THE INNERVATION AND ULTRASTRUCTURE OF BLOOD VESSELS SUPPLYING BENIGN AND MALIGNANT COLORECTAL TUMOURS

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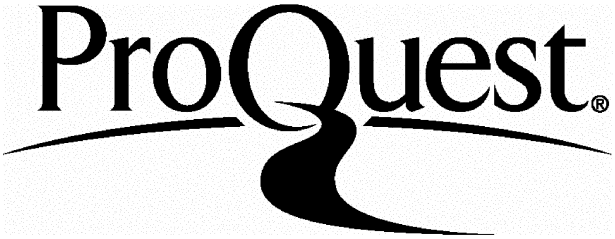

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Dedicated to my family,

in recognition of their support and encouragement.

CONTENTS OF THESIS

LIST OF TABLES AND FIGURES

Fig.2. Immunoreactivity to substance P and vasoactive intestinal polypeptide in nerve bundles and mucosa of controls and polyps.

Fig 3. Immunoreactivity to VIP in nerves of submucosa and mucosa of benign polyps.

Chapter 5, figures and legends: p. 140-148.

Fig 1A. Submucosal arteriole of the normal submucosa within 3 mm of colorectal cancer edge

Fig 1B. Submucosal arteriole of the normal submucosa at least 5 cms. from colorectal cancer

Fig 2. Ultrastructure of arteriolar smooth muscle cell within colorectal cancer (longitudinal section)

Fig 3A. Ultrastructure of an arteriole in normal tissue at 100 microns from the edge of a colorectal cancer

Fig 3B. Ultrastructure of an arteriole in colorectal cancer, within 100 microns from its edge.

Fig 4. Ultrastructure of arterioles in a benign colorectal polyp.

Fig 5. Smooth muscle structure in arterioles of polyps.

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Abstract

The regulation of vascular tone in vessels supplying colorectal tumours has important therapeutic implications. This study of the blood supply to benign and malignant colorectal tumours focuses on the neural and smooth muscle elements that control vascular calibre.

Particular emphasis was placed on the study of submucosal arterioles because of their crucial role in supplying colorectal tumours. They are the feeder vessels to the tumours and are the last vessels of resistance in the mesenteric circulation of the colon.

Innervation was studied by means of markers of neurotransmitter and vasoactive substances: neuropeptide Y (NPY), tyrosine hydroxylase (TH), vasoactive intestinal peptide (VIP), substance P (SP), calcitonin gene-related peptide (CGRP), and the general neuronal marker protein gene product 9.5 (PGP 9.5). The ultrastructure of blood vessels was determined by electron microscopy.

The study confirmed the absence of perivascular nerves in colorectal cancer and showed for the first time a decrease in perivascular innervation in the submucosa adjacent to cancers ($p<0.002$ for TH and NPY and $p<0.015$ for VIP).

Extrinsic nerve loss appeared greater with cancer of advanced Dukes' stages.

Perivascular nerve immunoreactivity around arterioles supplying benign tumours differed from controls. There was a decrease in sympathetic neural immunoreactivity (TH, NPY) and an increase in parasympathetic (VIP) and sensory neural immunoreactivity (CGRP) in the submucosa of polyps compared to controls. SP immunoreactivity did not differ significantly from controls.

Whereas at electron microscopy smooth muscle cells in blood vessels of normal mucosa and submucosa showed a contractile phenotype, in cancers the vascular smooth muscles were mainly of a secretory phenotype. In polyps, vascular smooth muscles showed a change towards a secretory phenotype.

Benign and malignant colorectal tumours appear to induce changes in the innervation and the expression of smooth muscle phenotypes in blood vessels within and adjacent to the tumours. Perivascular nerve changes may result from release of tumour factors from the tumour cells.

The differential expression of neurotransmitters and cell phenotypes in colorectal tumours could be of value as markers of disease and as pointers to targets for therapy.

Chapter 1: Introdution

1.0 General introduction & Outline of thesis

1.1 Microvascular architecture of the colon.

1.2 Control of normal blood vessels.

1.3 Innervation of microvasculature and mucosa.

1.4 Ultrastructure of blood vessels.

1.5 Nerve loss in diseases.

1.6 Consequences of nerve loss.

1.7 Colorectal neoplasia.

CHAPTER 1. INTRODUCTION

1.0 General Introduction.

Therapy aimed at altering tumour vasculature is based on exploiting differences between normal and tumour. Differences exist in the number of vessels present, their structure, and their response to vasoactive substances (Bouck et al. 1996, Mattsson et al. 1981, 1982).

Two types of vessels are associated with tumours: pre-existing vessels present in the tissue adjacent to the tumour providing blood to the tumour, and new vessels (neovasculature) that are usually present in the tumour mass itself (Jain 1988). Therapy aimed at new vessels (anti-angiogenic) prevents the expansion of the vascular network, hence the expansion of the tumour and the establishment of metastases. On the other hand the pre-existing vasculature can be modified to halt tumour progression (vascular targeting) (Denekamp 1993).

The regulation of tumour blood vessels differs from the normal. Perivascular nerves, which in the normal regulates vessel tone, are absent in the neovasculature of solid tumours, including both primary and metastatic colorectal malignancy (Mitchell et al. 1994a), (Ashraf et al. 1996). The endothelium and the type of smooth muscles, both determinants of vasomotor action in the

normal, show variation in tumours (Buttery et al. 1993, Andrade et al. 1992, Ashraf et al. 1997).

Previous studies of the vasculature of tumours have centred mainly on the vessels within the tumour mass. These intra tumoral vessels respond poorly to vasoactive agents and are usually maximally dilated (Mattsson et al. 1982). It has been shown however that blood vessels in tissue near tumours can respond to vasoactive agents. Experiments in a rat model carried out in a transparent chamber have demonstrated that pre-existing arterioles supplying implanted neoplasms respond to vasoactive agents (Hori et al. 1993). In human tumours, feeder vessels respond differently from those within the tumours and from the normal vasculature (Young et al. 1979).

Little is known about the factors that control these pre-existing vessels. They have been shown to provide the blood supply to the neovasculature in both experimental and human colorectal tumours (Skinner et al. 1990, 1995).

In our light microscopy study we therefore focused on the perivascular innervation of the arterioles in the submucosa adjacent to colorectal tumours, benign and malignant. The study next examined the ultrastructure of the blood vessels supplying colonic epithelial tissues ranging from normal to malignant tissue through their precursor lesions.

The study was performed to enable comparison of normal tissues with a range of neoplastic tissues. In colorectal

neoplasia the sequence of progression from dysplasia to cancer is well-established (Fearon and Vogelstein 1990).

In studying the structure of blood vessels in relation to the neural and other elements known to affect vascular tone, hence the blood supply, we aimed to shed light on some of the factors that affect blood flow to neoplastic tissues.

The presence, degree of innervation and types of nerves of blood vessels were studied with the use of a general neuronal marker and specific markers of neurotransmitter substances. The ultrastructure of the vessels was determined by electron microscopic study.

Outline of thesis.

In the introduction chapter, I review the current knowledge on the architecture of the blood vessels within the colonic wall both in the normal and in colorectal tumours (1.1), and discuss the factors regulating blood vessels in the normal (1.2). The innervation of the microvasculature and of the mucosa of the colon is reviewed. I focus on the perivascular nerves of the submucosal arterioles and discuss the innervation of the structures within the mucosa where the closely related mucosal cells are richly vascularised (1.3). I study the ultrastructural features of blood vessels with special reference to the smooth muscle elements, in normal and in neoplasia of the colon (1.4). I review the history of

perivascular nerve anomalies in tumours and other conditions (1.5). The effects of nerve loss on vessel ultrastructure are next discussed (1.6). The adenoma-carcinoma sequence, the steps and stages in the progression of colorectal tumours, their natural history, prognosis and treatment are reviewed (1.7).

In chapter 2 the methods used are described. Chapters 3, 4, 5 constitute the results section. In chapter 3, I focus on the perivascular innervation of submucosal vessels supplying colorectal cancers, and in chapter 4 that of their premalignant precursors mainly adenomatous polyps compared to controls.

In chapter 5, the results of the study of the ultrastructure of blood vessels in colorectal tumours are described with emphasis on the smooth elements of arterioles and their phenotypes in normal tissue and in colorectal tumours.

Chapter 6 is the general discussion. I discuss the main issues raised by the study and the possible future applications of my findings.

1.1 Microvascular architecture of normal colon and of neoplastic tissue

Microvascular architecture of normal colon

The human colonic wall is supplied by arterioles derived from mesenteric arteries. The arteries divide at the mesenteric border of the colon to join an arterial arcade that follows the mesenteric border. This mesenteric arcade gives rise to small arterioles (250-400 micrometers in diameter) that pass towards the colon.

Some of these small arterioles pass straight through the bowel wall to the submucosa, whilst others course from the mesenteric border to the antimesenteric border in a subserosal position. The latter then penetrate the bowel wall, coursing like their counterparts from serosa to submucosa (figure 1A). The arterioles supply the distributional networks of the subserosa, and of the muscular and mucosal layers (Wolfram-Gabel et al. 1986).

From their submucosal position the final network of arterioles provide the blood supply to the mucosal layer, dividing at the level of the muscularis mucosa whence they link to capillary loops in the lamina propria (figure 1A). Capillaries in the mucosa form a honeycombe-like appearance around each of the mucosal glands. Such an arrangement is established in various species including the rat and guinea pig (Aharinejad et al. 1992). In

humans a similar arrangement to the rat was shown to be present in a comparative study (Skinner et al. 1996).

Microvascular architecture of colon tumour

Electron microscope studies of resin casts of the microvasculature within primary colon tumours in rats have shown the following features. Arterioles do not pass beyond the submucosa, branching at the mucosa-submucosa junction to supply capillary networks. In tumours two distinct vascular regions are found, a central region of large vessels continuous with vessels of the submucosa and muscularis propria of the adjacent normal colon and a region of nutrient vessels continuous with the mucosal layer of the adjoining normal colon. There is no difference in vasculature architecture between benign polyps and small polypoid carcinomas, but large differentiated adenocarcinomas lacked a central pedicle of large diameter vessels (Skinner et al. 1990).

Further studies using human neoplastic tissue confirm that the general architecture of vessels supplying tumours has a similar pattern to normal colon and the arrangement is similar to the experimental model (Skinner et al. 1995). It appears therefore that, as in the experimental tumours, there are two distinct phases of vasculature development in colonic tumours: a phase when pre-existing vessels supply the tumour, and a phase of proliferation of new vessels. The latter are linked at all stages of their development to the pre-existing vessels.

Within the tumours themselves progressive changes in architectural arrangement occur. Microvascular corrosion casting combined with scanning electron microscopy of the colon showed that the regular hexagonal arrangement of capillaries is lost in transitional mucosa near malignant tumours, and the vessels have a slightly larger diameter. In adenomas above 3 mm the density of microvessels increased in the space between tumour cells, whilst within carcinomas the much increased density of vessels within the tumour resulted in clusters of capillaries with a tortuous course and with a mean diameter larger than normal (Skinner et al. 1995).

In the peripheral part of malignant tumours the vessels are disorganised and of varied morphology with claw like formations, widened sinuses, diverticula and appendixoid patterns. In the central areas of the malignant tumours there are no microvessels (Sun et al. 1992). This may be the result of necrosis at the centre of the tumour.

Blood flow through the tumour circulation was found to be increased in laser Doppler flow studies of rectal carcinoma (Zografos et al. 1990). The flow of blood through tumour circulation is the result of several features of the tumour neo-circulation, which include an increase in the number of vessels (mean vessel density), in the mean volume density and in the mean transverse

cross sectional area (Skinner et al. 1995, Tipoe and White 1995).

Histological and morphometric studies of density of vessels in colorectal tumours have shown an increase in mean vessel density of tumours compared to normal mucosa. The mean vessel density (MVD) within benign tumours is intermediate between normal mucosa and malignant tumours. These differences in colorectal tumours did not reach statistical significance (Tipoe and White 1995).

In another study of rectal carcinoma significantly higher angiogenic scores were seen in tumours with transmural penetration and higher scores were found in larger tumours (Saclarides et al. 1994). Other workers noted differences in vessel density between different colorectal cancers, but a low variability in vessel density within individual tumours (Vermeulen et al. 1995). In moderately differentiated carcinomas of the colon the mean value for surface density was only X 1.4 higher than the mean value for normal mucosa, suggesting a weak angiogenic response to these tumours. Surrounding host tissues did not show an increase in vascular density close to colorectal tumours (Pritchard et al. 1995).

1.2 Control of blood vessels in normal

Control of vascular calibre in normal

The control of vascular calibre in all organs appears to be dependent on both extrinsic and local influences. Extrinsic influences on blood vessels are the perivascular nerves and circulating hormones. Local factors released from active tissue usually result in vasodilatation. Smooth muscle cells respond to vasoactive substances released not only by perivascular nerves but also by the endothelial cells of the intimal layer of blood vessels (Burnstock and Ralevic 1994).

Sympathetic nerves

The majority of noradrenergic cell bodies that give rise to sympathetic nerve endings in the gut wall are in the prevertebral sympathetic ganglia. A few groups of cells exist scattered along the mesenteric nerves beyond the prevertebral ganglia in the human intestine (Furness and Costa 1987 p.208).

A dense plexus of noradrenergic axons is found forming a perivascular network around arteries that run through the mesentery to the intestine and around intramural arteries. The veins within the gut wall appear to be uninnervated by noradrenergic axons. Separate noradrenergic neurons supply separate tissue targets within the gut wall. Subpopulations of noradrenergic neurons that also contain neuropeptide Y supply intestinal

arterioles (Furness and Costa 1987 p 86, Nichols et al. 1994).

Parasympathetic nerves

The majority of parasympathetic postganglionic axons arise from intrinsic nerve cell bodies, which have been detected in both the myenteric and submucous plexus. It appears that there are no extrinsic vasodilator fibres supplying blood vessels of the gastrointestinal tract.

The main neurotransmitter in the parasympathetic postganglionic fibres is vasoactive intestinal polypeptide (VIP). VIP axons are associated with small blood vessels mostly in the submucosa and mucosa throughout the gastrointestinal tract, mainly in the small and large intestine (Ferri et al. 1988, Mazumdar and Das 1992). Coexistence of VIP and NOS in nerve terminal was detected around blood vessels in the gastrointestinal tract of mouse, rat, hamster, and guinea pig (Ekblad et al. 1988).

Sensorimotor nerves

Nerves containing Substance P and calcitonin gene-related peptide nerves are found around arterioles, and in the main have an extrinsic origin, being the peripheral endings of sensory neurons. Nerves containing these substances are numerous in the human intestine (Llewellyn-Smith et al. 1984, Timmermans et al. 1992).

1.3 Innervation of the microvasculature and mucosa of the colon in normal and tumours

Intrinsic and extrinsic nerves of the normal colon

The gut is innervated by intrinsic nerves which form interconnected networks with ganglion cells both in the submucosa termed Henle's intermediate and Meissner's plexuses, and between the circular and the longitudinal muscular layers (Auerbach's plexus). This system of neurons whose cell bodies are found within the walls of the gastrointestinal tract may collectively be defined as the enteric nervous system (Furness and Costa 1987 p.6).

