. 5.15)

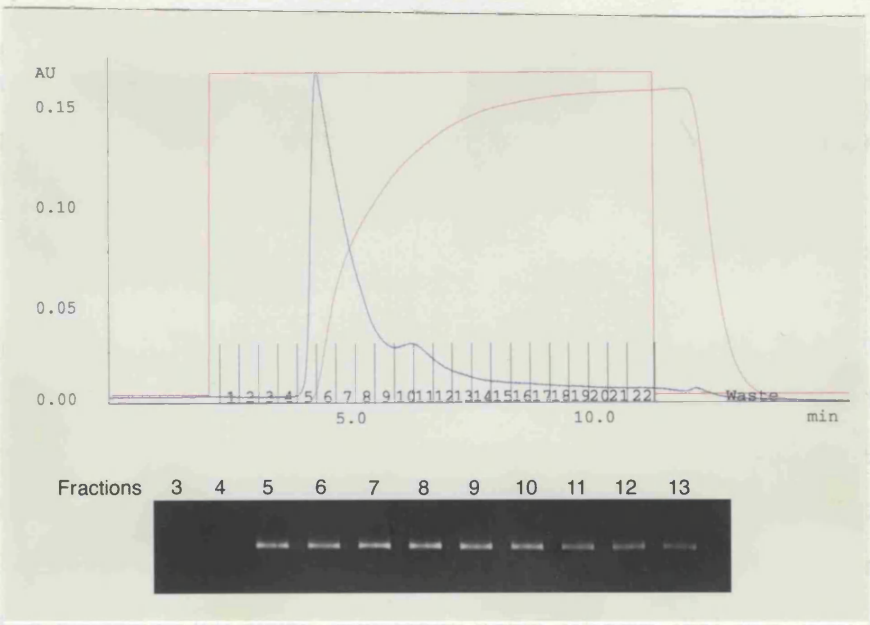


Fig. 5.14 Concentration of fractions 4-11 eluted from MonoQ at pH 6.5
Fractions 4-11 were pooled, diluted 1:2 in 50mM TEA (pH 7.5) and the entire material loaded. Elution was performed in a single step from 0-1000mM NaCl in 20mM TEA (pH 7.5). Prior to elution, mixer was washed in 1000mM NaCl and the flow rate reduced to 50µl/min to provide a sharp increase in salt concentration. Fractions (20µl) were taken, tested for proMMP-9 activity in a 1:200 dilution and analysed by zymography (loading: 2µl). Two protein peaks were detectable (fraction 6 and fraction 11). The proMMP-9 inducing activity was strong in fractions 5-8 and declined thereafter (blue: sample; pink: conductivity; red: planned elution).

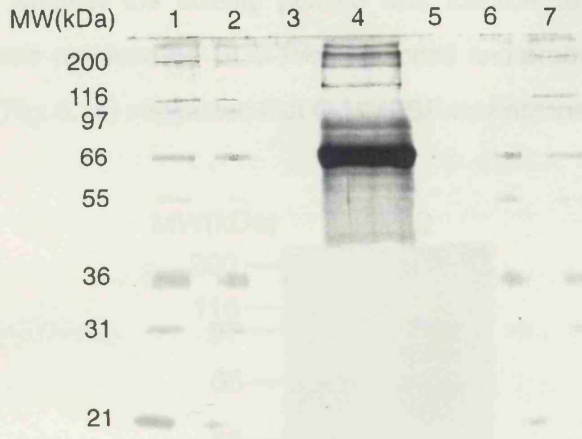


Fig. 5.15 Analysis of proteins by SDS-PAGE and silver staining
Fraction 6 (20µl) was resolved under reducing conditions by 10% SDS-PAGE and proteins stained with silver nitrate. Several bands with molecular weights from 40 to beyond 200kDa were detectable. A strong band co-migrated with BSA present as molecular weight marker (67kDa). (lanes 1, 2, 3, 5, 6, and 7: molecular weight marker, lane 4: sample)

5.3.8 Gel filtration of MMPSF from step 4 and Lectin blot

SDS-PAGE (Fig. 5.15) suggested that a number of proteins with distinct molecular weight could be Q-MMPSF. To analyse which contained the proMMP-9 inducing activity the samples 5+7 and 8+9 were pooled and resolved by gel filtration on the Superdex 200 column at a flow rate of 40µl/min. The column was equilibrated with PBS containing 0.01% Triton-100 (reduced, peroxide and carbonyl-free, Sigma) to prevent non specific binding of protein to the resin. Eppendorf tubes (Sigma) and tips (Life Sciences International, Basingstoke, UK) used in this experiment were siliconised to prevent losses. Fractions of 20µl were taken in the range comparable to that shown in Fig. 5.11 (close to the exclusion size to approximately 13kDa), tested by bioassay (dilution 1:100 in the first experiment and 1:50 in the second) followed by zymography. In the second experiment the range of sampling was extended beyond the exclusion size. In both gel filtration experiments one protein peak co-migrating with albumin (66kDa) was detectable (data not shown). Fractions containing this protein were analysed by SDS-PAGE and silver staining. A single protein co-migrating with BSA in the protein marker was detected

(data not shown). ProMMP-9 stimulating activity, however, was not recovered in either of the two gel filtration experiments (data not shown).

To analyse whether the activity purified was identical to WGA-MMPSF, a sample (fraction 6) was resolved by SDS-PAGE, blotted and probed with biotinylated WGA. The results (Fig. 5.16) suggested that Q-MMPSF was not identical to WGA-MMPSF.

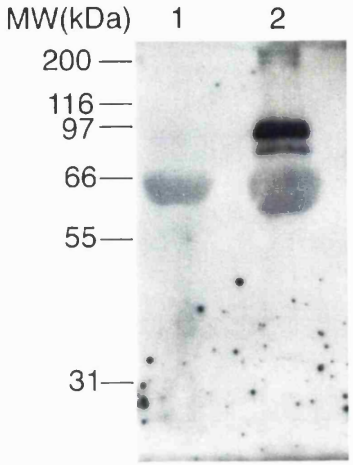


Fig. 5.16 Lectin blot of Q-MMPSF eluted at 80mM NaCl in histidine buffer at pH 6.5
A sample (fraction 6) obtained by ion exchange chromatography (80mM NaCl, histidine buffer pH 6.5) was resolved by 10% SDS-PAGE and blotted onto a PVDF-membrane. Lectins were detected with biotinylated WGA and strepavidin labelled horseradish peroxidase. PEO1 derived CM was used as positive control. No WGA-binding was seen in fraction 6 (lane 1) whereas several WGA-binding proteins were detectable in PEO1 derived CM (lane 2).

5.4 Discussion

In chapter 4 of this thesis a co-culture system of the ovarian cancer cell line PEO1 and the monocytic cell line THP-1 was analysed with respect to proMMP-9 production. Conditioned medium (CM) derived from the tumour cell line PEO1 was found to induce MMP-9 gene expression (Fig. 4.7) and proMMP-9 protein release (Fig. 4.1). In addition, the data suggested that the proMMP-9 inducing factor present in CM, tentatively named matrix metalloproteinase stimulating factor (MMPSF), was distinct from TNF-α, TGF-α, Fas Ligand, IL-6 receptor, p55 TNF-α-receptor, p75 TNF-α-receptor, stem cell factor, M-CSF, L-selectin and the thyrotropin receptor ectodomain. In this chapter MMPSF was further characterised.

A series of chromatography resins were tested for MMPSF binding and then tested for activity recovery. Two resins, wheat-germ agglutinin (WGA) and Q-Sepharose were found to bind proMMP-9 inducing activity (WGA-MMPSF and Q-MMPSF, respectively) and activity could also be eluted. WGA is a lectin that binds carbohydrates, in particular N-acetyl glucosamines (GlcNAc β1-4 GlcNAc β1-4 GlcNAc > GlcNAc β1-4 GlcNAc >> GlcNAc (208, 209)). Binding of MMPSF to WGA-Sepharose was repeatedly found to be partial and experiments performed to increase binding and elution efficiency were unsuccessful. Protein enriched with WGA-Sepharose was analysed by gel filtration and the fractions analysed by bio assay. ProMMP-9 inducing activity showed a molecular weight >100kDa by gel filtration. This was confirmed by lectin blotting showing two doublets of protein at a molecular weight of approximately 100 and 200kDa. For several reasons WGA-MMPSF was not further characterised. Binding of WGA-MMPSF was only partial and large amount of proMMP-9 inducing activity remained in the flow through (Fig. 5.1). This suggested that WGA-MMPSF was not the main proMMP-9 inducing activity present in PEO1 derived CM. This was supported by the observation that in comparison to Q-Sepharose much larger amounts of CM were required to obtain a similar proMMP-9 inducing effect (Fig. 5.1: WGA-MMPSF from 50ml CM, Fig. 5.2: Q-MMPSF from 1ml CM). In addition, lectins e.g. Phaseolus vulgaris agglutinin (PHA), have been to shown to induce monocytic TNF-α which is a strong inducer of MMP-9 release

(210, 211). Induction of TNF-α, however, was not seen in the stimulation experiments performed in chapter 4 (Fig. 4.7), suggesting that this was not the mode of action of MMPSF. Finally, the proteins isolated with WGA-Sepharose showed a molecular weight above 100kDa as determined by SDS-PAGE. This is a unusual molecular weight for a cytokine type protein which generally show a molecular weight below 30kDa in their monomeric form (212). Taken together, these findings indicated that CM probably contained several proteins which have the capacity to induce proMMP-9 production by THP-1 cells.

A second approach to characterise MMPSF was taken by ion exchange chromatography. Binding and elution of proMMP-9 inducing activity to Q-Sepharose (Q-MMPSF) was optimised with respect to the amount of resin required and the conditions for elution to obtain maximal concentration of Q-MMPSF. In small scale experiments (1ml CM) Q-MMPSF was enriched up to 20 fold whereas in the large scale (500ml CM) usually a 5 fold concentration of Q-MMPSF was achieved. It is not clear what caused the difference in the degree of concentration. It is possible that there was some variability in the composition (e.g. salt concentration) of CM from batch to batch and from small to large scale preparation of CM to which the purification protocol was sensitive. Alternatively, the efficiency of binding and elution might have been affected by the volumes processed (500ml CM was diluted to a volume of 5ltrs).

Eluates from Q-Sepharose were analysed by SDS-PAGE for protein content and strong protein contamination was detected. This experiment also suggested that a single protein was the major contaminant. Bovine serum albumin (BSA) was thought to be a likely candidate. BSA was present in the medium used for routine culture of PEO1 cells, which contained 10% foetal calf serum (FCS). It is possible that BSA adhered to PEO1 cells and was released into the FCS-free medium during the incubation period for the preparation of CM. Binding to the cells might be even enhanced through removal of cell surface proteins during trypsinisation of the cells and release favoured as soon as the cell surface was re-established. In addition, the isolelectric point of BSA was approximately at pH 5.6. Binding to Q-Sepharose was therefore expected at pH 7.5. A protein co-migrating with BSA was also detected in later purification steps by both gel filtration and SDS-PAGE. Experiments were performed to prevent accumulation or to remove this protein from PEO1 derived CM. Cells were detached and washed three

times in PBS prior to seeding and CM analysed by SDS-PAGE. In addition, removal of this protein from CM was attempted by chromatographic means (ion exchange, affinity or hydrophobic interaction chromatography). However, no significant reduction of this protein was achieved (data not shown).

Q-MMPSF concentrated in batch with Q-Sepharose was further enriched by ion exchange chromatography (MonoQ) on a HPLC/FPLC system. Most of the activity was eluted at 150mM NaCl but even after extended washing, elution of activity was incomplete at this salt concentration. More activity was found in a second elution step to 240mM NaCl. This might suggest that Q-MMPSF co-eluted with other proteins or that Q-MMPSF's binding to MonoQ was affected by other proteins. Fractions containing Q-MMPSF were pooled and analysed by gel filtration. Two peaks of proMMP-9 inducing activity were detected. One migrated at a molecular weight close to the exclusion size of the column (1300kDa) and a second peak was sized to 92±10kDa. Gel filtration was a very gentle method of protein separation by size and was not expected to disrupt multimers of a protein. It was therefore unlikely that the high molecular weight form of Q-MMPSF represented a multimer of low molecular weight Q-MMPSF. Samples were also analysed by ion exchange chromatography (MonoQ) using various elution protocols and different pH. At pH 8.5, Q-MMPSF separation was not improved in comparison to pH 7.5. At pH 6.5, however, a small peak containing strong proMMP-9 inducing activity could be separated from the majority of protein (Q-MMPSF, pH 6.5). This activity was concentrated and analysed by gel filtration but no proMMP-9 inducing activity was recovered. A sample resolved by SDS-PAGE and stained with silver nitrate revealed multiple protein bands ranging from approx. 40kDa to 200kDa. Particularly strong was a band co-migrating with BSA present in the protein marker. Lectin blotting of a sample showed that the activity present in these fractions was distinct from WGA-MMPSF.

Taken together these data suggested that PEO1 derived CM contained at least three proMMP-9 inducing proteins (WGA-MMPSF, high molecular weight Q-MMPSF and low molecular weight Q-MMPSF). It was not clear whether Q-MMPSF, pH 6.5 is identical to any of these three and whether WGA-MMPSF and high molecular weight Q-MMPSF were identical.

A major problem throughout the purification was the contamination of CM with a protein which seemed to be BSA. Attempts to separate this protein from MMPSF by

chromatographic means and by washing of the cells prior to seeding did not lead to a reduction of the protein. It seems likely that BSA was bound to the cells at the beginning of the incubation period and that it consequently detached from the cells. One possible approach to reduce the BSA contamination was therefore to wash the PEO1 cells after they have adhered, to add fresh FCS free medium and to use this conditioned medium for the purification of MMPSF.

6. Characterisation of a 85kDa form of MMP-9

6.1 Introduction

In chapter 4 of this thesis TNF-α and a tumour cell derived factor (MMPSF) were shown to stimulate the monocytic cell line THP-1 to release proMMP-9 (92kDa) into the cell culture supernatant. The activated form of MMP-9 (82kDa), however, was not observed. This raised the question whether activated MMP-9 might be associated with the cell rather than being released. Indeed, several groups reported recently the existence of a 92 and 85kDa cell surface or vesicle associated form of MMP-9 (91, 141, 142, 213). The 92kDa form has been consistently identified as proMMP-9 but conflicting data were reported with respect to the 85kDa form. Mazzieri *et al.*, Ginestra *et al.* and Nguyen *et al.* (141, 142, 213) concluded that the 85kDa band represented an activated species of MMP-9 whereas Toth *et al.* (91) presented data indicating it might be a glycosylation variant of proMMP-9 modified to bind to the cell membrane. This raised the question whether stimulated THP-1 cells also contained cell associated forms of MMP-9 and whether these were activated.

ProMMP-9 is proteolytically inactive and frequently associated with the tissue inhibitor of metalloproteases TIMP-1 (46, 214). The mechanisms by which proMMP-9 becomes activated are not completely understood but it is thought that a cascade of proteases, possibly including the plasminogen activators uPA and tPA, are involved (138, 141). *In vitro* studies using purified or recombinant protein have shown that proMMP-9 can be activated by bacterial proteinases and trypsin (119, 120), neutrophil elastase (215), MMP-1 (119), MMP-2 (132), MMP-3 (119, 216) and MMP-7 (134). In an initial step these proteases remove a part of the N-terminal pro-peptide leading to an intermediate form of MMP-9 with a molecular weight of approximately 84kDa. An autocatalytic process then yields the fully active MMP-9 (82kDa).

The aim of this chapter was to establish whether different MMP-9 forms were also associated with stimulated THP-1 cells and to resolve the controversy with respect to the activation of these forms.

6.2 Materials and Methods

Cell culture and LPS stimulation

THP-1 cells were cultured as described in chapter 4. Briefly, cells were harvested by centrifugation (200g, 5 min), resuspended in Aim V medium (Gibco) and the cell concentration adjusted to $1x10^6$ cells/ml. Cells were stimulated with LPS (*Salmonella minnesota*, Sigma) which is a natural stimulus for monocytic cells and a strong inducer of MMP-9 protein release. Cells and cell culture supernatant were harvested after an incubation period of 48 hours at 37°C, 5%CO_2. LPS was prepared as a stock solution of 1mg/ml in PBS and stored at -20°C. Once thawed the stock solution was kept at 4°C. LPS stock solution was vortexed to solubilise precipitated material prior to use.

Acetone precipitation

Acetone precipitation was performed in siliconised (SigmaCote, Sigma) eppendorf tubes. Sample and acetone (-20°C) were mixed at a 1:1 ratio and centrifuged for 30 min (30000g, 4°C). Pellets were dried under vacuum.

Triton-X-114 extraction of LPS stimulated THP-1 cells

Triton-X-114 (TX-114, Sigma) was prepared as 10% (v/v) stock solution in PBS and diluted to a 1.5% (v/v) working solution. Stock and working solution were stored at 4°C. Cell extractions were performed as published (217). Briefly, cells were pelleted by centrifugation, washed 3 times in ice cold PBS, resuspended in 1.5% TX-114 and vortexed to disrupt them. The cell lysate was incubated at 4°C on a roller for 20 min, briefly centrifuged, warmed to 37°C for 5 min and centrifuged at maximal speed (14000g, 2 min) to separate the upper aqueous and the lower detergent phase. The upper aqueous phase was removed and the lower hydrophobic phase resolubilised in ice cold PBS. To concentrate MMP-9, gelatin sepharose beads (Sigma) were added, the sample incubated for 20 min at 4°C on a roller, the beads were pelleted by centrifugation and MMP-9 eluted in 50mM Tris HCl (pH 7.6), 10mM EDTA, 10% DMSO. The eluate was tested for proteolytic activity by zymography or analysed by western blotting. Zymography was performed as described in chapter 3 (180).

Cell fractionation by sonication

Cells were pelleted and resuspended in ice cold lysis buffer (0.25M sucrose, 1mM EDTA, 10mM HEPES-NaOH, pH 7.4). Cell fractionation was performed by sonication (SoniPrep 150, MSE, Fisons, Loughborough, UK) at a wavelength of 10μm. Cell lysis was followed by phase contrast microscopy and continued until cells in excess of 95% were disrupted (1-5 min). The sample was cooled on ice to prevent warming.