Extrinsic nerves modulate the activity of the intrinsic innervation by parasympathetic stimulating nerves containing acetylcholine (Ach) and sympathetic inhibitory nerves containing noradrenaline (NA) and neuropeptide Y (NPY) and derived from abdominal plexuses.

In addition there are extrinsic sensory-motor nerves containing substance P (SP) and calcitonin gene-related peptide (CGRP) and derived from central nervous system neurones in cranial and spinal nerve nuclei. The nerves terminate in the bowel as tendril-like sensory endings. Their influence may be modulated by local factors particularly in the mucosa where mechanisms of autoregulatory escape exists to counteract the effects of neurogenic vasoconstriction (Furness and Costa 1987 p.220).

Paravascular and perivascular nerves

Two types of nerves are associated with arteries and arterioles of the gut: paravascular nerves that follow arteries, using the tract of arteries as a conduit, and perivascular nerves that lie in the adventitia of vessels.

Most of paravascular nerve fibres are destined to other structures including the enteric ganglia, intestinal smooth muscles and the mucosa. These nerves are like peripheral nerves elsewhere consisting of several fascicles, each of which is surrounded by a perineurial sheath. Within this the endoneurium contains myelinated fibres of various sizes. Large fascicles are divided by septa.

In addition a perivascular plexus, which forms a continuous network of anastomosing nerves consisting of both motor fibres and vascular sensory fibres, surrounds the arteries and arterioles (fig 1B). Veins also have perivascular nerves but these are almost restricted to the mesenteric veins; there are very few nerves in veins of the gut wall.

Perivascular nerves

Perivascular nerves travel a long distance along the arteries and arterioles in the adventitia where they run amid collagen fibres and small vessels including lymphatics and provide innervation to the vessel smooth

muscles. The nerves penetrate the adventitia and lie at the adventitia-media border, and in this situation influence vascular calibre by regulating the contraction of the smooth muscle cells. The nerve fibres do not penetrate the media as a rule and are never found in the intima. However nerve fibres are sometimes found close to the wall of capillaries.

Perivascular nerves are small bundles of axons, which are invariably unmyelinated and typically varicose. In the vascular adventitia these varicose areas are the terminal portions of axons and have a typical beaded appearance with expanded bulbous portion up to 1.5 micrometer in diameter and narrow intervaricose segments up to 0.2 micrometers in diameter (Ralevic and Burnstock 1996, p136). The intervaricose segments are occupied almost exclusively by a few microtubules.

Neurotransmitters

Neuroeffector transmission from perivascular nerves to vascular smooth muscles has until recently been considered to be solely secondary to noradrenaline (NA) in sympathetic and acetylcholine (Ach) in parasympathetic nerves. These agents act on receptors on smooth muscle cells after crossing the junctional cleft.

More recently nonadrenergic and noncholinergic (NANC) neurotransmitters have been identified. Some do not fulfil all the criteria for neurotransmitters and are termed putative transmitters. These NANC

neurotransmitters include vasoactive intestinal peptide (VIP), substance P (SP), neuropeptide Y (NPY), and calcitonin gene-related polypeptide (CGRP), all of which have been demonstrated in perivascular nerves (Lincoln and Burnstock 1990).

Thus the nerves to blood vessels contain not only cholinergic and adrenergic fibres but also peptidergic fibres. The origin of these perivascular nerves varies. Whilst peptide-containing nerves to the rest of the gut structures are mainly intrinsic (particularly VIP), perivascular nerves are largely extrinsic in origin. Evidence for this is derived from studies involving extrinsic denervation of the rat transverse colon (Ekblad et al. 1988) and small bowel transplantation in the piglet (Shen et al. 1993).

Nevertheless some VIP-containing nerves around blood vessels in the jejunum and SP-containing nerves around veins in the jejunum are intrinsic. Similarly in the rat rectum a large part of SP fibres seem to be intrinsic (Domoto et al. 1990, 1992), and in the rat colon some CGRP perivascular fibres are intrinsic (Ekblad et al. 1988).

Perivascular nerves of neoplastic tissue

In the normal the control of vascular tone in blood vessels is dependent on its perivascular nerves. Whilst these nerves are generally absent in the mass of primary and secondary tumours (Krylova 1969, Mattsson et al. 1977, Ashraf et al. 1996), perivascular innervation in the

tissue adjacent to tumours has been identified (Mattsson et al. 1977, Mitchell et al. 1994a). In the latter qualitative studies the greatest density of perivascular nerves was found in relation to arterioles as opposed to veins and capillaries.

In order for tumour to grow or establish itself as a metastasis, whether in the experimental situation of tumour implantation or in vivo, it must be able to establish a good blood supply. The mechanisms regulating the vessels of the tumour bed are therefore important for the development and growth of the tumour and may be a useful target for tumour therapy. The regulation of the vasculature of the tissue in the immediate vicinity of tumours however is largely unknown.

1.4 Ultrastructure of blood vessels in normal and neoplasia

Ultrastructure of arteries in normal colon

The wall of arteries consists of three coats: the intima, the innermost coat consists of the endothelium separated by a thin layer of loose connective tissue from the internal elastic lamina, a thick fenestrated cylinder of elastic fibres. The media consists of a tight spiral of smooth muscle cells, which lie, in a meshwork of elastic and collagen fibres. This stroma is scanty in the smallest arteries. An external elastic lamina separates the media from the adventitia, a thin layer of loosely arranged collagen and elastic fibres. The adventitia is rich in lymphatics and traversed by perivascular nerves, which supply the smooth muscles of the media.

The smallest arteries are resistance vessels and regulate blood flow by changes in their calibre. Smooth muscle cells are the predominant component of the media in these small arteries reflecting their function. The smooth muscle has a rich autonomic innervation, which together with circulating vasoactive hormones and factors produced by endothelial cells control the vessel calibre.

Ultrastructure of blood vessels in neoplastic tissue

When a solid tumour exceeds a few millimetres further growth becomes dependent on new vessel formation (Folkman and Cotran 1976, Fidler and Ellis, 1994). Angiogenesis, a process of sprouting of new blood vessels from pre-

existing host vessels, is induced by tumours through the production and release of multiple angiogenic factors (Folkman 1985). Angiogenesis occurs in benign as well as malignant tumours of the colon. Angiogenesis is most prominent within colorectal cancer tissue (Skinner et al. 1995, Bossi et al. 1995). By contrast host tissues close to colorectal cancer do not show a general increase in vascular density suggesting a weak angiogenic response in the adjacent tissue (Pritchard et al. 1995).

In adenomas, angiogenesis occurs in the later stages of development prior to the onset of malignancy. The microvessels of small adenomas have a similar ultrastructure to normal microvessels except that the endothelial cells are elongated (Wang and Campiche 1982, Skinner et al. 1995). Above 3 mm the density of microvessels increases in the space between tumour cells, whilst within carcinomas the much increased density of vessels within the tumour resulted in clusters of capillaries with a tortuous course and with a mean diameter larger than normal (Skinner et al. 1995).

Further studies have revealed changes in smooth muscle cells. Ultrastructural studies of tumour vasculature in liver metastases in an experimental model have revealed a lack of smooth muscle cells in blood vessels within the tumours (Ashraf et al. 1997). By contrast blood vessels are endowed with proliferative smooth muscle cells in human liver metastases (Ashraf et al. 1996).

Other structural changes have been described in the human liver; regression changes occur in the media of tumour vessels within hepatocellular carcinomas, and hypoplasia of smooth muscle in the media was a characteristic finding. To a lesser degree host arteries supplying hepatocellular carcinomas also showed regressional changes and were characterised by thinning of the media (Suzuki et al. 1987).

1.5 Nerve absence and loss in tumours and after denervation

Loss of perivascular nerves has been documented in several diseases and its effects studied following denervation. Nerve loss occurs in diabetes mellitus (Belai et al. 1988), and nerve absence is characteristic of blood vessels within malignant tumours.

Perivascular nerve loss in tumours

The absence of perivascular nerves within tumours was initially reported in experimental tumours in animal models, and innervation was found to be present but not quantified in host tissue (Krylova 1969, Mattsson et al. 1977). Since then the absence of nerves has been demonstrated both in experimental and clinical tumours, both benign and malignant.

Benign portwine tumours display a lack of perivascular autonomic nerves (Rosen and Smoller 1987). Malignant tumours arising from a number of tissue types, including breast, colon and liver have been shown to lack perivascular nerves in the main tumour mass (Mitchell et al. 1994a, 1994b). The absence of perivascular adrenergic nerves in human primary tumours (Mitchell et al. 1994a) and in metastases to the liver from colorectal cancer (Ashraf et al. 1996) was more recently identified. In none of these however has attention been focused on the tissue adjacent to the tumours.

The extent and effect of nerve loss in studies of denervation of the gut depends on the anatomy of the nerve supply. The length of intestine supplied by noradrenergic nerves following any one artery as it enters the gut wall is approximately coextensive with the area of supply of the artery and its branches. If the nerve following a particular artery is cut the noradrenergic terminals in the area of supply by its branches degenerate but there is little change in the noradrenergic innervation of adjacent areas (Furness and Costa 1987 p.211).

1.6 Effects of nerve loss

Effect of mesenteric nerve damage on blood flow.

Koch 1930 and Wright 1932 placed snares around the splanchnic nerves at operation and led out ligatures through small incisions. After the animals had recovered (1 hour for the rabbits by Koch, 0.5 to 7 hours for cats by Wright), the ligatures were pulled to break the nerves. Splanchnic vessel dilatation as a result of damage to extrinsic vasoconstrictor nerves led to a fall in systemic blood pressure of the order of 25-35% (Furness and Costa 1987, p.227).

In other experiments periarterial placement of Capsaicin induced an increase in intestinal blood flow. The increase in blood flow to the mucosa was higher than the increase in total blood flow. There do not appear to be any extrinsic vasodilator fibres innervating blood vessels of the gastrointestinal system directly. Intrinsic nerves can however be activated by enteric reflexes or by neuronal inputs from the pelvic nerves.

The response of blood vessels to vasoactive agents may also be affected by denervation. 5-HT normally causes both vasoconstriction and vasodilatation depending on arteriolar size and dose range. When periarterial nerves are cut however vasodilatation is elicited over the entire dose range (Fara 1976).

Effect of denervation on cell proliferation

Several lines of evidence suggest that nerves may directly influence cell proliferation both in the normal and in tumours. Sympathetic nerves play a part in the control of cell proliferation of the normal gut epithelium. Normal cells of the colon can uptake amines released by nerves in their vicinity.

Similarly adenocarcinoma cells in colonic tumours exhibit an admixture of endocrine and proliferative properties, and thus in addition to taking up amines from the vicinity may regulate their growth in an autocrine fashion. Chemical sympathectomy, which causes a loss of amines in nerves is associated with an increased mitotic rate in carcinomas of the mouse colon (Kennedy et al. 1985).

Hence the loss of nerves may influence the rate of growth of normal and tumour cells, perhaps favouring one cell population compared to the other. Furthermore cells may become more sensitive to circulating amines when they are deprived of innervation (Tutton and Barkla 1987).

The absence of nerves does not exclude the presence of receptors for transmitters they normally release or similar agents. Indeed as pointed out above both cells and vessels may be more sensitive to agents in the circulation. Hence SP receptors have been identified in the majority of peritumoral vessels in colorectal

carcinomas, suggesting that SP antagonists may be used in the treatment of tumours (Hennig et al. 1995).

Effects of denervation on blood vessel structure

Absence of nerves both in experimental and clinical tumours is associated with abnormality of vessel structure. In benign portwine tumours a lack of perivascular autonomic nerves is associated with ectasia of the blood vessels. Similarly malignant tumours lack perivascular nerves in the main tumour mass, with associated abnormality of structure and frequent saccular areas and dilatations.

The loss of perivascular nerves may produce changes in the smooth muscle elements of blood vessels. Sympathectomized ear artery in the rabbit developed thickening of the intima with dedifferentiation of smooth muscle cells (Kacem et al. 1997). Smooth muscle cells in denervated arteries display immature phenotype, with loss of thick myofilaments and an increase of organelles involved in the synthesis of extracellular matrix (RER, ribosomes), (Azevedo and Oswald 1986, Kacem et al. 1995). These changes may become detectable two months after surgical sympathectomy (Dimitriadov et al. 1988).

1.7 Colorectal neoplasia

The development of colorectal neoplasia

The development of colorectal cancer involves a sequence of progressive instability in the mucosal cell, with consequently adenoma formation, adenoma progression, and transformation to cancer. The premalignant phase is characterised by increased proliferative activity in the crypt cell of the mucosa.

This progression from normal to cancer through a premalignant phase, is the result of a cumulative acquisition of genetic defects involving the loss of tumour suppressor genes and oncogene activation, coupled with overexpression of growth factor. In familial adenomatous polyposis, the inherited defect is a mutation of the apc gene, while in hereditary non-polyposis coli syndromes, mutations of at least 4 DNA genes are involved. In both the inherited and the commoner sporadic cancers, however the ultimate genetic alterations are similar.

Deleted in Colon Cancer gene (DCC), tumorigenicity and neuronal differentiation.

Of the many genetic alterations that occur in the progression to colorectal cancer, the loss of the DCC gene is relevant to both tumour suppression and neuronal differentiation and is therefore considered here in greater detail. A frequent mutation in colon carcinomas is the loss of a specific region of chromosome 18q. This region is deleted in at least 50% of late adenomas and in

70% of carcinomas (Vogelstein et al. 1988). Molecular cloning of this region on chromosome 18q identified the DCC gene.

The DCC gene encodes an immunoglobulin superfamily and sequence analysis showed that DCC is almost homologous to the neural cell adhesion molecule member of the immunoglobulin superfamily such as NCAM 1(neural cell adhesion molecule 1) (Fearon et al. 1990, Johnson and Allegra 1995). DCC seems to function as a tumour suppressor gene and the loss of the gene is correlated with tumour progression perhaps through the disturbance of normal cell to cell interactions, which control growth.

Experiments involving the DCC gene illustrate its functions. The insertion of chromosome 18 into colorectal carcinoma cells, with subsequent re-expression of DCC at the cell surface decreased the tumorigenicity and metastatic potential of the cells (Goyette et al. 1992). When exposed to nerve growth factor for several days, cells of the phaeochromatocytoma cell line (PC-12) developed long dendrites. This morphological change was associated with an up-regulation of DCC. Furthermore the neurone-like phenotype was reversed when DCC expression was inhibited in nerve growth factor-differentiated cells. Thus DCC is involved in neural differentiation (Lawlor and Narayanan 1992).

Benign colorectal polyps

The term polyp is descriptive (Latin 'polypus' signifies 'many feet') and refers to any circumscribed elevated lesion projecting into the bowel lumen. Polyps may have a well-formed stalk and described as being pedunculated, they may be flat or sessile with a broad base (fig 2).

A polyp may result when neoplasia, inflammation, or dysmaturation (metaplasia) alters normal cell replication and differentiation in the crypt of Lieberkuhn. The most important types of polyps are neoplastic, mostly adenomas because they may develop into colorectal cancer. Some metaplastic polyps may also be precancerous as discussed later (Fenoglio-Preiser 1988, Otori et al. 1997).

Colonoscopy studies of asymptomatic individuals, selected on the basis of faecal occult blood tests, have reported an incidence of polyps of around 1.4% (Farrands et al. 1985). The incidence of polyps increases with age, and varies with sex being more common in males.

Adenomas of the colon and rectum

Colonic adenomas are common, occurring in around 50% of men and women over the age of 55 years (Rickert et al. 1979). In patients over the age of 55, around 50% of all colonic polyps are adenomas, and adenomas constitute 25% of all rectal polyps (Berg 1988). Adenomas may cause symptoms, usually bleeding or less commonly severe electrolyte losses.

In contradistinction with metaplastic polyps, also known as hyperplastic polyps, the abnormality of cell proliferation in adenomas occurs not only in the proliferative zone but also throughout the whole length of the crypt of Lieberkuhn and on the free surface.

In view of existing confusing terminology that exists the WHO (World Health Organisation) has recommended a classification of adenomas on the basis of histology (Morson and Sobin 1976). The three types are tubular, villous, and tubovillous depending on the microscopic features. All types however are considered to be macroscopic and microscopic variants of neoplasia; these neoplastic polyps display the following anomalies: the features of dysplasia or atypia; disorder of cell turnover; disorder of cell maturation; DNA abnormalities, and chromosome abnormalities may be present (Williams 1993).

Dysplasia is characterised by the following features: atypia, that is, atypical gland formation, pleomorphic nuclei, and an increase in mitosis (Williams 1993, Riddell et al. 1983). Adenomas can display varying degrees of dysplasia from mild to moderate or severe.

Many of the factors considered to be of etiological importance in the development of colorectal carcinoma are implicated in the formation of adenomas. Indeed most carcinomas are thought to originate in adenomatous polyps. The evidence for such adenoma-carcinoma sequence is based

on epidemiological studies where carcinomas were found to be more common in populations where adenomas were also common and carcinomas affected an older age group compared to adenomas. Anatomical studies have also showed that the site of predilection of adenoma (ascending colon, sigmoid colon) and their distribution (75% distal to the splenic flexure) matched those of carcinoma in the colon in most studies (Muto et al. 1975).

Factors affecting prognosis in adenomas

Large, villous and severely dysplastic adenomas have increased malignant potential (Morson and Bussey 1985). The chances of finding carcinomas in an adenoma depends on its size, a sharp increase occurs in adenomas over 1cm, as well as the type of adenoma, carcinomas being more commonly found in villous compared to tubular adenomas (Bond et al. 2000). Of the factors known to affect the risk of transformation to malignancy size over 1.5 cm proved to be the most important factor for adenomas in a prospective study of over 20,000 polyps (Nusko et al. 1997).

The histological type of polyps is another important criterion in assessing risks of development of carcinoma. Whilst tubular adenomas carry a low risk of carcinoma (<3%), and villous adenoma a higher risk (15 to 25%), tubovillous adenomas are an intermediate group, which may be difficult to define if histological type alone is considered. Similarly size and type of adenomas influence

the risk of recurrence after excision (Muto et al. 1975, Atkin et al. 1992).

Malignant polyps

Invasion of the submucosa by neoplastic cells marks the transition from benign to malignant disease (Haggitt et al. 1985). Lymph node involvement occurs in about 10% of adenomas in which submucosal invasion is present (Wilcox et al. 1986).

The final diagnosis of lymph node involvement in individual cases can only be reached after excision of the lymph nodes, so that lymph node status cannot be assessed in patients who have had their tumour treated by colonoscopy or minimally invasive methods. However it has been suggested that the use of CEA as a marker in tissue specimens may predict the presence or absence of metastases (Tokonaga et al. 1994).

Metaplastic (hyperplastic) polyps

In metaplastic polyps, also termed hyperplastic polyps the abnormality of cell proliferation is within the proliferative zone of the crypt. Metaplastic polyps were initially regarded as completely non neoplastic until a group of metaplastic polyps with features of adenomas, termed mixed metaplastic adenomatous polyps was recognised (Fenoglio-Preiser 1988). In addition other workers have identified both adenomatous and carcinomatous change in metaplastic polyps (Cooper 1998).

Whilst the sequence of genetic alterations which includes activation of the ras oncogene and loss of tumour suppressor genes on chromosomes 5, 17, 18, has been established about a decade ago for adenomatous polyps, evidence of genetic alterations in metaplastic polyps have only recently become available. K-ras gene mutations, which were identified in colorectal adenomas and carcinomas, are thought to play an important role in colorectal carcinogenesis (Fearon and Vogelstein 1990). It has been suggested that K-ras mutations precede the emergence of malignant phenotype. In a study of genetic changes in metaplastic polyps, K-ras gene mutations were detected in 47% of metaplastic polyps (n=19), and in 56% of adenomatous polyps (n=9) (Otori et al. 1997).

Furthermore the staining patterns of mucins in metaplastic polyps suggests that they display similar phenotypes to serrated adenomas. Serrated adenomas are characterised by epithelial neoplasia combining the architectural features of a metaplastic polyp with the cytological features of an adenoma (Yao et al. 1999). The similarity in staining patterns and genetic changes suggest that a metaplasia-adenoma-carcinoma sequence may exist.

Treatment of polyps

The removal of neoplastic polyps by mechanical means (polypectomy), electro-coagulative (cautery), and biochemical means is considered to be a secondary form of

prevention since these lesions are precursors of colorectal cancer.

The asymptomatic phase pror to the development of colorectal cancer is calculated to be of several years' duration, during which time its precursor lesion, usually an adenoma, grows and progresses to a malignant form. In this visible but asymptomatic phase it is possible to prevent cancer development and cure early cancer by screening and treatment by colonoscopy.

The treatment of the malignant polyp, that is an adenoma partly replaced by carcinoma, by polypectomy can be curative. Recurrence after polypectomy may however be of the order of 30% (Neugut et al. 1985). Studies of risk factors implicated have identified polyps at a high risk of recurrence. Invasion of the polyp stalk and its submucosa by cancer cells, poor differentiation of the neoplasm, and the presence of invasion of blood vessels or lymphatics carry a high risk of local and nodal recurrence. In these cases surgical resection locally or by colectomy is indicated (Haggitt et al. 1985).

On the other hand if polypectomy is complete, the margins of excision are free from malignancy on histology, and the above adverse risk factors are absent the mortality of large bowel resection may exceed the risk of lymph node metastasis after polypectomy for the malignant polyp (De Cosse 1984).

Malignant tumours of the colon and rectum

Carcinoma of the colon is the second commonest malignancy of the Western world; in the United Kingdom it accounts for over 18,000 deaths per year (Silverberg 1983, OPCS 1993). Colorectal cancer is the second commonest cause of death in the United Kingdom. 60% of patients die within 5 years of presentation (Mc Ardle et al. 1990).

Types of malignant tumours

The vast majority of colorectal tumours are epithelial in origin. The WHO recognises 7 types of malignant cancers of the colonic epithelium; they are in majority adenocarcinomas (Morson and Sobin 1976 p. 57-58). Rarely tumours arise from neuroendocrine or other cell types (DiSario et al. 1994). Squamous cell carcinomas invading from the anal epithelium or by spread of primary malignancy from adjacent organs may occur. Secondary metastases to the colon and rectum from distant organs are rare.

Adenocarcinoma of the colon usually arises from a precursor lesion, most commonly from an adenomatous polyp. Carcinomas may also arise from flat adenomas (Minamoto et al. 1994), from colonic mucosa affected by ulcerative or Crohn's colitis, or in previously irradiated rectum. The risk of carcinomatous change varies according to the type of precursor lesion.

In the case of colorectal adenomas the risk factors are the presence of multiple polyps, polyp size of > 1.0

cm, a villous type on histology, severe dysplasia (Morson 1984, Stryker et al. 1987), or mixed histological features (Muto et al. 1975, Nusko et al. 1997).

In ulcerative colitis follow up suggests that patients with extensive colitis for more than 10 years are at increased risk of developing carcinoma. Patients with proctitis only do not carry an increased risk (Lennard-Jones 1985). The risk of carcinoma in ulcerative colitis increases four-fold in patients who have a history of primary sclerosing cholangitis. Patients with Crohn's colitis have a 4 to 20 fold increase in risk of developing colorectal carcinoma (Wyatt et al. 1987).

The majority of adenocarcinomas however arise from adenomas through a series of genetic changes, and transformation to a malignant phenotype may take several years (Morson and Bussey 1985). Carcinomas in ulcerative colitis may follow an alternative pathway for tumour progression (Bell et al. 1991).

The commonest of epithelial cancers are adenocarcinomas. Carcinomas are evaluated and graded according to their degree of differentiation. Colonic cancers are graded into one of four groups: well differentiated, moderately differentiated, poorly differentiated adenocarcinoma and undifferentiated carcinoma. Well-differentiated cancers have cellular and histological features closely resembling normal epithelium, whilst poorly differentiated cancers barely

resemble normal epithelium. A moderately differentiated carcinoma is intermediate between well differentiated and poorly differentiated.

In view of considerable overlap in the criteria and in variation in reporting the adenocarcinomas are classified as well/moderately differentiated adenocarcinomas or poorly differentiated in some studies (Blenkinsopp et al. 1981). Furthermore survival is similar in patients with well-differentiated and moderately differentiated cancers, being 77% and 61% respectively, whilst in poorly differentiated tumours survival is 29% only (Dukes and Bussey 1958).

Histology of cancers: extent of spread

Full histological assessment of resected cancers of the colon and rectum gives a good indication of prognosis. Tumour stage is the most important predictor of survival in colorectal cancer and of local recurrence of rectal cancer (Bokey et al. 1999). Dukes' and other staging systems such as the TNM and the Astley-Coller systems are based on a number of histological criteria including the depth of invasion of the bowel wall, invasion of adjacent organs, and lymph node metastases. Further refinements have been included in the staging systems with subdivision of the Dukes' C stages for instance according of the site and number of lymph nodes involved.

Lymph node involvement

The original Dukes' classification, describing A, B, and C stages (Dukes 1932), does not take into account distant spread since it is based on the evaluation of the resected cancer and its mesenteric lymph nodes. Nevertheless it remains the gold standard by which other prognostic indicators and methods of classification are judged since lymph node involvement is a very important prognostic factor in colorectal cancer.

The number of lymph nodes involved was found to have an independent influence on survival in a retrospective study of 309 rectal resections. Survival over 5 years decreased from 65% in patients with 3 or less positive nodes to 42% in patients with 4 or more positive lymph nodes (Jass et al. 1987).

The presence of cancer in the apical lymph node worsens prognosis. Apical node positivity for cancer was associated with a 5-year survival of 14% only compared to 41% in patients with apical node-negative cancers where other lymph nodes were positive (Dukes and Bussey 1958).

Dissemination of cancer is associated with an increase in morbidity and mortality (Lewis 1988). The lymphatics of the large bowel constitute one of the main pathways of spread of colorectal cancer. The lymphatic vessels issuing from the bowel course up into the mesentery in close association with mesenteric blood vessels as these supply and drain the large bowel and rectum. The

lymphatics encircling the wall of major arteries form a rich and irregular plexus (Sacchi et al. 1990), closely related to the adventitia (Kato et al. 1993) and thus also to the perivascular nerves, as these course in a network arrangement around the blood vessels.

In the symptomatic phase, colon cancer proximal to the sigmoid presents as an emergency in about 34% of cases, most commonly as intestinal obstruction, acute bleeding, perforation and abscess. The vast majority present with the primary symptoms of abdominal pain and alteration of bowel habit (66%). Iron deficiency anaemia or an abdominal mass is present in 61% of patients. Rectal blood loss and change in bowel habit in combination occur in 64% of patients with rectal or sigmoid cancer. 21% of patients with sigmoid and rectal cancer present with the symptom of change in bowel habit alone. Rectal bleed in the absence of anal symptoms accounts for 10% of patients. Non specific symptoms or rectal mass without other symptoms are rarer modes of presentation (2 to 3%) (Thompson 1999).

Currently prevention of morbidity and mortality from colorectal cancer rests largely on the identification of the precursors of colorectal cancers and their early removal. To identify adenomas and early cancers some high-risk groups can be identified and offered screening. A history of previous adenomatous polyp, inflammatory

bowel disease, and previous pelvic irradiation increase an individual's risk of developing colorectal cancer.

Treatment of colorectal cancer

The treatment of a neoplasm of the colon and rectum depends on the site of the lesion, its histology, and the stage of the disease. At the time of presentation the majority of patients have advanced Dukes stages B, C, and D cancers. Patients affected by liver metastases rarely survive 5 years. Overt metastases to the liver are detectable in 25% of patients presenting with colorectal cancer. Up to 30 % of patients who show no evidence of metastases on routine testing have been calculated to have micrometastases detectable by serial investigations. Preoperative variables related to the development of liver metastases include sex, Log10 serum alkaline phosphatase (Taylor et al. 1990). Patient factors such general status should also be considered.

The aim of curative treatment is to eradicate disease and prevent relapse from local and distant recurrence. Resection remains the most important means of eradicating disease. Cure is likely when resection is complete and disease is localised, as in Dukes A cancers.

A number of adjuvant treatments have become increasingly important, the main aims of adjuvant treatment are to improve survival and minimise recurrence of disease. The main modalities of treatment include the use of

chemotherapeutic agents and radiotherapy. Distant spread at presentation or on follow up, usually in the form of liver and lung metastases may be treated with improvement in survival.

Surgery

Surgery remains the main modality of curative treatment in colorectal cancer, although primary and secondary prevention may in future play an increasingly important role. Curative resection and therefore improved prognosis can be achieved by clearance of the tumour at the initial operation.

Surgical factors during tumour excision affect recurrence rates. Synchronous tumours should be excluded, and implantation of tumour cells at the time of surgery should be prevented by the use of cytocidal solutions and careful surgical technique (Zirginbl et al. 1990, Karanjia et al. 1994).

The importance of clear proximal and distal longitudinal margins of resection has long been recognised. Resection with a margin of at least 5 cm of normal bowel is advocated, although lesser margins of clearance of 2 cms may be adequate at the distal end of a low anterior resection (Williams 1983, Heald 1992). Microscopic involvement beyond 2 cms from the macroscopic edge of the tumour does not seem to occur except in some Dukes C or anaplastic tumours (Phillips 1992, Vernava and Moran 1992). Significantly higher local recurrence rates

were found in patients with distal resection margins of < 2 cms in a study using stapled anastomosis (Laxamana et al. 1995).

More recently the involvement by cancer cells of the lateral margin, that is the mesorectum and perirectal tissues, was shown to be an important factor predisposing to recurrence. In a meticulous study of specimens from rectal cancer resections, tumour involvement in the lateral resection margin was found to be present in 14 of 52 patients. All 14 developed local recurrence subsequently. The involvement of circumferential margin predicted local recurrence with a sensitivity of 92% and a specificity of 95% (Quirke et al. 1986).

In rectal cancer the lateral or circumferential margin of clearance should be at least 1 mm on microscopic examination (Shepherd et al. 1995). This can be achieved by total mesenteric excision (TME) with a reduction in recurrence compared to conventional surgery (Heald and Ryall 1986, Heald 1995, Havenga et al. 1999).

Adjuvant Treatment

Colorectal cancer recurs in a significant proportion of patients despite adequate surgery with curative intent. Whilst recurrence occurs in the abdomen in colon cancer, in rectal cancer recurrence is usually localised to the pelvis.