Large scale purification of proMMP-9 from cell culture supernatant

MMP-9 was purified with small modifications as described elsewhere (116). Briefly, THP-1 cells ($2.4x10^9$ cells) were stimulated with 5ng/ml LPS (*S. minnesota*) and incubated in roller bottles (Falcon, Greiner Labortechnik Ltd., Stonehouse, UK) for 48 hours. Cells were pelleted (5 min, 200g, 4°C) and the supernatant cleared from cell debris by centrifugation (1000g, 10 min, 4°C). Pellets were discarded, 0.02% (w/v) sodium azide (Fisons, Loughborough, UK) was added and the supernatant incubated in batches of 300ml with 25ml gelatin sepharose (Sigma) on a roller (4°C, 15 min). Sepharose beads were harvested by centrifugation (10 min, 100g, 4°C), the supernatant (flow through) removed and another aliquot of culture medium added. This procedure was repeated until the entire volume was processed. Sepharose beads were washed extensively in wash buffer (50mM Tris HCl (pH 7.6), 10mM EDTA, 1M NaCl) and MMP-9 eluted in elution buffer (50mM Tris HCl (pH 7.6), 10mM EDTA, 10% DMSO). Elution was performed in batches of 25ml. Cell culture supernatant, samples of the flow through and eluates were tested for MMP-9 by zymography and protein identity confirmed by western blotting. 12ml of the second eluate (E2) was concentrated by acetone precipitation, dried, resuspended in loading buffer and resolved by 10% SDS-PAGE under reducing conditions followed by staining with Coomassie-Blue (PhastGel Blue, Pharmacia). The precipitate of the remaining 12ml of E2 was N-terminally sequenced.

Preparation of membrane associated MMP-9 from LPS stimulated THP-1 cells

THP-1 cells were stimulated with 5ng/ml LPS and incubated for 48 hours. In analytical experiments $2\text{-}10x10^6$ cells were used. For N-terminal sequencing of membrane

associated MMP-9 forms $10x10^9$ cells were grown in roller bottles (Falcon). Adherent and non-adherent cells were harvested, washed 3 times in ice cold PBS, resuspended in lysis buffer (0.25M sucrose, 1mM EDTA, 10mM HEPES-NaOH, pH 7.4) and sonicated as described. The cell lysate was centrifuged for 15 min (3000g, 4°C) to pellet cell nuclei and unlysed cells. The supernatant was removed and the pellet resuspended in lysis buffer and spun as before. The supernatants were pooled and the resulting total cell lysate centrifuged for 20 min (17000g, 4°C). The supernatant was removed and the pellet resuspended in lysis buffer and spun as before. The supernatants were pooled and spun at 100000g (1 hour, 4°C). The pellets remaining after the centrifugation at 3000g, 17000g and 100000g were extracted with 1.5% TX-114, the aqueous phase prepared and MMP-9 purified as detailed in the purification of MMP-9 from cell culture supernatant. Eluate containing MMP-9 forms was concentrated by acetone precipitation, the pellet dried, resuspended in loading buffer and resolved by 8% SDS-PAGE under reducing conditions followed by staining with Coomassie-Blue (PhastGel Blue, Pharmacia). A further precipitate was N-terminally sequenced.

N-terminal sequencing of MMP-9

N-terminal sequencing was kindly performed by B. Dunbar in the laboratory of Prof. Dr. John E. Fothergill, Department of Molecular and Cell Biology, University of Aberdeen, Institute of Medical Sciences, Foresterhill, Aberdeen, Scotland AB25 2ZD.

Western blotting

Samples were resolved by 8% or 10% SDS-PAGE under reducing conditions. Blotting and detection of MMP-9 was performed as described in detail in chapter 4 of this thesis.

Other materials

The synthetic MMP inhibitor BB-94 (gift of British Biotech Pharmaceuticals Ltd., Oxford, UK) was prepared in DMSO as a 100mM stock solution and used at a concentration of 1μM. This broad spectrum inhibitor blocks MMP activity in the lower nanomolar range (25, 218). Purified proMMP-9 was purchased from TCS Biologicals (Botolph Claydon, UK).

6.3 Results

6.3.1 LPS stimulates proMMP-9 release in a dose dependent manner

THP-1 cells ($1x10^6$ cells/ml) were stimulated with various doses of LPS from three microbial sources (*E.coli* 055:B5, *E.coli* 0111:B4 and *Salmonella minnesota*). Cells were incubated for 48 hours and the proMMP-9 activity in the cell culture supernatant analysed by zymography. As shown in Fig. 6.1 all three types of LPS induced proMMP-9 production in a dose dependent manner. The strongest induction (90 fold of control) was seen with 200ng/ml LPS from *Salmonella minnesota*. At concentrations below 0.1ng/ml LPS the induction of proMMP-9 was below 2 fold that of unstimulated THP-1 cells for all the three types of LPS. Endotoxin from *Salmonella minnesota* was used for all further experiments.

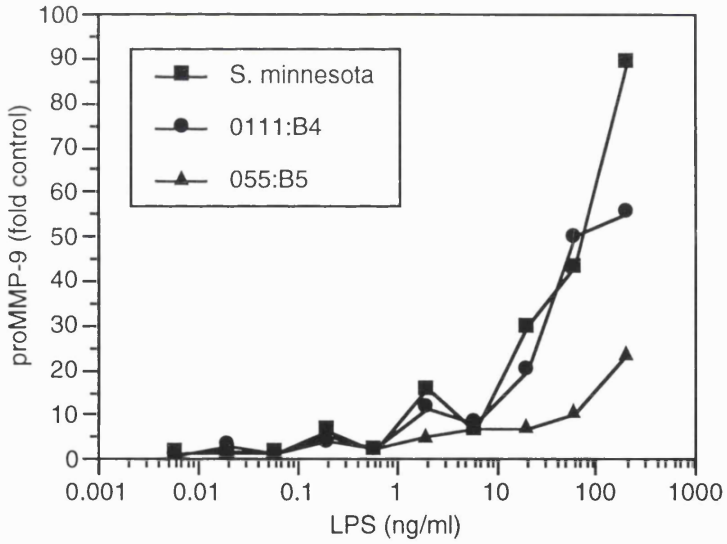


Fig. 6.1 MMP-9 production in response to endotoxin
THP-1 cells were stimulated with endotoxin (LPS) from different microbial sources and proMMP-9 production was assessed by zymography. All types of LPS induced proMMP-9 production. Strongest induction was seen with LPS from *Salmonella minnesota*. These data were representative of three independent experiments.

6.3.2 Detection of cell associated gelatinolytic activity in LPS stimulated THP-1 cells

THP-1 cells ($2x10^6$ cells) were stimulated with 100ng/ml LPS (*S. minnesota*) and incubated for 48 hours at 37°C, 5%CO_2. The cell culture supernatant was harvested, the cells extracted with Triton-X-114 (TX-114) and MMP-9 concentrated from both the aqueous and the detergent phase with gelatin-sepharose beads. Cell culture supernatants and eluates were analysed by zymography (Fig. 6.2). LPS induction led to a strong gelatinolytic activity in the cell culture supernatant. This activity co-migrated with commercially available proMMP-9. Very little gelatinolytic activity was detectable in the PBS used to wash the cells prior to lysis with TX-114. Three bands of gelatinolytic activity were detectable in the aqueous phase. The upper band (92kDa) co-migrated with proMMP-9 detected in the cell culture supernatant. The two other bands showed a molecular weight of approximately 85 and 80kDa. No proteolytic activity was detectable in the hydrophobic phase of TX-114 extractions (Fig. 6.2).

To analyse whether metalloproteases caused the gelatinolysis observed on the gels, the matrix metalloproteinase inhibitor BB-94 (1μM) was added to the collagenase buffer during the development of the zymogram. All three bands of gelatinolytic activity disappeared (data not shown).

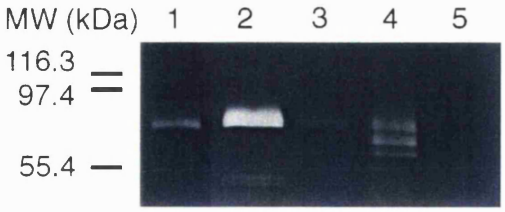


Fig. 6.2 Gelatinolytic activity in extracts of LPS stimulated THP-1 cells
LPS stimulated THP-1 cells were incubated for 48 hours and the supernatant harvested. Cells were washed, lysed in Triton-X-114 (TX-114) and gelatinolytic activity concentrated with gelatin sepharose from both the aqueous and detergent phase. There was a strong gelatinolytic activity in the culture medium of LPS stimulated THP-1 cells. Three bands of proteolytic activity were detectable in the aqueous phase of TX-114 extracts but none in the hydrophobic phase.
Lane 1: purified proMMP-9 (1ng); lane 2: culture medium from LPS stimulated THP-1 cells; lane 3: sample of third PBS-wash of cells prior to lysis with TX-114; lane 4: aqueous phase of TX-114 extracts; lane 5: hydrophobic phase of TX-114 extracts. This is a typical result of four experiments performed.