Recurrence of disease following apparently curative treatment for colorectal cancer is more common in patients

with more advanced disease for example in Dukes stages B or C and in poorly differentiated cancers. This may occur either locally or regionally such as in the pelvis in rectal cancer or at more distant sites, commonly in the liver, or in a combination of sites.

In a study of 177 patients with rectal cancer, local recurrence in the pelvis occurred in 45% of patients (30% solely in the pelvis). Recurrence correlated with histologic grade of the tumour, transmural penetration, or invasion of adjacent viscera or structures. For patients with > 5 lymph nodes involved regional failure rate was 50% (Willett et al. 1984).

Many regimens of chemotherapy have been used in an attempt to improve survival and prevent recurrence of disease. The best agent known at present is 5-fluorouracil, which is most effective when used in combination with folinic acid. Combination chemotherapy with 5-fluorouracil and folinic acid leads to improvement in survival for patients with node positive (Dukes C) colon cancer (Francini et al. 1994). The benefits of chemotherapy for node negative colon cancer or rectal cancer is debated and currently being evaluated.

Radiotherapy

In rectal cancer recurrence is usually localised to the pelvis. Radiotherapy therefore has a well-defined place in the treatment of rectal cancer. In addition to its role in the control of advanced irresectable disease radiotherapy also has a valuable role as an adjunct in resectable cancers to reduce recurrence and improve survival (Wheeler et al. 1999, Steele and Sebab-Montefiore 1999).

Radiotherapy may be used in combination with chemotherapeutic agents, and may be offered pre or postoperatively. Postoperative radiotherapy allows more accurate staging of the cancer by histology of the resected specimen, and patients can be spared unnecessary treatment. However an increased amount of small bowel in the pelvis is exposed to postoperative radiotherapy when the rectum has been removed. Delays in treatment may also occur whilst the patient recovers from the operation.

Although current methods of investigation are of value in staging colorectal cancers (Brown et al. 1999a,b), there is a need to search for factors that can predict prognosis more accurately. Attempts to identify subgroups of patients who would benefit from other forms of treatment in addition to surgery have led to studies at molecular level.

The absence of the DCC protein for instance has a negative influence on survival in rectal cancer and is a

predictor of distant metastases after curative surgery (Reymond et al. 1998). Such patients may benefit from adjuvant chemotherapeutic treatment.

The expression of enzyme sucrase-isomaltase increases the risk of death by 1.83 fold. The enzyme was found to be an independent prognostic marker and was not associated with the expression of other clinicopathological variables (Jessup et al. 1995).

Of the serum markers of colorectal cancer, Carcino-embryonic antigen (CEA), which was first isolated in foetal tissues and adult colorectal cancer (Gold and Freedman 1965), is a good marker of the presence of colorectal cancer. However, elevated levels may occur in inflammatory bowel disease, in hepatic impairment and in other cancers such as breast and gastric cancer.

In relation to prognosis CEA is a poor marker. One third of patients do not have abnormal CEA levels at any stage of their disease whilst others who are disease free may show transient rises (Northover 1985). There is therefore, still a need to search for better markers of prognosis and of response to treatment.

FIGURE LEGENDS

1A. Blood supply of the wall of the human colon.

The colon is supplied by arteries of the mesentery (MT). Arterioles derived from these supply the bowel wall in a segmental fashion. At the level of the serosa these arterioles divide and are termed 'straight arteries'.

1=straight arteries within the mesocolon 1a, running straight through the bowel wall; 1b and 2, running a subserosal course. 3=Straight arteries penetrating the full thickness of the colonic wall.

9=Network of submucosal arterioles (red layer). Note the segmental supply from the straight arteries.

10=arterioles supplying the mucosa are derived from the submucosal network.

7=intermuscular network of arterioles.

8=intermuscular arterioles.

MT=Mesentery of the colon. TSM=Submucosal layer. CC=Circular muscle. CL=Longitudinal muscle. GO=Greater omentum. SS=Subserosal layer.

Reproduced from Wolfram-Gabel et al., Acta Anatomica, 1986, fig 1 p67, with permission from Karger, Basel, Switzerland.

1B. Perivascular nerves, which innervate blood vessels, and paravascular nerves shown in relation to arteries of the intestinal wall.

Note the close association of perivascular nerves (red) to the arterial wall throughout the course of the blood vessel. Paravascular nerves (black) by contrast course alongside the blood vessel to innervate the other structures of the bowel wall.

Reproduced from Furness and Costa, Neuroscience 1980 (5): 1-20, with permission from Elsevier Science.

Figure 2. Diagrammatic representation of a pedunculated (a) and of a sessile polyp (b).
The presence of neoplastic tissue through the muscularis mucosa into the submucosa (arrowhead) is a sign of invasiveness of the tumour, and defines it as an invasive carcinoma.
Reproduced from Haggitt et al., Gastroenterology 1985 89:329, fig 1, with permission from the American Gastroenterological Association.

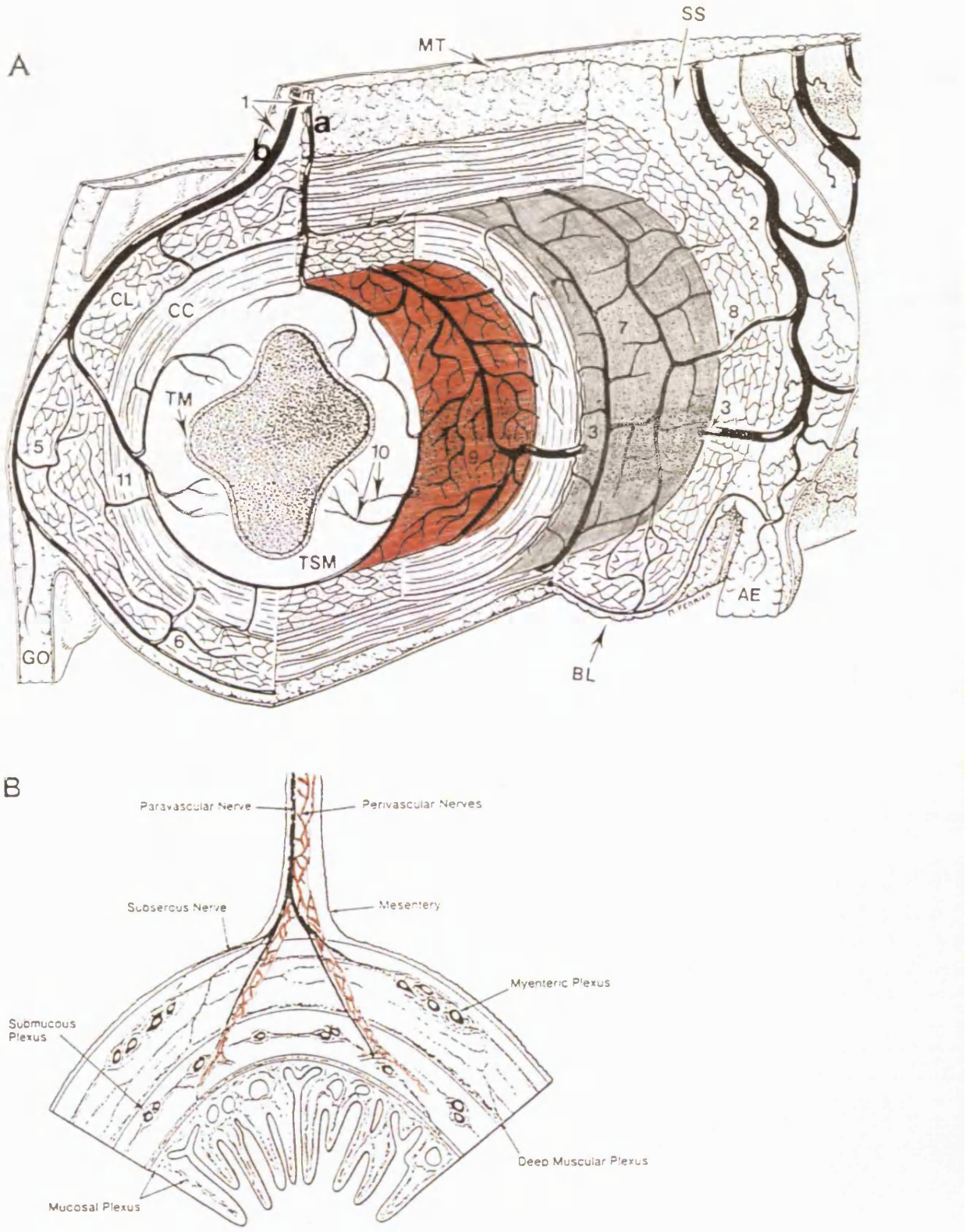
A
SS
MT
1
a
b
2
CL
CC
7
8
TM
3
5
10
11
9
3
TSM
AE
GO
6
BL
B
Paravascular Nerve
Perivascular Nerves
Subserous Nerve
Mesentery
Myenteric Plexus
Submucous Plexus
Deep Muscular Plexus
Mucosal Plexus

Figure 1

Pedunculated and sessile adenoma: Morphology and levels of invasion

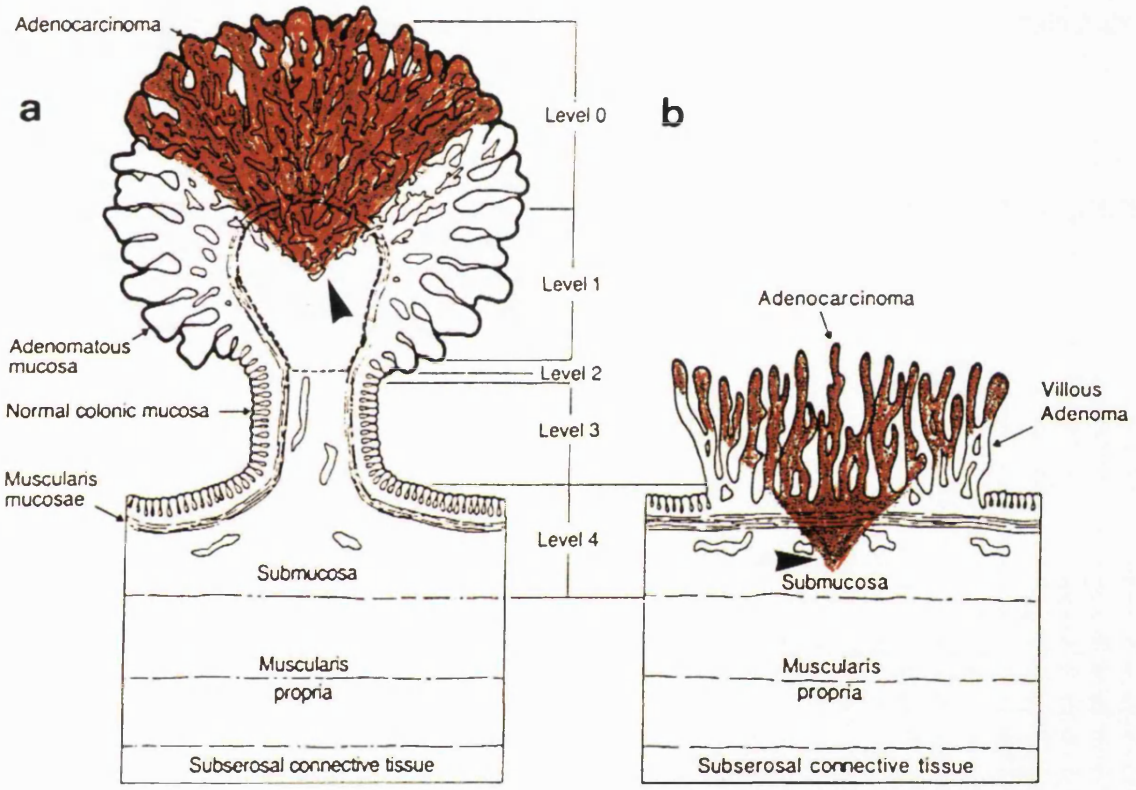

Figure 2

Chapter 2: Materials and Methods.

2.1 Materials

2.2 Methods light microscopy

2.3 Methods, electron microscopy

2.4 Substances studied

2.5 Source of antibodies to neurotransmitters

2.6 Statistical analysis

CHAPTER 2. MATERIALS AND METHODS.

2.1 Light Microscopy, Materials.

Colorectal Cancers and controls

Cancer and normal samples were obtained from 14 patients (7 males and 7 females) with colorectal cancer (age range 55 to 83 years, mean age 67).

There were 13 adenocarcinomas. One patient with a rectal cancer provided normal control sample only. Left sided carcinomas were predominant (n=11), of these 6 were from the rectum.

The samples were collected from resected operative specimens as follows: the bowel was opened longitudinally, and samples were taken longitudinally across the tumour to include the tumour edge and the adjacent bowel wall. Normal controls (n=14) were taken in the same orientation at least 5 cms from the tumours.

Colorectal polyps and controls

Samples were obtained from 15 patients. The mean age of the patients was 63.1 years (range 45-78); there were 9 females and 6 males.

Polyp samples were obtained from 11 of these patients (15 polyps). They were collected from resected colonic segments (10 polyps in 8 patients) or during colonoscopy (5 polyps in 3 patients). The samples were taken at sites more than 5 cms from any synchronous cancers and other

area of disease. The polyps (9 adenomas and 6 metaplastic polyps) were analysed quantitatively and qualitatively.

Control samples obtained in 8 patients were subjected to quantitative analysis. The samples were taken at least 5 centimetres from diseased segments of bowel, from normal areas of colon resected for carcinoma (n=6) or for diverticular disease (n=2). All operative specimens were taken in the longitudinal axis.

Normal mucosa obtained at colonoscopy (n=2) was used in the qualitative analysis.

Histology

After the initial reporting of the histology, the H&E slides were reported by another independent histopathologist from a different hospital.

2.2 Methods: Light microscopy

Tissue processing

The tissue samples were immediately placed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS).

Sections of 10 micrometers were cut on a cryostat and air dried onto gelatine coated glass slides for immunohistochemistry. Adjacent sections were stained with Haematoxylin and Eosin.

Immunohistochemistry

Before each step of the following immunoprocedure, the preparations were washed 3X 10 mins in PBS.

The slides were incubated for 18 hours with primary polyclonal rabbit antibody to each of the following neurotransmitters and markers individually at a dilution of 1:1000. That is, to tyrosine hydroxylase (TH) (Affiniti, Exeter, UK.), neuropeptide Y (NPY) (Biogenesis, Poole, UK.), vasoactive intestinal peptide (VIP) (Incstar, Wokingham, UK.), substance P (SP) (Genosys, Cambridge, UK.), calcitonin gene-related peptide (CGRP) (Affiniti, Exeter, UK.), and protein gene product 9.5(PGP 9.5) (Ultraclone, Isle of Wight, UK.). For negative controls the primary antibody was omitted.

This was followed by incubation at room temperature with secondary donkey anti-rabbit biotinylated species-specific antibody (Amersham life science, Amersham, U.K.) at a dilution of 1:250, for one hour.

Immunostaining was performed using streptavidin fluorescein (Amersham life science) at a 1:100 dilution for a further hour. The preparations were finally washed in PBS and mounted in citifluor (City University, London, U.K) and stained sections were stored at 4-8 degrees centigrade. Normal colonic tissue samples were processed simultaneously as positive controls.

The immunoreactive fibres were visualised using a Zeiss microscope equipped for viewing blue light immunofluorescence. Both qualitative and quantitative studies were carried out.

Vessel Count

The submucosa of the control samples, of polyps and and the submucosa adjacent to the cancers were studied. The total number of arterioles of normal controls and pathological specimen slides were counted in 3 fields per slide at X 250 magnification. Arterioles cut obliquely in sections were excluded from the count. Within these three fields the arterioles showing any degree of immunofluorescence in the perivascular nerves was also counted. The proportion of immunoreactive arterioles was recorded.

Interobserver variation

An experienced observer verified the count of transversely cut arterioles and there was complete agreement between the two observers. As a reference we used negative controls, that is samples processed and

immunoreacted in the same way as described above except for the omission of the antibodies to the neurotransmitter substances.

Three fields were studied in the normal submucosa adjacent to the cancers. The first field studied was the first area of normal submucosa away from the most advanced edge of the tumour. The second and third fields were the next ones containing arterioles moving further away from the tumour edge.

Thus the count was carried out moving progressively away from the microscopic edge of the tumour until three adjacent fields containing arterioles had been visualised. The distance from the edge of the cancer to the distal edge of the last field studied was measured. The maximum distance was 3.0 mm from the tumour edge (mean 1.76 mm).

In the colorectal cancers we furthered our study by next examining the immunoreacted samples to establish how far changes in perivascular innervation occurred in relation to distance from the cancer edge. Slides in which the adjacent mucosa did not exceed 3.0 mm from the edge of the cancer or was discontinuous were excluded from the study.

For NPY-immunoreacted samples 8 samples were suitable, 11 samples for TH and 7 samples for VIP. SP-immunoreactivity was reduced by 10% in the overall tumour group compared to controls and CGRP staining was sparse so that meaningful measurements could not be carried out in

relation to distance from the tumour edge. The lateral distance from the tumour edge was measured to the level at which the percentage of immunoreactive arterioles reached control levels. Measurements and counts were made without knowledge of the Dukes' stage.

To verify the presence of immunoreactivity in the perivascular nerves as noted by the observer, studies were performed using image analyser. Using the method described below in the image analysis section arterioles recorded as showing immunoreactivity by the observer were subjected to image analysis. Samples from the main groups, that is normal controls adenomas and cancers, were studied. Photographic comparisons were also made and discussed. The author was detecting immunoreactive nerves, as opposed to autoimmunofluorescence in the arterial wall.

The presence of perivascular innervation was also verified at transmission electron microscopy. All normal submucosal arterioles were found to have perivascular nerves. By contrast, of a total of 21 arterioles examined specifically in tissue adjacent to cancers 12 did not show perivascular nerves and 7 arterioles had a reduced density of perivascular nerves.

Image analysis

Image analysis (Seescan Imaging, Cambridge U.K) was used in order to verify the detection of immunoreactive perivascular nerves as described above.

We also measured periarteriolar immunoreactivity in relationship to the distance from the edge of the tumour in 2 tumour samples. In one Dukes A and in one Dukes C images were obtained from NPY-immunoreacted sections because the difference between Dukes A and Dukes B/C tumours detected at vessel count (described above) was striking.

During the process of image analysis the image under study was captured, the background of the microscope slide was then subtracted from the captured image to enhance the immunofluorescence. Following its capture the image was further enhanced by an automatic process allowing the immunofluorescence to be thresholded and highlighted before measurement was made.

The immunofluorescence of the arterial wall itself was excluded by drawing around the area of autofluorescence and excluding the area from the measurements. The final edited image was then quantified using the following parameters: total area under frame and area of red in frame, the latter representing the area of immunofluorescence being measured. The percentage of each frame with immunofluorescence (percentage of red in frame) was thus obtained.

2.3 Electron microscopy, Materials and Methods.

Samples were collected as for light microscopy, and initially 1-micrometer sections were stained with toluidine blue to pinpoint areas of interest. The areas selected for study were the stroma of the tumour, the peripheral part of the tumour, as well as the tissues adjacent to the tumours. Comparison was made with corresponding areas in controls.

There were 7 control samples, 5 well differentiated adenocarcinomas (Dukes A to C) and 3 polyps (One with a focus of adenocarcinoma) collected from 7 patients (3 males, 4 females, mean age 60 years).

Samples were cut into 1 to 1.5 mm slices and fixed in 2% glutaraldehyde overnight. To wash excess aldehyde the tissue was rinsed in phosphate buffer x2 for half-hour.

In order to preserve lipid membranes the tissue was fixed secondarily in 1% Osmium in 0.1M phosphate buffer at 4 degrees centigrade in a darkened fridge. Osmium also acts as a metal stain for electron microscopy. The tissue was further washed in 0.1 M phosphate buffer for 10 minutes.

After x2 washes in 0.1M sodium acetate for 10 minutes, en bloc staining was performed in 2% Uranyl acetate in 0.1M sodium acetate buffer for 45 minutes at 4 degrees centigrade. Further washes in distilled water X2 for 10 minutes were carried out.

To prevent damage resulting from excessive changes in solvent concentration dehydration was carried out in a graded series of Ethanol (25% to 50% to 70% to 90%) for 10 minutes each followed by 100% Ethanol x4 changes of 10 minutes each. This process removes all water and replaces it with Ethanol.

Embedding was effected by x3 changes in propylene oxide, which mixes well with the resin. A 50-50% resin - propylene medium was used initially followed by 100% resin (10 g diodecmyl succinice anhydride, 10g araldite CY1212, 0.8g plasticizer, plus 0.4 mls benzyldimethylamine when mixed and moulded), left overnight on a rotator at room temperature. Fresh resin was used for replacement in the morning.

The polymerised blocks were trimmed to 1-mm square and semi-thin sections of 1- micrometer thickness were cut on the ultramicrometer in order to choose the areas for study. The quality of fixation and embedding was assessed. Ultrathin sections of 80 nanometers were then cut and collected on copper grids of 200-400-mesh size. Sections were stained with lead citrate.

2.4 Substances studied

Protein gene produt 9.5 (PGP) is a soluble protein first isolated from the human brain. The demonstration of PGP 9.5 is useful for the delineation of nerves at all levels of the nervous system (Rode et al. 1985, Wilson et al. 1988). However antibodies directed against the protein can also cause staining of epithelial tissues (Mitchell et al. 1994b).

Tyrosine hydroxylase (TH) is a precursor of adrenaline and noradrenaline. The hydroxylation of tyrosine is regarded as the rate-limiting step in the biosynthesis of catecholamines and tyrosine hydroxylase (TH) is activated following the stimulation of adrenergic nerves.

Noradrenaline is found in the perivascular plexus around arterioles and venules of the intestine in the rat colonic mucosa but not around other types of vessels of the microcirculation (Dikranjan et al. 1992).

Nearly all the NA content of adrenergic innervated organs is confined to the postganglionic sympathetic fibres. In the human rectum mucosal catecholamines increase in inflammatory bowel disease (Pentilla et al. 1975).

Neuropeptide Y (NPY) a 36-amino acid peptide. The neurotransmitter participates in the neuromodulation of blood flow, motility, and secretion in the mammalian intestine.

Rich sympathetic perivascular nerve fibres containing NPY are extrinsic in origin and innervate the submucosal microvasculature (Nichols et al. 1994), (Ferri et al. 1988). NPY has profound pressor effects. NPY-immunoreactive nerve fibres densely innervate blood vessels, being more numerous around arteries than around the corresponding veins.

The tissue content of NPY can be depleted by surgical sympathectomy and by treatment with reserpine. The effect of the latter treatment is dependent on intact nerve activity. Reserpine pre-treatment depletes the peripheral tissue stores of NA while leaving those of NPY largely unaffected if the neuronal activity has been impaired pharmacologically by guanethidine or clonidine or surgically by denervation (Lundberg et al. 1991).

NPY is synthesised by the nerve cell body where it is stored in large dense cored vesicles and transported to peripheral nerve by axonal transport. NPY is generally co-stored with noradrenaline. The co-stored substances NPY and NA interact reciprocally in modulating their release from sympathetic nerve terminal, NA inhibits NPY release, and conversely NPY inhibits NA release.

NPY shares considerable sequence homology with pancreatic polypeptide produced by cells of the endocrine pancreas and belongs to the pancreatic polypeptide family. NPY-immunoreactive cells are present in the colonic mucosa (Nichols et al. 1994).

Vasoactive intestinal peptide (VIP) is a 28 amino acid peptide present both in the central nervous system and the peripheral nervous system.

VIP was first discovered in the gut (Said and Mutt 1972), and is found in all layers colon preferentially in the circular muscle (Surrenti et al. 1993).

VIP is involved in the peristaltic reflex by relaxing smooth muscle. VIP stimulates water and electrolyte secretion and is also thought to alter immune cell function.

VIP is a powerful vasodilator. Immunoreactivity to VIP is present in the wall of normal blood vessels of man (Mazumdar and Das 1992), (Polak and Bloom 1978).

In disease states VIP- immunoreactivity may be altered: it is decreased in all locations in ulcerative colitis (Mazumdar and Das 1992) and in the wall of blood vessels both in ulcerative colitis and Crohn's (Koch et al. 1987). Decreased immunoreactivity in blood vessels in the lamina propria was more prominent in the severe forms of active ulcerative colitis and Crohn's disease (Koch et al. 1987). Kimura found an increased VIP-immunoreactivity only in hypervascular areas along proliferating blood vessels in the lamina propria in ulcerative colitis (Kimura et al. 1994).

Substance P (SP), an 11 amino acid peptide of the tachykinin family (Pascual et al. 1994) is a sensory-motor neurotransmitter found in all layers of the human colon.

SP-immunoreactivity is found in the submucosa, muscularis mucosa, circular muscle, and lymphoid follicle of the normal human colon. It is in addition found in epithelial cells of the basolateral domain.

SP is a vasodilator and has an excitatory role in motility, maintenance of haemodynamics, and ion transport (Brodin et al. 1983 and Llewellyn-Smith et al. 1984). In inflammatory bowel disease a decrease in SP-immunoreactivity was noted, particularly in Crohn's disease in the walls of arterioles and venules of the lamina propria (Koch et al. 1987).

Calcitonin gene-related peptide (CGRP) is a 37 amino acid peptide. It is found in many systems and is a neuromediator of importance in the cardiovascular system where it causes systemic vasodilatation. It relaxes the taenia coli and increases the electrolyte and water flow in the colon.

CGRP is present in perivascular nerve fibres and coexists with intrinsic VIP fibres and extrinsic SP fibres (Timmermans et al. 1992), (Ekblad et al. 1988).

2.5 Source of antibodies to neurotransmitter substances and neuronal markers.

TH (Affiniti, Exeter, UK.)

NPY (Biogenesis, Poole, UK.)

VIP (Incstar, Wokingham, UK.)

SP (Genosys, Cambridge, UK.)

CGRP (Affiniti, Exeter, UK.)

PGP (Ultraclone, Isle of Wight, U.K.)

2.6 Statistical analysis

The data was entered for statistical analysis using the Stata version 6.0 package for comparison of each data set, that is cancers versus controls (chapter 3) and polyps including adenomas versus controls (chapter 4).

For clarity the results for the numbers of arterioles positive for immunoreactive nerves were expressed as percentages of the total count.

For analysis of statistical significance we used non-parametric tests since the data was highly skewed. These included the Mann-Whitney and Kruskall-Wallis tests for two and three samples respectively.

Figure Legends

1A. Colonoscopic view of the normal colonic mucosa. The submucosal vessels are clearly visible (arrow head); note their segmental pattern.

1B. Colonoscopic view of a colonic cancer (arrow head)

2. The normal submucosa adjacent to colorectal cancer contains arterioles with several layers of smooth muscle cells (small arrows). Cancer cells at the invading edge are shown (large arrows). Haematoxylin and eosin stain. Calibration bar 10 microns.

3. Immunoreactivity to neurotransmitter substances around submucosal arterioles, showing the presence of perivascular and paravascular nerves.
A. NPY-immunoreactivity in sympathetic perivascular nerves (thick arrow) in control submucosa. Note autofluorescence of the arterial wall (thin arrows). B.Immunoreactivity to VIP in paravascular nerves (thick arrow) near a submucosal arteriole. Autofluorescence is present in the arteriolar wall (thin arrow). Calibration bar 25 microns.