6.3.3 *Western blotting confirmed the 92kDa and the 85kDa band to be a forms of MMP-9*

Western blotting was used to test whether the gelatinolytic activities detected in the supernatant of LPS stimulated THP-1 cells and in the aqueous phase of TX-114 extracts were forms of MMP-9. Eluates from gelatin sepharose were pooled, resolved by 10% SDS-PAGE, electroblotted on a nitrocellulose membrane (Hybond ECL, Amersham) and MMP-9 detected as described in the Materials and Methods. Both monoclonal anti-MMP-9 antibodies (CA-209 and AB-2) detected one band (92kDa) in the supernatant of LPS stimulated THP-1 cells and two bands (92 and 85kDa) in the aqueous phase of TX-114 extracts (Fig. 6.3). The third band (80kDa) was not detected with the antibodies used.

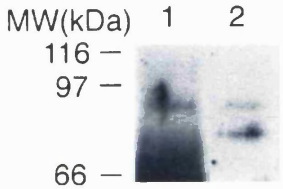


Fig. 6.3 Detection of MMP-9 by western blotting in culture medium and in the aqueous phase TX-114 extracts of LPS stimulated THP-1 cells
A single band corresponding to proMMP-9 was detectable in the cell culture supernatant of LPS stimulated THP-1 cells (lane 1). Two forms of MMP-9 (92kDa and 85kDa) were detected in the aqueous phase of TX-114 extracts from LPS stimulated THP-1 cells (lane 2). This result was obtained with the monoclonal antibody CA-209 and was reproduced with the monoclonal antibody Ab-2. The data shown are typical for three experiments performed.

6.3.4 *Triton-X-114 extractable forms of MMP-9 could be pelleted at 100 000g*

To analyse the cellular location of 92 and 85kDa MMP-9, subcellular fractions from LPS (100ng/ml) stimulated THP-1 cells we prepared by differential centrifugation (219). The pellets were extracted with TX-114, MMP-9 concentrated from the aqueous phase with gelatin sepharose beads and samples analysed by zymography (Fig. 6.4). Most MMP-9 was detectable in the fraction obtained at 100000g, very little in the 3000g and 17000g fractions. No MMP-9 was detectable in the remaining supernatant after 100000g centrifugation (data not shown). These results suggested that the 92kDa and 85kDa MMP-9 were associated with the plasma or intracellular membranes.

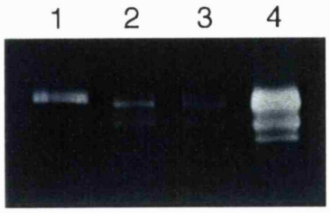


Fig. 6.4 Subcellular fractionation of THP-1 cells by differential centrifugation
THP-1 cells were stimulated with LPS (100ng/ml), incubated for 48 hours, washed and lysed by sonication. The cell lysate was fractionated by centrifugation (3000g, 17000g, 100000g), the pellets lysed with TX-114, the aqueous phase prepared and MMP-9 concentrated with gelatin-sepharose beads. The eluates were analysed by zymography. The majority of 92 and 85kDa MMP-9 was detectable in the 100000g fraction.
Lane 1: medium from LPS stimulated THP-1 cells; lane 2: 3000g fraction; lane 3: 17000g fraction; lane 4: 100000g fraction.

6.3.5 Optimisation of cell culture conditions to obtain maximal yield of cell associated MMP-9 forms

THP-1 cells were stimulated with different concentrations of LPS (*S. minnesota*), incubated for 48 hours and the number of viable cells assessed by phase contrast microscopy. The cells were then lysed in TX-114, the aqueous phase prepared, MMP-9 concentrated as described before and the eluates tested by zymography. As shown in Fig. 6.5 A, the yield of 92 and 85kDa MMP-9 was maximal at a LPS concentration of 5 and 10ng/ml LPS and declined at higher and lower LPS concentrations. The cell counts showed that LPS at concentrations higher then 5ng/ml resulted in a reduction in cell number whilst lower LPS concentrations had not effect (Fig. 6.5 B). The presence of cell debris observed in the cultures with LPS concentrations above 5ng/ml suggested toxicity of the reagent. This was confirmed by electron microscopy. THP-1 cells stimulated with 100ng/ml showed strong signs of necrosis whereas cells stimulated with 5ng/ml LPS appeared to be intact (data not shown). For all further experiments a LPS concentration of 5ng/ml was used.

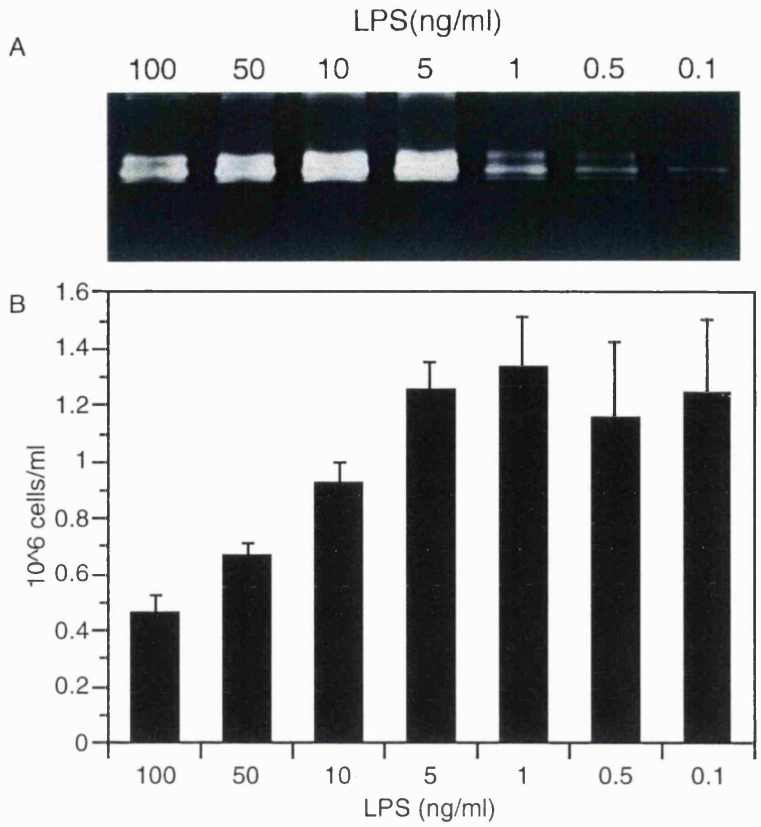


Fig. 6.5 Optimisation of the production of cell associated forms of MMP-9
THP-1 cells were stimulated with different amounts of LPS and the number of viable cells was determined by phase contrast microscopy after an incubation period of 48 hours. Then the cells were extracted with TX-114, MMP-9 in the aqueous phase was concentrated with gelatin sepharose and eluates were analysed by zymography. The error bars reflect the standard deviation of the three cell counts done per condition.

6.3.6 Purification of proMMP-9 (92kDa) from cell culture supernatant of LPS stimulated THP-1 cells

Soluble proMMP-9 was purified from medium of LPS stimulated THP-1 cells ($2.4x10^9$ cells). A sample of concentrated eluate was resolved by 10% SDS-PAGE under reducing conditions and the gel stained with Coomassie Blue (Fig. 6.6). A sample prepared in parallel was N-terminally sequenced. Two bands with a molecular weight of 92 and 66kDa were detectable. The 92kDa band was N-terminally sequenced and the protein identified as proMMP-9 (accession number in the SWISS-PROT sequence data base: P14780). The 66kDa band is probably bovine serum albumin and did not show activity on zymograms (data not shown). Interestingly, TIMP-1 (28kDa) was not co-purified with proMMP-9 (Fig. 6.6).

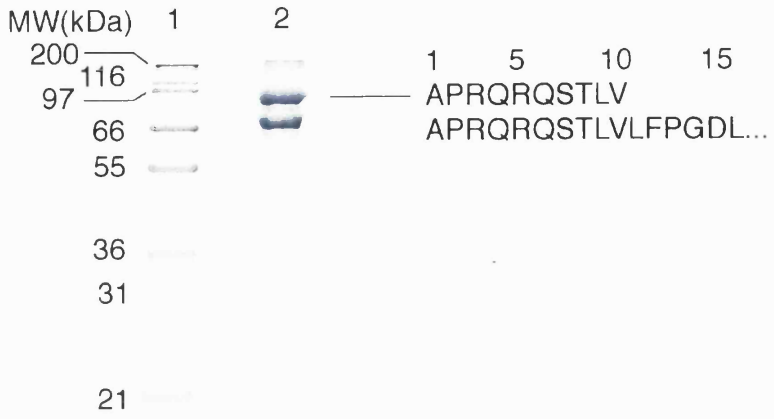


Fig. 6.6 SDS-PAGE of MMP-9 purified from cell culture supernatant
MMP-9 was purified from cell culture supernatant of LPS stimulated THP-1 cells and a sample resolved by SDS-PAGE. Coomassie Blue staining revealed two bands of 92 and 66kDa. The 92kDa band was identified as proMMP-9 by N-terminal sequencing.