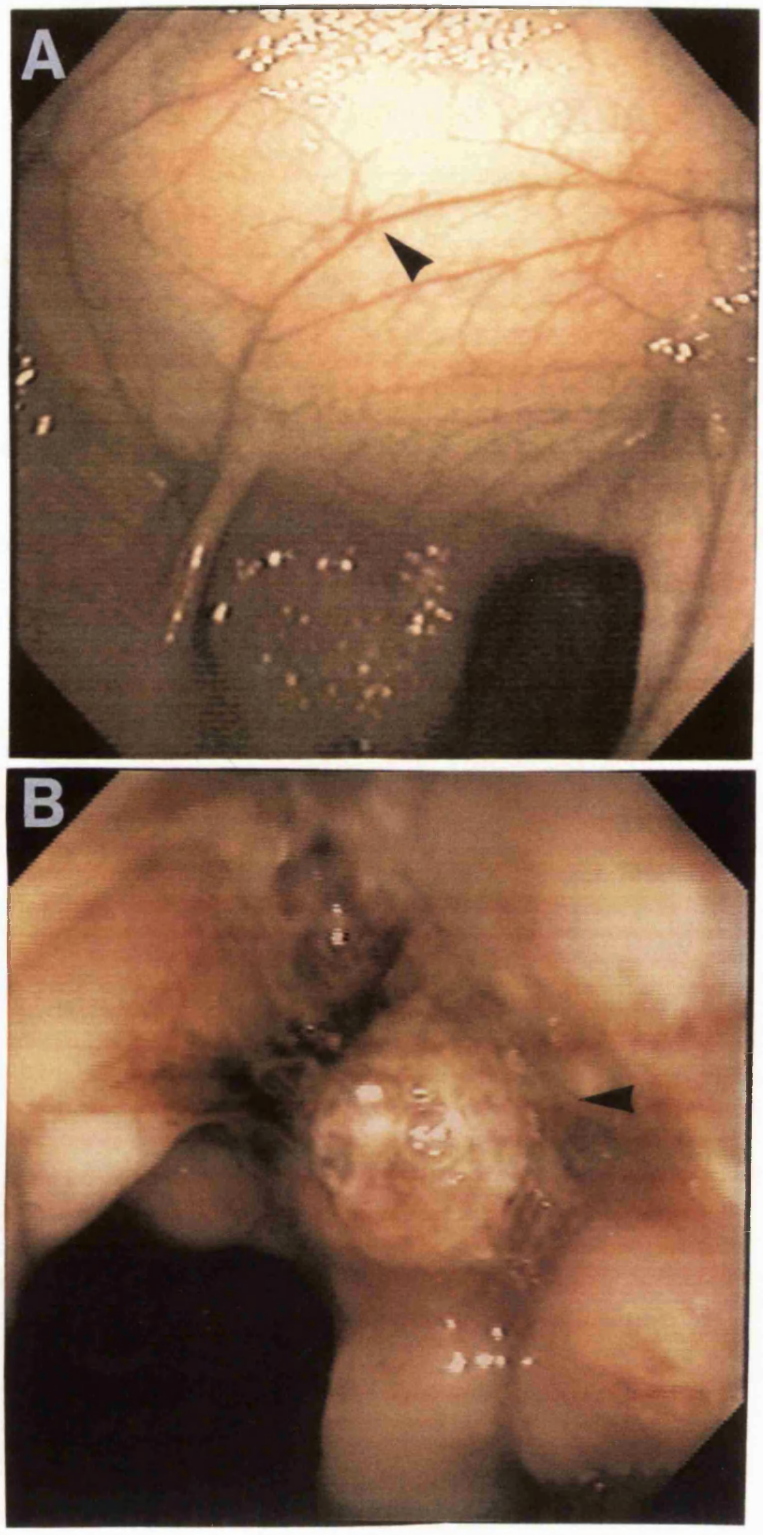

Figure 1

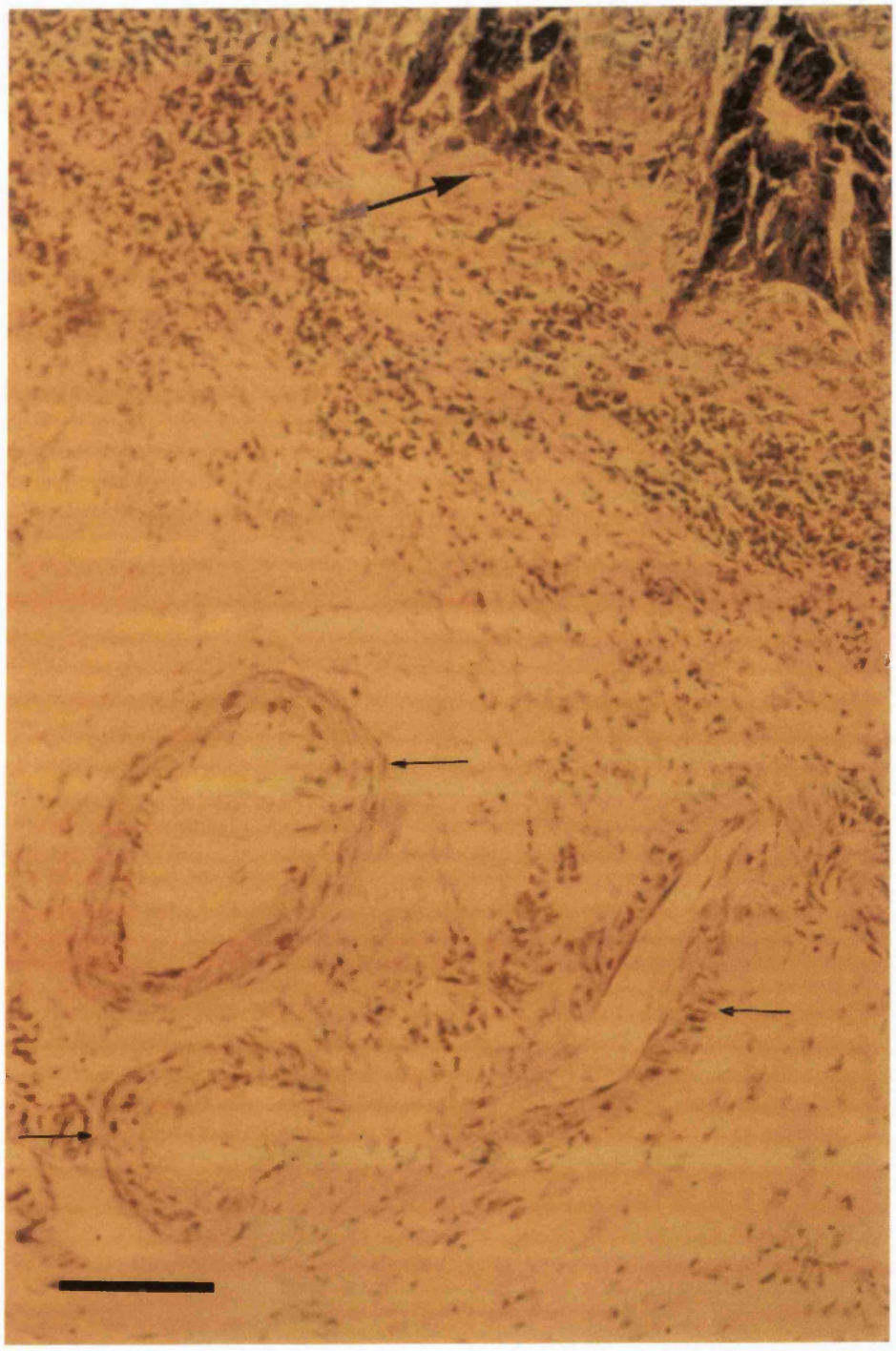

Figure 2

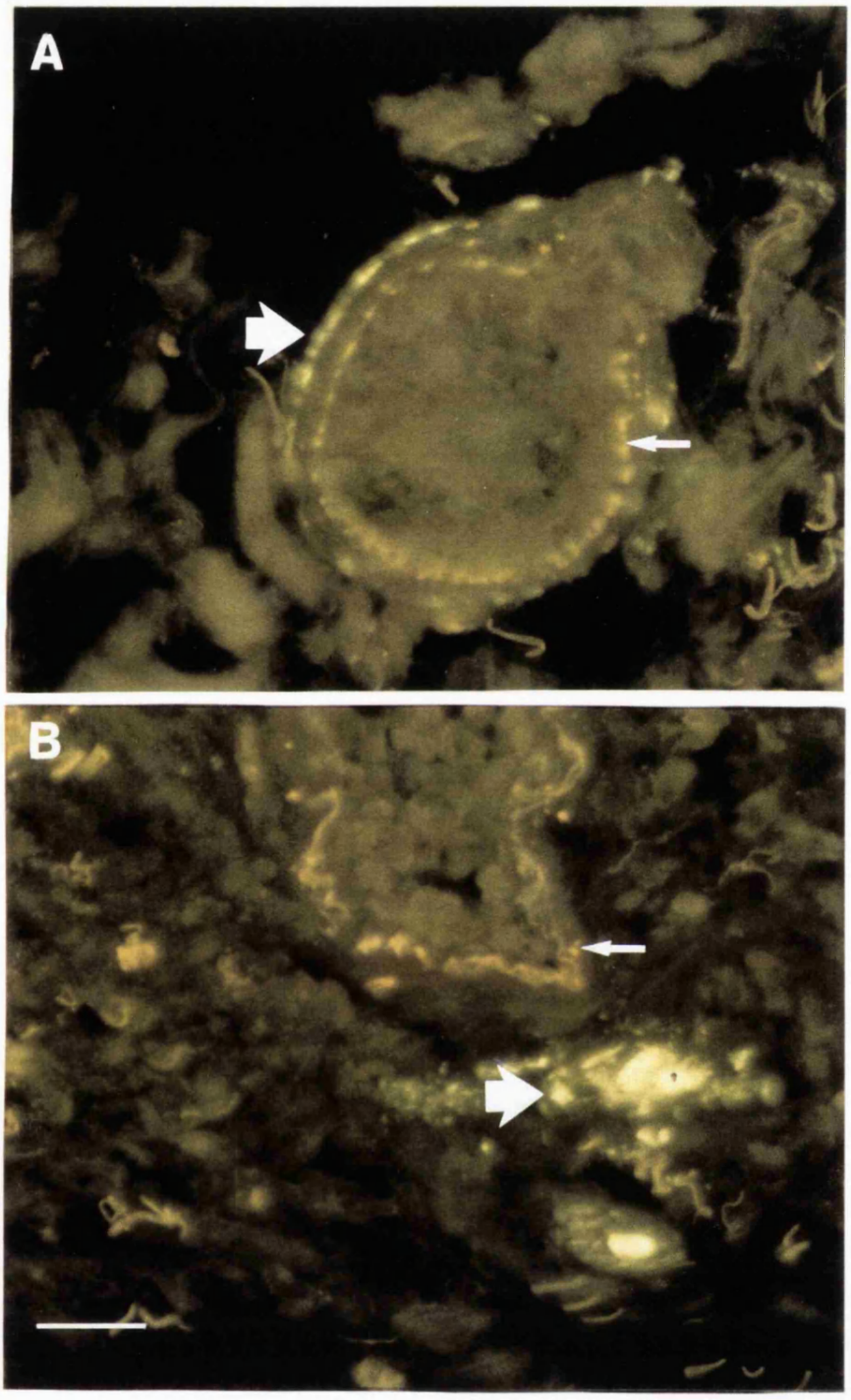

Figure 3

Chapter 3: Perivascular nerves in colorectal cancer.

3.1 Introduction

3.2 Results: Counts of submucosal arterioles

Relation to distance from the cancer

Image analysis

3.3 Discussion

CHAPTER 3. Perivascular nerves in the submucosa adjacent to colorectal cancer

Abstract

Various cancers lack perivascular innervation. No quantitative study of perivascular innervation of the adjacent submucosal tissue exists. In studying the neural elements known to affect vascular tone, we aimed to shed light on some of the factors that affect blood flow through the submucosal arterioles that supply colorectal cancer.

Methods

Markers of neurotransmitters, neuropeptide Y (NPY), vasoactive intestinal peptide (VIP), substance P (SP) and calcitonin gene-related peptide (CGRP), and tyrosine hydroxylase (TH) as a marker for catecholamines were studied. Perivascular immunoreactivity in submucosa adjacent to colorectal cancer and within the cancers was compared with that of normal tissue at least 5 cm away from the cancers (controls).

Results

There was absence of perivascular nerves within colorectal cancers and loss of perivascular innervation in submucosa adjacent to the cancers. The loss comprised all neurotransmitter substances in submucosa adjacent to cancers (n=13) except for CGRP where the mean was less than 1% in both controls and cancers (TH controls 69%

versus cancers 6%, NPY 95% versus 27%, VIP 50% versus 17%, SP 39% versus 22%). These differences were statistically significant for TH ($p<0.002$), NPY ($p<0.002$), and VIP ($p<0.015$)

The pattern of loss varied for different transmitters when the cancers were analysed by Dukes' stage. The loss was progressively greater with advancing tumour stage for NPY (controls 95%, Dukes A 68%, Dukes B 17%, and Dukes C 6%), and VIP (50%, 23%, 14%, and 17%). For TH there was extensive loss of innervation around tumours of all stages (69%, 5%, 9%, 0%). SP-immunoreactive periarteriolar nerves was similar in control tissue (39%) and adjacent to Dukes A tumours (40%) but fell to 20% and 0% in tissue adjacent to Dukes B and C tumours, respectively. In none of the tissues was CGRP-immunoreactivity above 4%.

The mean distance over which there was reduced NPY-immunoreactivity from the edge of the adenocarcinomas was 2.43 mm for Dukes A/B tumours compared with 7.20 mm for Dukes C tumours; for VIP-immunoreactivity this distance was 5.22 mm for Dukes A/B tumours and 5.52 mm for Dukes C tumours.

Conclusion

There was a loss of neurotransmitters in perivascular plexuses in submucosa adjacent to colorectal cancer. A progressive loss of neurotransmitters was found with disease staging in terms of vascular nerve immunoreactivity both within a defined distance (3mm) and

within increasing distances from the tumour edge (maximum distance 8.2 mm).

These results suggest that the tumour itself may influence neural integrity in perivascular plexuses, perhaps via the secretion of an inhibitory factor.

3.1 Introduction

Invasion and growth of solid tumours are dependent on their blood supply which is derived from two sources: blood vessels recruited from the pre-existing host vascular network and those resulting from the angiogenic response to cancer cells (Jain 1988, Jiang et al. 1994, Fidler and Ellis, 1994).

In the tissue adjacent to the tumour where the former type of vessels are found, innervated blood vessels have been observed (Mitchell et al. 1994a) but detailed study of the vasculature in this area has received little attention.

The importance of the study of the tissue adjacent to the tumour receives support from histological studies. It has been shown in studies on lymphatic involvement in colorectal cancers that prognosis relates to the aggressiveness of the advancing edge of the cancer rather than to the cells in the tumour centre, even in early cancers (Hase et al. 1995).

Arterioles show the densest neural immunoreactivity compared with veins and capillaries; in the submucosa they form the final resistance vessels and thus control blood flow to the gut wall and surrounding tissue (Jiang et al. 1994). When a tumour grows, the tumour circulation becomes linked to the arterioles of the submucosa (Hori et al. 1993, Skinner et al. 1995).

Therefore, the study of the pattern of tumour vascular supply and its neural control may allow therapeutic modulation of tumour invasion and spread.

In this study, we compared the pattern of innervation of arterioles in the submucosal tissue adjacent to colorectal Dukes A, B, and C cancers with healthy control tissue at least 5 cm away from the tumour, using specific markers for neurotransmitter substances.

3.2 Results

Pathological Groups

There were 13 adenocarcinomas and 14 normal samples in 14 patients. The adenocarcinomas were classified as Dukes A stage in 4 cases, Dukes B in 6 cases, Dukes C in 3 cases.

Total arterial count

The mean total number of arterioles cut transversely was 7.2 (range 3-17) in all the control sections, and 5.7 (range 2-12) in the submucosa adjacent to the cancers (n=13). Furthermore there was no significant difference in the mean total number of arterioles in tissue adjacent to cancers related to Dukes staging.

Arterioles with perivascular immunoreactivity

Normal Controls

Immunoreactivity to neurotransmitter substances in perivascular nerves was present around all types of vessels in the control submucosa (fig 1). In the normal control submucosa, perivascular immunoreactivity to the general neuronal marker PGP 9.5 was present in all arterioles examined.

The mean percentage of arterioles with perivascular nerve immunoreactivity to NPY was 95 ± 3%, and for TH 69 ± 8%, VIP 50 ± 9%, SP 39 ± 9% (Table 1). For CGRP it was less than 1%. Perivascular nerves situated at the adventitia-media border were much finer than paravascular nerves in the normal submucosa (Figure 2).

Cancers

While no perivascular nerve immunoreactivity was observed around the vessels within cancer tissue both perivascular and paravascular nerves were seen in the submucosa adjacent to malignant tumours, particularly in Dukes A cancers.

There was reduced immunoreactivity in perivascular fibres to TH, NPY, VIP, SP in the region adjacent to the malignant tumours compared with controls and except for SP the decrease was statistically significant (Table 1). The mean percentage of immunoreactive arterioles in the submucosa adjacent to the cancers (n=13) was 5.9 ± 2.6% for TH (controls 69%), 27.3 ± 10% for NPY (controls 95%), 17.4 ± 6.5% for VIP (controls 50%), 21.5 ± 8.3% for SP (controls 39%).

The difference between tissue adjacent to cancers and controls reached statistical significance for TH (p <0.002), for NPY (p <0.002), and for VIP (p <0.015). There was no significant difference between controls and cancers for SP. Only a few fibres were immunoreactive to CGRP (< 1% in controls and cancers) therefore comparison was not possible (table 1,page 109).

Relationship to Dukes' stage

In addition, there appeared to be a relationship between perivascular immunoreactivity to TH, NPY, VIP, SP and the stage of the adenocarcinoma (Dukes A, B, C). The more advanced tumours showed greater loss in the adjacent

tissue. This was most marked for NPY where 68% of arterioles in submucosa adjacent to Dukes A, 17% in Dukes B, and 6% in Dukes C, were immunoreactive. The loss of TH-immunoreactivity was more marked in all tumour stages than for NPY; only 5% of arterioles in submucosa adjacent to Dukes A tumours, 9% in Dukes B, and none in Dukes C were immunoreactive to TH.

VIP-immunoreactivity in perivascular nerves decreased progressively with tumour stage; 23% in Dukes A, 14% in Dukes B tumours and 17% in Dukes C. SP-immunoreactive perivascular nerves were present in 40% of arterioles adjacent to Dukes A tumours, compared with 20% adjacent to Dukes B tumours, and 0% adjacent to Dukes C tumours.

SP- immunoreactivity for Dukes A was similar to controls (controls 39%, Dukes A 40%), but was decreased in Dukes B and C tumours (20% and 0% respectively).

In the control samples of individual patients (appendix IV) NPY was the predominant neurotransmitter present around submucosal arterioles of normal mucosa, followed by TH. A similar predominance of NPY-immunoreactive fibres compared to the other neurotransmitters was present in all Dukes A cancers, whilst in Dukes B/C group cancers either showed no periarteriolar immunoreactive nerves to any of the neurotransmitters tested or showed a predominance of VIP-immunoreactive fibres, albeit reduced compared to controls.

Relationship to distance from the edge of the cancer

To investigate how far the loss of perivascular nerves extended from the tumour edge, we measured the percentage of arterioles with perivascular immunoreactivity to NPY, TH and VIP in the submucosa in relation to distance from the cancer edge. Results for tumours of stages A and B were combined to obtain equal groups for comparison and because A and B groups represent invasion of the bowel wall and surrounding tissue as opposed to C group where mesenteric lymph nodes were involved by the tumour.

For all tumours the mean lateral distance from the tumour edge at which the percentage of arterioles in the submucosa reached control levels was 4.47 mm (± 1.38, n = 7) for NPY and 5.34 mm (±1.74, n = 7) for VIP. In NPY-immunoreactive samples, the mean distance was 2.43 mm (±0.88, n = 4) for Dukes A/B tumours, 7.20 mm (±2.31, n = 3) for Dukes C tumours. For VIP the distances were 5.22 mm (±1.94, n = 4) for Dukes A/B tumours, and 5.52 mm (±2.92, n = 3) for Dukes C tumours.

The mean distance for TH could not be determined because the extent of loss of perivascular immunoreactivity sometimes exceeded the length of sample available for analysis. The maximum length of adjacent tissue available on all 11 samples (8 A/B tumours, 3 C tumours) suitable for analysis did not exceed 8.2 mm. In 4 of these 11 samples control levels were reached at 4.0,

6.1, 7.2, and 7.6 mm. All 4 were A/B tumours (that is 4/8 of A/B tumours), whilst in none of the C tumours (0/3) were there arterioles with perivascular immunoreactivity within 3.6, 6.7, and 7.9 mm.

Image Analysis

Two adenocarcinomas were specifically studied with the image analyser to measure the density of NPY-immunoreactive bundles in relation to the distance from the tumour edge. An increase in the intensity of perivascular immunoreactivity was noted as the distance from the tumour edge increased.

In a Dukes C tumour the image analysis for PGP-immunoreactivity showed a similar trend of increasing immunoreactivity with distance. The immunoreactivity to PGP around the arterioles clearly increased with distance from the tumour edge.

3.3 Discussion

In experimental and human colorectal cancer, tumour circulation is supplied from arterioles in the submucosa and the muscular layers, as well as from vessels in the adjacent mucosa (Skinner et al. 1990, 1995). The adjacent submucosal arterioles provide blood to the tumour either directly or indirectly by supplying the adjacent mucosa to the tumour. In carcinoma of the colon prognosis in terms of overall survival and local recurrence relates to the depth of invasion of the bowel wall by tumour (Dukes 1932). Since growth and invasion depend on blood supply the control of the tumour blood supply is of relevance.

Control of vascular tone in normal blood vessels is dependent on its perivascular nerves and endothelial cells (Burnstock and Ralevic 1994). Whilst perivascular nerves are generally absent in the mass of primary and secondary tumours (Krylova 1969, Mattsson et al. 1977, Ashraf et al. 96), perivascular innervation adjacent to tumours has been identified (Mattsson et al. 1977, Mitchell et al. 1994a). In the latter qualitative studies, arterioles in tissue adjacent to tumours were found to be more densely innervated compared with veins and capillaries, a pattern that is also seen in normal tissue.

In this study, the number of arterioles with perivascular immunoreactivity to specific neurotransmitter markers (TH, NPY, VIP, SP, and CGRP) has been quantified.

Whilst the study confirmed the absence of perivascular nerves within colorectal cancers, it also demonstrated for the first time a decrease in the percentage of immunoreactive perivascular nerves in submucosal tissue adjacent to colorectal cancer, despite the small number of samples studied.

This finding suggests that malignant tumours may be responsible for the degeneration of nerves via the release of tumour factors, in a manner similar to the way tumours influence the formation of new vessels by the release of angiogenic factors. Tumour angiogenesis factor is known to produce changes not only in newly formed vessels but also in pre-existing ones (Jain 1988, Folkman and Cotran 1976). The mechanism of tumour factor release is supported by our finding of perivascular nerve loss in areas adjacent to cancers, even early ones.

A significant variation in the loss of immunoreactivity to markers of different nerve types, and in the distance from the tumour over which loss occurred, in relation to tumour stage has been observed. The changes observed in tissue adjacent to Dukes C tumours may relate to the proximity of the lymphatics to the perivascular nerves as they run along the arteries and veins in the mesentery (fig 1, chapter 1). Release of tumour factor in lymph nodes of the mesentery would thus be responsible for more extensive nerve degeneration, coextensive with the distribution of arterial supply to

the bowel, as opposed to the immediate vicinity of the tumour in the bowel wall in the earlier cancers. Alternatively, greater loss of immunoreactivity with more advanced cancers may be related to a larger production of a putative substance with inhibitory effects on perivascular nerves over longer distances.

The pattern of neurotransmitter loss described in this study warrants discussion. Both extrinsic and intrinsic nerves supplying the gut contain the peptides NPY, VIP, SP, CGRP. Therefore, it is not plausible to relate the non-conformed loss of the different peptide-containing perivascular nerves to extrinsic or intrinsic origins.

However, it is of interest that both NPY- and SP-containing perivascular nerves, largely extrinsic in origin, were preserved in Dukes A tumours compared with more advanced tumours, in contrast to VIP- containing nerves which are intrinsic in origin. CGRP containing nerves were too few to allow valid comparison.

Further explanation is required regarding the more profound loss observed in nerves containing TH, an enzyme used in the synthesis of the amines, adrenaline and noradrenaline. Specific neurotransmitters may be affected differentially both under experimental conditions and by disease processes. In diabetes analogous changes occur, and several studies have demonstrated that certain nerve types can be selectively damaged (Belai et al. 1988, Loesch et al. 1986).

Thus, whilst a decrease in immunoreactivity and tissue levels of noradrenaline were present in the colon of streptozotocin-diabetic rats, a resistance to change was demonstrated in neuropeptide-containing nerves (Belai et al. 1988). In human diabetic sensorimotor neuropathy the small diameter sensory and autonomic fibres which bear the tyrosine kinase A receptor become dysfunctional (Dyck 1996). In malignant colorectal tumours, as in diabetes, there seems to be a non-conformed loss of nerve types.

Tumour growth is influenced by biogenic amines released by nerves (Tutton and Barkla 1977). Chemical sympathectomy, which leads to loss of neural amines such noradrenaline in those nerves is associated with an increased mitotic rate in DMH induced carcinomas of mouse colon (Kennedy et al. 1985) and in rat carcinomas (Tutton and Barkla 1987).

A loss of sympathetic nerves and the decreased availability of amines, as demonstrated in the loss of TH-immunoreactivity, were associated with advanced cancers in the present study. However noradrenaline has been shown to promote cell proliferation both in colonic tumour cell lines as well as normal intestinal epithelium (Tutton and Barkla 1987). On the other hand, it is conceivable that diminished sympathetic regulation of the submucosal arterioles resulting in vasodilatation and increased blood flow, is a more important factor promoting tumour growth.

Autoradiographic studies have demonstrated the presence of receptors for the vasodilator SP in the peritumoral vessels in colorectal carcinomas, suggesting that SP antagonists may be used in the treatment of tumours as their binding to vascular receptors may decrease tumour blood supply (Hennig et al. 1995). SP-immunoreactivity in host veins in the area around colorectal tumours is upregulated, with an increase of 3 to 5 times compared with controls (Reubi et al. 1996). This is consistent with the proposal that modulation of the haemodynamics of neoplastic tissue is possible via peritumoral rather than intratumoral targeting (Hennig et al. 1995, Reubi et al. 1996).

In our study there also appeared to be a differential loss of nerve types, at least with time, with greater preservation of VIP and SP, both vasodilators, in the submucosa adjacent to Dukes A and B tumours. Since this pattern was not observed in Dukes C cancers the preservation of vasodilatory influence may be a feature of the earlier tumours.

Hence neurotransmitters influence tumour growth either by a trophic effect or by virtue of their influence on blood vessels in the submucosa adjacent to the tumour from which the tumour derives its blood supply. Thus adjuvant treatment based on the knowledge of the range of neurotransmitters that are expressed at the tumour-normal

junction may provide a means of reducing rates of local recurrence and a basis for individualising treatment.

FIGURE LEGENDS

Figure 1 Immunoreactivity to NPY (thick arrows) is present around all types of vessels in the submucosa of normal controls: a, artery and vein; b, arteriole; c, vein. Immunoreactivity is most dense around arterioles. Note autofluorescence of vessel walls (thin arrows). Calibration bar 50 μm

Figure 2 Perivascular immunoreactivity (green) is intense around arterioles (thick arrows) in normal submucosa and in some arterioles adjacent to Dukes A cancers. Note the yellow autofluorescence especially in intima. Calibration bar 25 μm