6.3.7 Purification and N-terminal sequencing of cell associated forms of MMP-9

THP-1 cells ($10x10^9$ cells) were stimulated with 5ng/ml LPS and incubated for 48 hours. The cells were harvested and MMP-9 forms purified. A sample was resolved by 8% SDS-PAGE under reducing conditions and the gel stained with Coomassie Blue. A further sample was N-terminally sequenced. Coomassie Blue staining of the gel revealed several bands in addition to three with a molecular weight of 92, 85 and 80kDa (Fig. 6.7). N-terminal sequencing was successful for the 92 and the 85kDa band. The sequences obtained from these two bands were identical and also identical to that of proMMP-9 isolated from the cell culture supernatant (Fig. 6.6 and 6.7). These data showed that both cell associated forms of MMP-9 were the proteolytically inactive pro-forms of MMP-9.

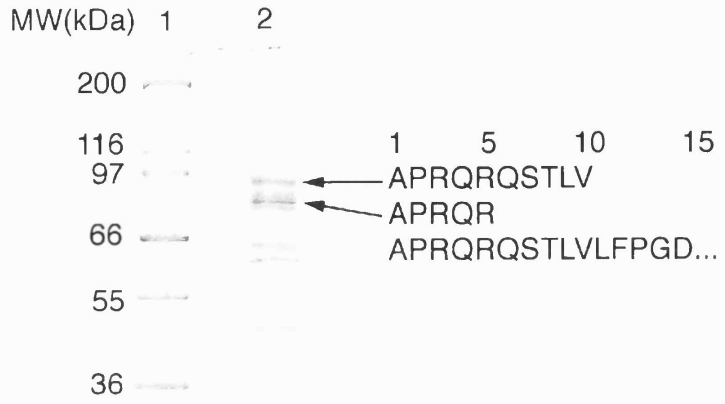


Fig. 6.7 SDS-PAGE of MMP-9 forms purified from cell extracts
MMP-9 was purified from TX-114 extracts of LPS stimulated THP-1 cells and a sample resolved by SDS-PAGE. Coomassie Blue staining revealed several protein bands. The proteins indicated were N-terminally sequenced and their N-termini found to be identical to that of soluble proMMP-9.

6.4 Discussion

The aim of this part of the thesis was to investigate whether activated forms of MMP-9 were produced by stimulated monocytic cells. As shown in chapter 4, TNF-α and tumour cell derived conditioned medium induced MMP-9 production from THP-1 cells. However, only the proteolytically inactive pro-form of MMP-9 was detectable in the cell culture supernatant raising the question whether activated MMP-9 was cell-associated. A similar observation has been made for MMP-2 which is a close homologue to MMP-9 in terms of protein structure and substrate specificity (Table 1.3, Fig. 1.1). Activation of proMMP-2 was associated with a membrane type MMP, MT-1-MMP and activated MMP-2 could be detected in both cell culture supernatant and associated with cell membranes (93). It was therefore possible that the activation process of MMP-9 was also localised to the cell membrane but that activated MMP-9 was retained on the cell surface. Indeed, several recent studies showed the existence of two MMP-9 forms which were associated with the surface of cells or vesicles (91, 141, 142, 213). A 92kDa form was consistently identified as proMMP-9 but conflicting data were reported with respect to a 85kDa form. Mazzieri *et al.*, Ginestra *et al.* and Nguyen *et al.* (141, 142, 213) concluded that the 85kDa band represented an activated species of MMP-9 whereas Toth *et al.* (91) presented data indicating it might be a glycosylation variant of proMMP-9 modified to bind to the cell membrane.

In this study THP-1 cells were stimulated with LPS, a natural stimulus for monocytes and strong inducer of MMP-9 gene expression. Stimulated cells released the proteolytically inactive pro-form of MMP-9 (92kDa) into the cell culture supernatant. Cell extracts of stimulated THP-1 cells contained three bands of proteolytic activity (92, 85, 80kDa) all of which were metalloproteases. Western blotting confirmed the cell associated 92 and 85kDa band as forms of MMP-9 and cellular fractionation followed by differential centrifugation showed that 85 and 92kDa MMP-9 could be precipitated at 100000g but not at the lower g-forces of 17000g or 3000g. These data suggested that 85 and 92kDa MMP-9 were associated either with the cell membrane or localised to intracellular vesicles (219). There were precedents for both options. Toth *et al.* (91), Mazzieri *et al.* (141) and Ginestra *et al.* (142) showed by cell surface biotinylation that the MMP-9 forms localised

to the surface of cells or vesicles whilst Nguyen *et al.* (213) showed by immunogold staining presence of MMP-9 in intracellular vesicles. Full localisation of the 92 and 85kDa form of MMP-9 in LPS stimulated THP-1 cells remained to be established.

Activation of the proteolytically inactive pro-form of MMP-9 has been analysed extensively *in vitro* and it is widely accepted that proteolytic processing of the N-terminal pro-peptide leads to an intermediate form of MMP-9 (approx. 84kDa) which autocatalytically converts into the fully activated MMP-9 (approx. 82kDa) (76). To analyse whether the cell associated 85kDa MMP-9 was activated, this form was purified and N-terminally sequenced. The results showed that both membrane associated MMP-9 species (92 and 85kDa) had the same N-terminal amino acid sequence and that it was also identical to that of soluble 92kDa proMMP-9. Taken together these data showed that both cell associated forms were proteolytically inactive. This result was in keeping with the findings by Toth *et al.* who used an antibody specific for the N-terminus of proMMP-9 (91) but contradicted the interpretation by Mazzieri *et al.* (141), Ginestra *et al.* (142) and Nguyen *et al.* (213). The latter, however, had based their conclusion only on differences in molecular weight observed by zymography rather then a more detailed analysis.

Interestingly, TIMP-1 (28kDa) was not co-purified with proMMP-9 from the cell culture supernatant of LPS stimulated THP-1 cells (Fig. 6.8). This was in contrast to results obtained by others who purified proMMP-9 from phorbol ester stimulated THP-1 cells (116). In addition, attempts to detect TIMP-1 by western blotting in medium of LPS stimulated THP-1 cells using a monoclonal or polyclonal anti-TIMP-1 antibody were unsuccessful. The protein was, however, readily detectable in medium of phorbol ester (300ng/ml) stimulated THP-1 cells suggesting that phorbol ester induced TIMP-1 protein production whereas LPS did not (C. Scotton, unpublished results). Taken together, these data suggested that TIMP-1 did not play an important role in the formation of 85kDa proMMP-9 and its association to the cell. In addition, the lack of TIMP-1 protein was consistent with findings by Nguyen *et al.* who provided data for the existence of intracellular TIMP-1-free MMP-9 (213).

Further work is required to fully localise the cell associated forms of MMP-9 and to characterise their biochemical differences. The data presented showed that the N-termini

of the intracellular 92 and 85kDa forms of MMP-9 were intact and therefore not the cause of the difference in molecular weight. A further possibility was a variation in the degree of glycosylation since MMP-9 released from U937 has been shown to be N- and O-glycosylated (111). Toth *et al.* provided data showing that such a modification was the cause for the molecular weight difference in their system consisting of the phorbol ester stimulated breast cancer epithelial cell line MCF10A (91). Alternatively, proteolytic processing of the C-terminus represented also an option to create the difference in molecular weight. Some preliminary results were obtained through the application of tunicamycin and Benzyl 2-acetoamido-2-deoxy α-D-galactopyranoside which prevent the formation of N and O-linked glycosylations, respectively. The data suggested that soluble 92kDa proMMP-9 and cell associated 92 and 85kDa proMMP-9 forms were not extensively O-glycosylated. In presence of tunicamycin, however, soluble 92kDa proMMP-9 migrated at a lower molecular weight (87kDa). This form was also detectable in cell extracts of tunicamycin treated THP-1 cells. More work, however, is required to determine whether tunicamycin also affected the formation of the 85kDa form of proMMP-9. Completion of this work appears to be important since it might also shed some light on the pathway leading to the formation of 85 and 92kDa cell associated MMP-9 and finally on their biological activities. In view of the increasing evidence showing the existence of cell associated but not activated MMP-9 it is possible that proteolysis might not be the only activity of MMP-9. In this respect the fibronectin-, collagen- and hemopexin-like domains might be of particular importance (Fig. 1.2). Although no function of the collagen-like domain has been reported to date, the fibronectin-like domain was shown to bind to native and denatured type I collagen, denatured type IV and V collagens and elastin (113-115) and the hemopexin-like domain to TIMP-1, pro- and active MMP-1 and MMP-9 (117, 118). Therefore, the role of MMP-9 might also include localisation of other proteins such as MMP-1 to components of the extracellular matrix.

7. Summary and future plans

The aim of this thesis was to contribute to the understanding of the role of MMPs in human ovarian cancer. Gene expression of a number of MMPs and TIMPs was analysed in tissue samples of normal, benign and malignant ovaries and compared to that detected in ovarian cancer cell lines and xenograft models of human ovarian cancer. The main result was that MMPs and TIMPs could be classified according to their frequency of expression (always, variably or rarely expressed). Resulting from this work and also from work previously done in the laboratory, the regulation of MMP-9 was analysed in an *in vitro* system of tumour stroma interaction. For this purpose a method (zymography) for the detection and quantitation of MMP-9 was optimised and validated. Then the interactions of the ovarian cancer cell line PEO1 and the monocytic cell line THP-1 were analysed with respect to MMP-9 production. A soluble, tumour derived factor (MMPSF) induced monocytic proMMP-9 production in synergistic action with auto- or paracrine TNF-α. This led to investigations on the nature of MMPSF present in tumour cell derived conditioned medium (CM). The results showed that CM contained at least three factors which were able to induce proMMP-9 production from THP-1 cells. The major problem preventing full characterisation of MMPSF was found to be contaminating BSA present in CM and a protocol for its reduction was proposed. Finally, as only proMMP-9 was detectable in supernatant of stimulated THP-1 cells, cell extracts were analysed for the existence of cell associated forms of MMP-9 and these characterised with respect to their status of activation. Two forms of MMP-9 with a distinct molecular weight of 92 and 85kDa were localised to LPS stimulated THP-1 cells. Both contained the fully intact N-terminal pro-peptide, hence were proteolytically inactive pro-forms.

Resulting from this work several lines of investigation could be pursued. The main interest should certainly be the identification of MMPSF. The experience gained during the work done so far (chapter 5) would certainly be valuable for a further attempt to characterise this protein. In addition, the essential tools are easily accessible. This includes the conditioned medium, endotoxin free reagents, an enclosed FPLC/HPLC system and a well established bioassay for the detection of the MMP-9 inducing

activity. In addition, it seems that a main obstacle in the purification of MMPSF, a strong contamination of CM with BSA, was identified and a protocol to avoid this problem was suggested. The continuation of this line of work seems particularly promising since the observation that MMPSF induced proMMP-9 production in a synergistic fashion with autocrine/paracrine TNF-α has not been reported yet for any known inducer of MMP-9 gene expression. Finally, the identification of MMPSF and its interaction with TNF-α might also help in understanding the role of TNF-α in the tumour microenvironment. Previous studies from the laboratory implicated endogenous TNF-α in stromal development and cell invasion human ovarian cancer [e.g. (170, 220)]. Promising results have been obtained in trials using TNF-α neutralising strategies in inflammatory disease [for review (221)]. Taken together these data may provide a rational for the extension of anti-TNF-α approaches to malignant disease.

Another interesting line of work which should be continued is the further characterisation of the cell associated 92 and 85kDa MMP-9 forms. It seems particularly important to define their biochemical differences. In particular the options of a C-terminal modification and/or a variation in the degree of glycosylation should be investigated. The former could be resolved by western blotting of cell associated MMP-9 using a commercially available monoclonal antibody to the C-terminus of MMP-9 and the latter through application of glycosylations inhibitors such as tunicamycin to prevent N-linked glycosylations and Benzyl 2-acetoamido-2-deoxy α-D-galactopyranoside (Benzyl-GalNAc) a O-linked glycosylation inhibitor and analysis by western blotting and/or zymography. These experiments should also throw some light on how the 85kDa form of MMP-9 is produced. With these results in hand it should then be clearer whether this form was, as suggested by Toth *et al.* (91), a side product of proMMP-9 synthesis, a specifically modified form of the pro-enzyme or even a degradation product of proMMP-9. This work might also help to understand MMP-9's bioactivity. It seems that proteolysis might not be the only function of MMP-9. To our knowledge there has been no convincing report of *in vivo* data showing activated MMP-9 and considering the presented results a difference in molecular weight differences seems no longer to be sufficient to claim the detection of activated MMP-9.

From the more general perspective on MMPs and TIMPs taken in chapter 2 of this thesis, another series of questions might be asked. One observation made in this chapter was that the genes of the tissue inhibitors of metalloproteases TIMP-1, -2 and -3 were always expressed. This was particularly interesting since the data were consistent with published results detecting TIMP-1 and -2 protein by reverse zymography in all normal and malignant biopsies samples of the human ovary (222) and raised the question whether correlation studies exclusively analysing MMPs provide meaningful results. Most of these studies were based on zymography which permitted detection of MMP-2 and MMP-9 and the results were correlated to stage and tumour grade (39-41). Zymography, however, revealed total potential activity of MMP-2 and -9 since the proteases are stripped of their inhibitor (chapter 3, (180)). Similarly, MMP-1 levels were correlated to poor prognosis by immunohistochemistry without investigating TIMPs (223). It seems therefore that an assay which does not prevent the interaction of MMPs and TIMPs might reflect better the net proteolytic activity in the tissue. *In vitro* degradation of, for example, radiolabelled collagen type I or IV, could give a better reflection of the *in vivo* situation with respect to proteolytic activity. Preliminary results obtained in the laboratory using this method suggested that there was little to none MMP related proteolytic activity detectable in protein extracts of ovarian cancer biopsies. This suggests that local activation of MMPs and ECM degradation, for example at the cell surface of invadopodia might be the critical event (217, 224-226). Whilst techniques based on tissue extracts such as RT-PCR, zymography and *in vitro* degradation of collagens give a global view on the enzymes/inhibitors present in a tissue, discrete changes localised to small fractions of the cell surface of a single cell may ultimately control matrix remodelling and invasion.

8. List of publications

1. Leber TM. and Balkwill FR. "Zymography: A single-step staining method for quantitation of proteolytic activity on substrate gels". Analytical Biochemistry (1997), *249*, 24-28 (reprint enclosed).

2. Leber TM. and Balkwill FR. "Regulation of monocyte MMP-9 production by TNF-α and a tumour derived soluble factor (MMPSF)". Accepted for publication in the British Journal of Cancer on the 5th of March 1998 .

3. Leber TM., Boyd R. and Balkwill FR. "Tumour cell-stromal cell interactions: proteases and protease inhibitors". Chapman & Hall Medical (London, UK), Ovarian Cancer 4, (in press).

4. Leber TM. and Negus RPM. "Detection and quantitation of matrix metalloprotease mRNA and protein". Humana Press (Totowa, NJ, USA), Methods in Molecular Medicine: Ovarian Cancer, (submitted).

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ANALYTICAL BIOCHEMISTRY 249, 24–28 (1997)
ARTICLE NO. AB972170

Zymography: A Single-Step Staining Method for Quantitation of Proteolytic Activity on Substrate Gels[1]

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Received January 27, 1997

Zymography is an electrophoretic method for measuring proteolytic activity. The method is based on an sodium dodecyl sulfate gel impregnated with a protein substrate which is degraded by the proteases resolved during the incubation period. Coomassie blue staining of the gel reveals sites of proteolysis as white bands on a dark blue background. Within a certain range the band intensity can be related linearly to the amount of protease loaded. Although the method is widely used, crucial points concerning quantitation of proteolytic activity have not been rigorously addressed. In this report we describe a new staining protocol which converts the independent staining and destaining procedure into a single step. This leads to fast and reproducible staining of zymograms permitting reliable quantitation of proteolytic activity. As shown for proMMP-9 (type IV gelatinase-b) proteolytic activity can be quantitated in a linear manner in as little as 1 h of zymogram staining. We established the detection limit for proMMP-9 (32 pg), the linear range (below 1000 pg), and the reproducibility of the assay (coefficient of variation <15%). This improved protocol is fast and reproducible. Its linear range of almost two log scales permits assay of proteolytic activity in a wider range than current methods.

Zymography is a simple, sensitive, quantifiable, and functional assay for analyzing proteolytic activity. It is already widely used for research on extracellular matrix degrading enzymes, in particular the matrix metalloproteases (MMPs)[3] (1).

The standard method is based on SDS gels impregnated with a protein substrate, in particular gelatin or casein. Proteases, which have the ability to renature and exert proteolytic activity on a copolymerized substrate upon removal of the SDS, can be analyzed by this method (2). MMP-2 and MMP-9 can be detected on gelatin zymograms and MMP-7 on casein gels (2, 3). Copolymerization of plasminogen together with gelatin allows detection of the plasminogen activators uPA and tPA (4). Fluorescent or radioactively labeled substrates and overlay techniques have also been used (5).

Zymography offers several features which render it particularly useful with respect to alternative methods such as ELISA: No expensive materials are routinely required (e.g., antibodies) and several proteases showing activity on the same substrate can be detected and quantified on a single gel. MMPs are released from cells in a proteolytically inactive pro-form (zymogen) which is about 10 kDa larger than the activated form. Since the pro-form becomes activated during the process of renaturation after gel electrophoresis, both forms can be detected on zymograms (1, 6). In addition, MMPs in solution are often associated with their corresponding tissue inhibitors of metalloproteases (TIMPs). During electrophoresis the inhibitors dissociate from the MMP and do not interfere with detection of the enzymatic activity (1). Total proteolytic activity will therefore be determined. Finally, zymography has been shown to be extremely sensitive. Levels of less than 10 pg of MMP-2 have been detected on gelatin zymograms comparing favorably with ELISAs (1).

As shown previously, proteolytic activity on zymograms can be quantified by computer-supported densitometry scanning and image analysis (1). In a certain range of the assay a linear relationship exists between the amount of enzyme loaded and the activity detected (1, 2). However, quantitation of zymograms remains difficult because (i) the limited number of wells per gel does not allow a full standard curve and several samples to be run on the same gel and (ii) the two-step staining/destaining method is not reliable and is difficult to reproduce. We have shown that the staining/destaining step in particular is crucial for accurate

[1] This project was supported by a grant of the European Union (Grant BIO4-CT96-5050).
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[3] Abbreviations used: MMP, matrix metalloproteases; ELISA, enzyme-linked immunosorbent assay; CV, coefficient of variation.

0003-2697/97 $25.00

measurements of proteolytic activity. Traditionally gels are stained in a 0.5% (w/v) Coomassie blue solution for 3 h (1, 2). This is followed by a destaining step of undefined length, usually several hours, until a satisfactory background/band staining is achieved. There are, however, major drawbacks to these undefined protocols. Overstaining of the gels reduces the assay sensitivity because bands of low activity will become undetectable. Excess destaining can also "bleach" the bands so that their intensity cannot be assessed in the linear range of the assay.

In this report we present an improved single-step staining/destaining protocol which leads to reproducible staining of gels for quantitation. Using this method we established the linear range, the reproducibility, and the sensitivity of the assay for proMMP-9 on standard gels (140 × 130 × 1 mm). We conclude that a single-point standard is sufficient for the determination of proteolytic activity.

MATERIALS AND METHODS

Materials required for gel preparation were purchased from National Diagnostics: AccuGel 29:1 (30% acrylamide, 29:1 acrylamide:bis-acrylamide), ProtoGel stacking gel buffer, and ProtoGel running gel buffer. Chemicals for the running buffer [25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS], gelatin (Porcine skin type I, bloom 300), ammonium persulfate, TEMED, Triton X-100, and Brij-35 were obtained from Sigma. Highly purified human proMMP-9 was purchased from TCS-Biologicals. The slab gel apparatus was from Genetic Research.

Preparation and running of gels. Zymogram gels, loading buffer [50 mM Tris–HCl (pH 6.8), 10% (v/v) glycerol, 1% (w/v) SDS, 0.01% (w/v) bromophenol blue], and collagenase buffer [50 mM Tris–HCl (pH 7.6), 0.2M NaCl, 5 mM $CaCl_2$, 0.2% (v/v) Brij-35] were prepared as described elsewhere (7). Briefly, 11% running gels (100 × 130 × 1 mm) containing 0.12 mg/ml gelatin were overlaid with a 4% stacking gel (12 wells, 40 × 130 × 1 mm), and samples were loaded and run at 180 V until the dye front ran off the gel. Gels were removed from glass plates and soaked for 1 h in 2.5% Triton X-100 on a shaker followed by two brief washes in collagenase buffer in which the gels were thereafter incubated for 18 h at 37°C.

Staining of gels. Prior to staining, gels were briefly rinsed in distilled water. PhastBlue tablets were purchased from Pharmacia and a 0.2% stain stock solution was prepared according to the manufacturer's instructions. Prior to storage at 4°C, stain stock solution was filtered through No. 54 Whatman filter paper to remove undissolved stain particles. A stain working solution (200 ml per gel) was prepared by mixing 10 ml of stain stock solution with 200 ml of destain (1:3:6 glacial acetic acid:methanol:distilled water) and gels were stained for the periods of time indicated on a shaker at room temperature. After completion of staining, gels were briefly rinsed in distilled water and carefully wrapped in Saran wrap.

Scanning of gels and quantitation of bands. Gels were scanned using a Umax (MagicScan) flatbed scanner driven by the NIH Image 1.58 image analysis program on a Macintosh computer. This program, which can be obtained through the National Institutes of Health, was also used for quantitation of the bands. Gels were scanned in gray scale (mode: transmissive; gamma = 1; shadow = 0; range = 1–255). The density of each pixel is encoded on a scale ranging from 1 (clear) to 255 (opaque). The resolution was set to 150 dots per inch. Pilot experiments showed that this resolution yields satisfactory results without leading to excessively large files. Further, rescanning of the same gel did not affect the outcome of the quantitation procedure, which is consistent with observations made by others (1). Determination of the band intensity was done as described in the handbook of NIH Image 1.58. Briefly, the scanner was calibrated using the step tablet No. 2 (Kodak) and the density measurements were linearized using the Rodbard curve-fitting function within the NIH Image 1.58 program. The results were stored in the form of a file for later use. Calibration of the scanner proved to be important since there was no linear relationship between the defined optical density of the step tablet and the measurements taken by the flatbed scanner. These findings were reproduced on a different flatbed scanner (Agfa, Arcus II).

To assess a zymogram, the file containing the scan of a gel, the calibration file, and the "gel-plotting macro" were loaded. Using the rectangular selection tool, an area consisting of the band and sufficient (at least 2 cm) background above and below the band was selected. Using the commands offered by the gel-plotting macro, the pixel intensity in each lane was plotted. The default setting of Image 1.58 was modified to obtain plots on which highest densities (opaque) were represented on the bottom end of the y-axis. The area under the curve was closed using the line tool by joining the horizontal background line before and after the peak. The area under the curve was evaluated by applying the wand tool on the enclosed area to determine the integrated band intensity.

Preparation of proMMP-9. Purified human proMMP-9 (TCS Biologicals) was diluted to 1000 pg/ml in 60 mM Tris–HCl (pH 7.2), 15 mM $CaCl_2$, 80 mM $NaCl_2$, and 0.1% bovine serum albumin (BSA) and stored at −20°C. For zymograms twofold dilutions were prepared in the same buffer and 8 μl was loaded onto a gel.

RESULTS

Time course of zymogram staining. Zymogram gels were poured as described under Materials and Meth-

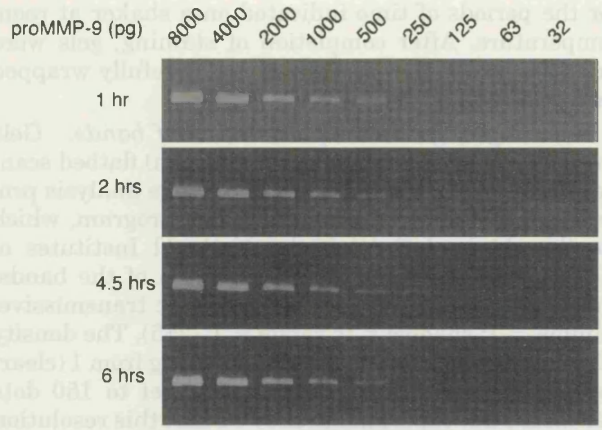

FIG. 1. Time course of zymogram staining. Purified human proMMP-9 was diluted in doubling dilutions and loaded in the range between 8000 and 32 pg/well of protein. Gels were run as described under Materials and Methods and stained for the times indicated. A darkening of the background staining over time can be seen.

ods, and samples of proMMP-9 were prepared by two-fold dilutions from a stock solution (1000 pg/μl) and loaded in the range from 32 to 8000 pg/well. Gels were scanned after 1, 2, 4.5, and 6 h of incubation in the staining solution. Figure 1 shows a typical zymogram at the four time points. A darkening of the background staining over time can be seen. It should be noted that bands at the lower end of the dilution curve are barely visible. Although they can be detected by the scanner, they do not photograph well. This finding is consistent with those made by others (1). The integrated area measured for each amount of proMMP-9 per time point is shown in Fig. 2A. For a particular amount of proMMP-9 the area increases with time. This is due to the darkening of the background staining. However, this effect is diminished after between 4.5 and 6 h of staining, indicating that a point of saturation of background staining is approached. Expression of the integrated area for an individual amount of protein as percentage value of that of the highest amount of proMMP-9 loaded (8000 pg = 100%) shows that the increase in background staining over time (Figs. 1 and 2A) does not alter the relative band intensities within a dilution series (Fig. 2B). Both Figs. 2A and 2B indicate that a linear relationship exists between the amount of protein loaded and the band intensity in the range below 1000 pg of proMMP-9. This range is shown in Fig. 2C and indeed confirms that they are all linear with regression coefficients of 0.99 for all four time points of staining.

Intergel reproducibility of linear range and detection limit. The time course experiment of zymogram staining was done three times and the observations made were found to be consistent with those shown in Figs. 1 and 2A–2C. The areas measured for the range from

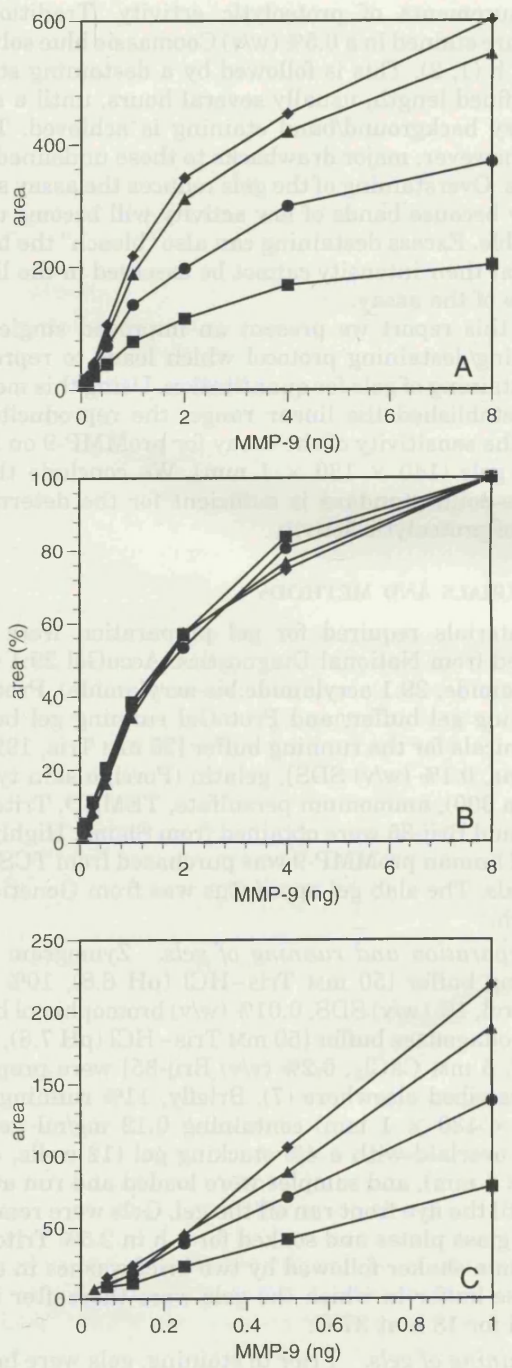

FIG. 2. Quantitation of the time course of zymogram staining (Fig. 1). (A) shows the area determined for the complete range of proMMP-9 loaded at all four time points of quantitation [1 (■), 2 (●), 4.5 (▲), and 6 (◆) h]. The value determined for a particular amount of protein increases with increasing staining time. This effect is diminished between time point 4.5 and 6 h. In (B) the individual values are expressed as percentage values of that found for 8 ng of proMMP-9. All periods of zymogram staining lead to the same curve. A linear range between the area measured and the amount of proMMP-9 loaded exists in the range of 1 ng proMMP-9 and below. This range is shown in (C).

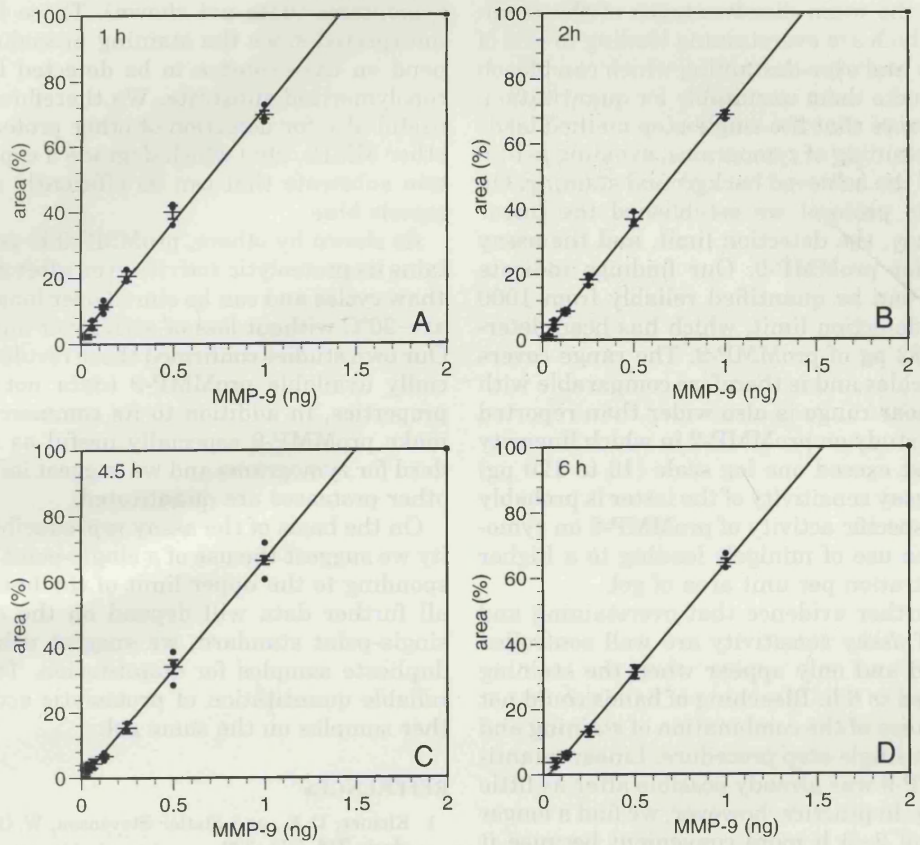

FIG. 3. Intergel reproducibility of the linear range for proMMP-9. The values shown are expressed as percentage values of that found for 2 ng of proMMP-9. For all four time points (1, 2, 4.5, and 6 h) linearity between the amount of protein loaded and the area measured exists in the range of 1 ng proMMP-9 and below. (●, individual data; —, averages; error bars, standard deviations).

32 to 2000 pg proMMP-9 expressed as percentage areas of that found for 2000 pg proMMP-9 (=100%) are shown in Figs. 3A–3D. For all four time points there is a linear relationship between the amount of protein loaded and the area measured in the range of 1000 pg proMMP-9 and below. Detection of the lowest amount of MMP-9 loaded (32 pg) was possible in two of three experiments for incubation periods of 1 and 2 h and in three of three experiments for a staining period of 4.5 h, and failed in all three cases for a staining period of 6 h. This indicates that the detection limit of the assay is at approximately 32 pg of proMMP-9.

Intragel variation of proMMP-9 quantitation. Four gels were poured from a single stock solution and on each gel eight times the same amount of proMMP-9 was loaded (1000, 500, 125, and 62.5 pg). The gels were stained as described under Materials and Methods for 3 h. Data and statistics are shown in Table 1. For all four amounts of protein, the coefficient of variation (CV) remains below 15% of the mean value, indicating a good intraassay reproducibility.

DISCUSSION

In this report we present an improved staining protocol merging the traditionally separated staining and destaining steps of zymograms into a single procedure.

TABLE 1

Intragel Reproducibility of the Linear Range for proMMP-9

	ProMMP-9			
	62.5	125	500	1000
n	8	8	8	8
Mean (area)	6.43	15.96	53.38	133.36
stdev (area)	0.74	1.69	7.97	8.62
CV (%)	11.58	10.60	14.94	6.46

Note. ProMMP-9 was diluted in doubling dilutions and each of the amounts was loaded eight times on a single gel. The zymograms were run as described under Materials and Methods and the gels stained for 3 h. For all four amounts of protein the coefficient of variation (CV) remains below 15% of the mean value.

This overcomes the main disadvantages of the traditional method which are overstaining leading to loss of assay sensitivity and over-destaining which can bleach the bands and make them unsuitable for quantitation. We provide evidence that the single-step method leads to reproducible staining of zymograms, avoiding gel-to-gel judgments of the achieved background staining. On the basis of this protocol we established the linear range of the assay, the detection limit, and the assay reproducibility for proMMP-9. Our findings indicate that proMMP-9 can be quantified reliably from 1000 pg down to the detection limit, which has been determined to be at 32 pg of proMMP-9. The range covers almost two log scales and is therefore comparable with ELISAs. The linear range is also wider than reported in a comparable study on proMMP-2 in which linearity was found to just exceed one log scale (10 to 120 pg) (1); the higher assay sensitivity of the latter is probably due to a higher specific activity of proMMP-2 on zymograms and to the use of minigels leading to a higher protease concentration per unit area of gel.

We provide further evidence that overstaining and therefore loss of assay sensitivity are well controlled with our method and only appear when the staining period is extended to 6 h. Bleaching of bands could not be observed because of the combination of staining and destaining into a single-step procedure. Linear quantitation of proMMP-9 was already possible after as little as 1 h of staining. In practice, however, we find a longer staining period of 2–3 h more convenient because it leads to more intensive background staining. Nevertheless, the procedure remains faster than other protocols reported and requires no additional handling of the gel.

In terms of reproducibility we were able to show that inter- and intragel reproducibilities are satisfactory. The linear range was maintained over several independently prepared zymograms. Experiments performed to investigate the intragel reproducibility showed that for the whole range of linearity, the CV remains below 15% of the mean value.

This staining protocol was also useful for detection of MMP-2 on gelatin zymograms and MMP-7 on casein zymograms (data not shown). These findings are not unexpected since the staining procedure does not depend on the protease to be detected but only on the copolymerized substrate. We therefore expect it to be useful also for detection of other proteases (uPA, tPA, other MMPs, etc.) which degrade a copolymerized protein substrate that can be efficiently stained by Coomassie blue.

As shown by others, proMMP-9 is very stable. It retains its proteolytic activity even after repeated freeze–thaw cycles and can be stored over long periods of time at −20°C without loss of activity or autoactivation (8). Our own studies confirmed these results using commercially available proMMP-9 (data not shown). These properties, in addition to its commercial availability, make proMMP-9 especially useful as a general standard for zymograms and we suggest its use, even when other proteases are quantitated.

On the basis of the assay reproducibility and linearity we suggest the use of a single-point standard corresponding to the upper limit of the linear range. Since all further data will depend on the outcome of this single-point standard, we suggest using the mean of duplicate samples for quantitation. This will allow a reliable quantitation of proteolytic activity in 10 further samples on the same gel.

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