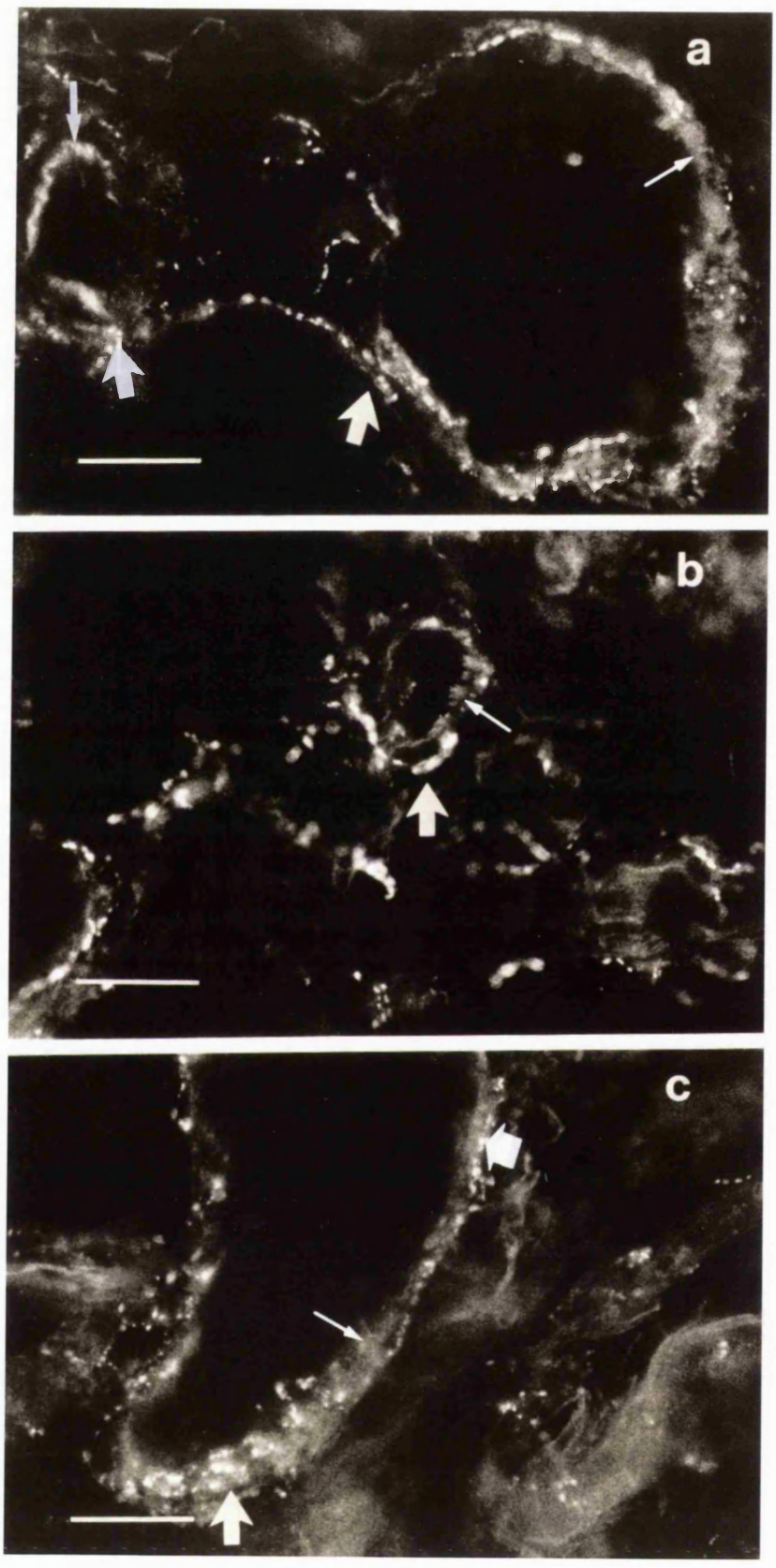

Figure 1

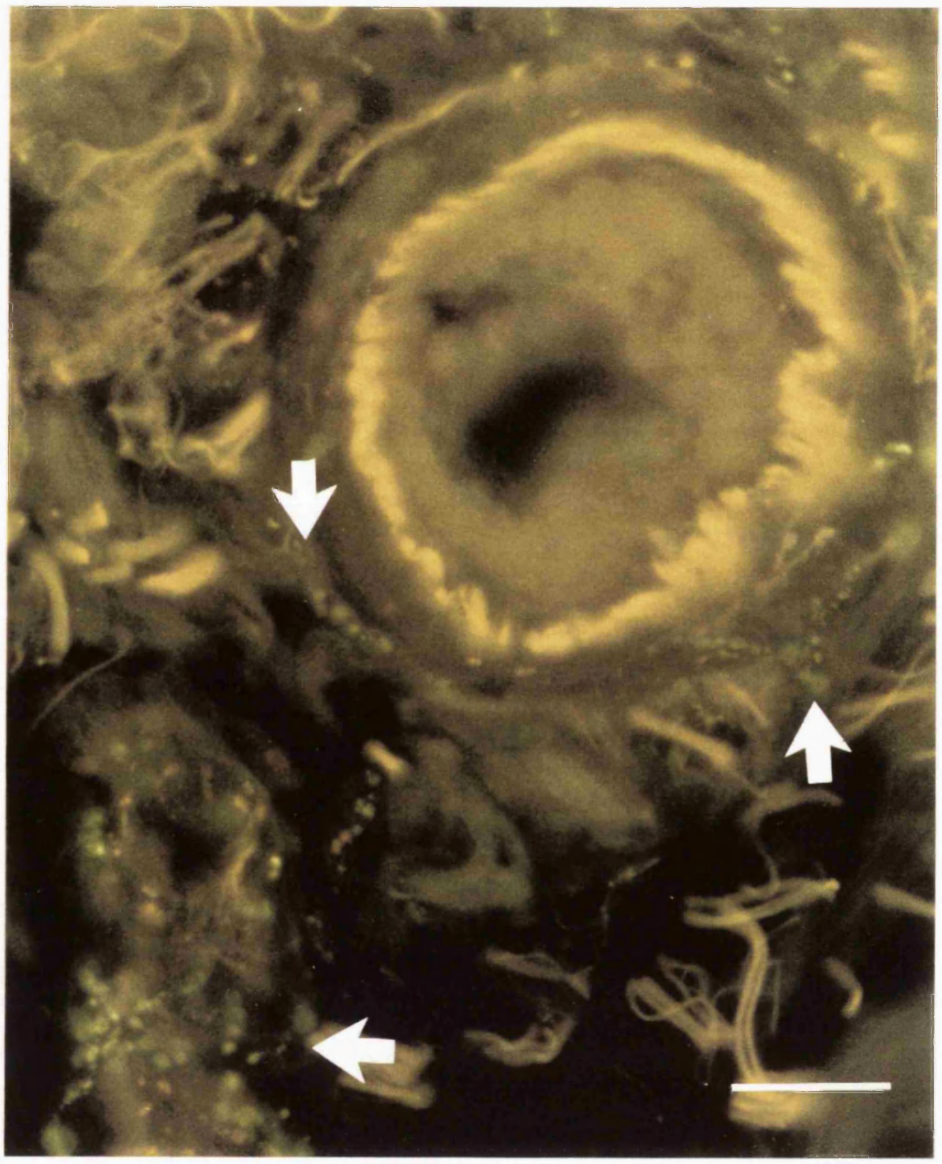

Figure 2

Table 1. Comparison of the percentage of arterioles showing perivascular neural immunoreactivity to TH, NPY, VIP, SP, CGRP in controls and in tissue adjacent to adenocarcinomas. Data are presented here as means ± S.E.M for clarity.

P values* are given in the right hand side for all cancers versus controls. N.S=not significant (p>0.05).

	Controls (n =14)	Submucosa adjacent to adenocarcinomas (n=13)	P values*
TH	68.7 ± *8.1*	5.9 ±*2.6*	P<0.002
NPY	94.9 ± *3.0*	27.3±*10.3*	P<0.002
VIP	50.0 ± *8.6*	17.4±*6.5*	P<0.015
SP	38.8 ± *9.2*	21.5±*8.3*	N.S
CGRP	0.8 ± *0.8*	0.96±*0.96*	N.S

Chapter 4: Innervation of colorectal polyps

CHAPTER 4.The innervation of benign colorectal polyps

Abstract:

We have previously demonstrated an absence of perivascular innervation in colorectal cancers and a loss of perivascular nerves in the adjacent submucosa. In this study we examine the changes that occur in the immunohistochemistry of vasoconstrictor and vasodilator neurotransmitters in nerves supplying colorectal polyps, the precursors of colorectal cancer.

Methods

We studied the perivascular innervation of submucosal arterioles of colorectal polyps (n=15) and the innervation of the epithelial layer the polyps compared with control specimens (n=8) using immunohistochemical markers of the neurotransmitters.

Using neuropeptide Y (NPY) and tyrosine hydroxylase (TH) as markers for sympathetic nerves, vasoactive intestinal peptide (VIP) for parasympathetic nerves, substance P (SP) and calcitonin gene-related peptide (CGRP) for sensorimotor nerves, we focused on the perivascular innervation of submucosal arterioles.

Results: submucosal arterioles

Relative to normal controls, the percentage of arterioles in the submucosa of polyps showing perivascular immunoreactivity was lower to TH (controls 81% vs polyps 41%) and to NPY (97% vs 80%). By contrast VIP and CGRP immunoreactive arterioles were more frequent in polyps (VIP 51% controls vs 69% polyps, and CRGP 0 vs 13%). There was no difference in immunoreactivity to SP (44% vs 43%).

When the adenomas (n=9) and metaplastic polyps (n=6) were analysed separately a similar trend of decrease in TH and NPY with an increase in VIP and CGRP compared to controls was found. There was a decrease in SP in metaplastic polyps and an increase in adenomas compared to controls.

Results: Lamina prppria

In the mucosal layer of benign polyps where nerves of the lamina propria were in close contact with both vascular and cellular elements, neural immunoreactivity to VIP and SP was more intense than in normal colon. There did not appear to be any difference in immunoreactivity to the TH and NPY in benign polyp mucosa and lamina propria compared with normal tissue. CGRP immunoreactivity was rarely encountered.

Conclusion

In summary there was a selective decrease in perivascular immunoreactivity to TH and NPY, sympathetic vasoconstrictor neurotransmitters in polyps compared to controls. By contrast, there was an increase in the vasodilator neurotransmitters, with increased immunoreactivity to VIP and CGRP in adenomas and metaplastic polyps and to SP in adenomas. These results suggest a predominantly vasodilatory neural influence in benign polyps, perhaps indicating a mechanism that maintains polyp growth.

4.1 Introduction

The growth of benign colorectal polyps, precursors of colorectal cancer, can be slowed down by modulating their blood supply. Inhibition of angiogenesis (new vessel formation) in adenomas by agents such as prostaglandin synthase inhibitors (e.g. sulindac, aspirin) are reported to be effective (Marnett 1995). The efficacy of these drugs in larger polyps and in sporadic polyps, as opposed to familial cases of polyposis, has been questioned (Ladenheim et al. 1995).

In this study we consider the feeder vessels of colorectal polyps, as opposed to the new vessels. Both the normal colonic mucosa and polyps are supplied by arterioles that branch at the mucosa/submucosal border (Wolfram-Gabel et al. 1986, Skinner et al. 1995). These feeder submucosal arterioles are the final resistance vessels in the enteric circulation and are endowed with the richest innervation of the microcirculation.

Although within colorectal cancer perivascular nerves are mainly absent, the feeder vessels in tissue adjacent to the tumour are innervated (Mitchell et al. 1994). Using specific neurotransmitter markers we have previously shown that in submucosa adjacent to colorectal cancers, there is a decrease in autonomic perivascular nerves and

the innervation profile is different when compared to normal submucosa (Chamary et al. 1998, 2000a).

In the present study we examined the immunoreactivity to the neurotransmitters neuropeptide Y (NPY) and tyrosine hydroxylase (TH) (sympathetic nerve markers), vasoactive intestinal peptide (VIP) (parasympathetic), substance P (SP) and calcitonin gene-related peptide (CGRP) (sensory-motor). We focused on immunoreactivity in the feeder submucosal vessels and in the lamina propria of colorectal polyps.

The study was designed to examine the differences between polyps and controls with a view to determining whether therapeutic manipulation of the blood supply of colorectal polyps might be a feasible approach to reducing tumour growth and development.

4.2 Results

Pathological groups

15 polyps (9 adenomas, 6 metaplastic polyps) and 8 normal controls were analysed quantitatively.

Submucosal arterioles: total vessel count

The mean total number of submucosal arterioles was 6.5(±0.3) for the normal controls obtained from the tumour resection specimens, 6.0(±0.3) for the adenomas, and 6.1(±0.3) for the metaplastic polyps (3 fields, x250 magnification). No significant difference was found between the pathological groups.

When the total count was analysed in relation to the neurotransmitters used no significant difference was found. The means were as follows: 6.0(±0.4) for TH, 6.1(±0.5) for NPY, 6.2(±0.3) for VIP, 6.0(±0.3) for SP and 6.3(±0.5) for CGRP.

Submucosal arterioles: perivascular innervation

Comparison of perivascular immunoreactivity was made between controls and polyps. Both perivascular and paravascular nerves were present around the arterioles of normal controls and polyps. However, the intensity of perivascular immunoreactivity to NPY in polyp arterioles was generally weaker than in controls (fig.1).

The percentage of arterioles showing perivascular immunoreactivity to the neurotransmitters differed in polyps compared to controls. We analysed our data by comparing all benign polyps with the controls and also subdividing the polyps into metaplastic and adenomatous polyps.

Benign polyps (n=15) versus normal controls (n=8)

Benign polyps comprised the adenomas and the metaplastic polyps grouped together (n=15). There was a decrease in the parasympathetic neurotransmitters in benign polyps compared to controls, TH (controls 81% benign polyps 41%) and NPY (97% versus 80%). By contrast immunoreactivity to VIP (51% versus 69%) and to CGRP (0% versus 12.9%) increased in polyps (table 1). No difference was noted in perivascular immunoreactivity to substance P (SP) (44% versus 43%).

Adenomas (n=9) and metaplastic polyps (n=6)

When the adenomas and metaplastic polyps were analysed separately a similar trend of decrease in immunoreactivity to TH compared to controls (controls 81%, adenomas 46%, metaplastic polyps 35%) and to NPY (97%, 76%, 84%) was found. An increase in immunoreactivity to VIP (51%, 69%, 69%) and CGRP (0%, 15%, 8%) was present. There was a decrease in SP in metaplastic polyp and an increase in adenomas compared to controls (table 1).

Innervation of the mucosa

Normal mucosa

In the normal mucosal layer, nerve fibres were present in the lamina propria coursing towards the apex of the colonic glands, and were frequently found in close association with the cells of the glands (fig. 2).

The intensity of these fibres immunoreactive to VIP, SP, and NPY were high. In samples where the glands were cut in transverse section fine SP fibres were visualised forming a hexagonal pattern around the colonic cells. Similarly NPY-immunoreactive fibres were seen in close apposition to the cells.

The cytoplasm of colonic cells throughout the glands immunoreacted to SP but not to the other neurotransmitters.

Polyps

Examination of the lamina propria of both adenomatous and metaplastic polyps revealed increased neural immunoreactivity to VIP and SP compared with normal controls (fig. 3). The VIP-immunoreactivity was high throughout the depth of the periglandular tissue and at the base near the muscularis mucosa (fig.4). Immunoreactivity was intense to SP within the lamina propria of many polyps with fibrillar appearance similar to normal controls but often more intense.

In some samples the muscularis mucosa immunoreacted well to CGRP. Immunoreactivity to TH was present in a few polyps within the muscularis mucosa but rarely was this evident elsewhere within the lamina propria. There did not appear to be any change from normal in NPY-immunoreactivity.

The epithelial cells showed diffuse cytoplasmic immunoreactivity to SP in the majority of polyps.

4.3 Discussion:

Studies of benign and malignant colorectal tumours both in the experimental situation in animal models and in the human colon reveal that the circulation in colorectal tumours is linked to the adjacent submucosa via submucosal arterioles (Skinner et al. 1990, 1995). Our previous study demonstrated a decrease in the innervation of arterioles in the submucosa adjacent to colorectal cancer (Chamary et al. 1998, 2000a). The finding of altered perivascular immunoreactivity in arterioles supplying polyps warrants discussion.

Immunoreactivity to TH and NPY, vasoconstrictor substances, was decreased in arterioles supplying both metaplastic and adenomatous polyps. This difference observed between polyps, precursors of colorectal cancer, and controls is of interest, since it parallels the difference seen between normal submucosal arterioles and those supplying carcinomas in our previous study (Chamary et al. 2000a). It is hypothesised that polyps acquire the ability to affect the sympathetic perivascular nerves in their progression towards becoming carcinomas.

The higher levels of perivascular immunoreactivity to vasodilator substances in submucosal arterioles supplying both adenomas and metaplastic polyps suggest that there is a major vasodilatory influence early in the development of

colorectal tumours. The vasodilatory influence exerted by VIP, a potent vasodilator, would also be maintained by the increase noted in CGRP and SP around the arterioles of the submucosa of polyps.

Arteriolar dilatation resulting from an increase in VIP, and CGRP in all polyps, as well in SP in adenomas, would lead to an increase in blood supply locally, an effect that would maintain polyp tissue growth. Such growth is open to therapeutic manipulation.

However the drugs which are currently available are effective in controlling the growth of adenomas of small size mainly (Nugent 1995, Ladenheim et al. 1995). Our studies suggest that an alternative approach to the treatment of polyps may be based on the neurotransmitters involved in the control of polyp arterioles.

Furthermore VIP-immunoreactivity was also higher in the lamina propria of benign polyps compared with normal mucosa. In the normal colon, VIP is implicated in the nerve-mediated and atropine-resistant electrolyte and mucus secretion by the mucosa (Phillips et al. 1984, Waldman et al. 1977). The increase in VIP- neural immunoreactivity in the lamina propria close to the mucosal cells suggests that VIP may play an important role in the pathophysiology of polyps. VIP may therefore be responsible for the increase in fluid and electrolyte

loss, particularly potassium, and in mucus secretion observed in adenomas especially those of villous types (Lee and Keown 1970 and Shnitka et al. 1961).

Thus the inhibition of VIP-dependant vasodilatation may not only alter growth through its actions on the submucosal arterioles but also modify the pathological effects of adenomas, acting through local mechanisms on the mucosal cells.

Table 1. Mean percentage (± S.E.M.) of arterioles with perivascular immunoreactivity to tyrosine hydroxylase (TH), neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), substance P (SP) and calcitonin gene-related peptide (CGRP).

		Polyps	
	Normal Tissue	Adenomas	Metaplastic Polyps
TH	81.3±10.8 (8)	45.6±13.2 (9)	35.2±17.3 (6)
NPY	97.1 ±3.3 (6)	76.3±12.9 (9)	84.6±5.6 (6)
VIP	51.3 ±8.7 (8)	69.4±11.5 (9)	69.3±14.4 (6)
SP	44.3±16.6 (7)	52.2±14.2 (8)	30.2±11.8 (6)
CGRP	0 ± 0 (7)	15.9±11.5 (9)	8.33±8.33 (6)

FIGURE LEGENDS

Figure 1 NPY-immunoreactivity in perivascular nerves of control tissue and benign polyps. Immunoreactivity to NPY (large arrows) is more intense (indicated by larger photographic exposure times) in controls (**a, b**) relative to benign polyps (**c, d**). Note small arrows indicating autofluorescence of the arterial wall. Calibration bar 50 μm.

Figure 2 Immunoreactivity to SP and VIP in nerve bundles and in mucosa of colorectal polyps. Immunoreactivity to SP (**a, c**) and VIP (**b, d**) in nerve bundles is intense (arrows). Arrow heads denote diffuse immunoreactivity to SP in the cytoplasm of polyp cells, and localised immunoreactivity in the base of polyp cells to VIP. Calibration bar 50 μm

Figure 3 Neural immunoreactivity to VIP around submucosal vessels and mucosal glands in benign polyps. Perivascular immunoreactivity is intense (arrows), fig 3A and 3B, around submucosal vessels (arrowheads), and in association with mucosal cells (g) at the base of polyps, fig 3B, and in the lamina propria, fig 3C. m=muscularis mucosa, g=glandular tissue. Calibration bars in A and C=20 μm, in B=50 μm

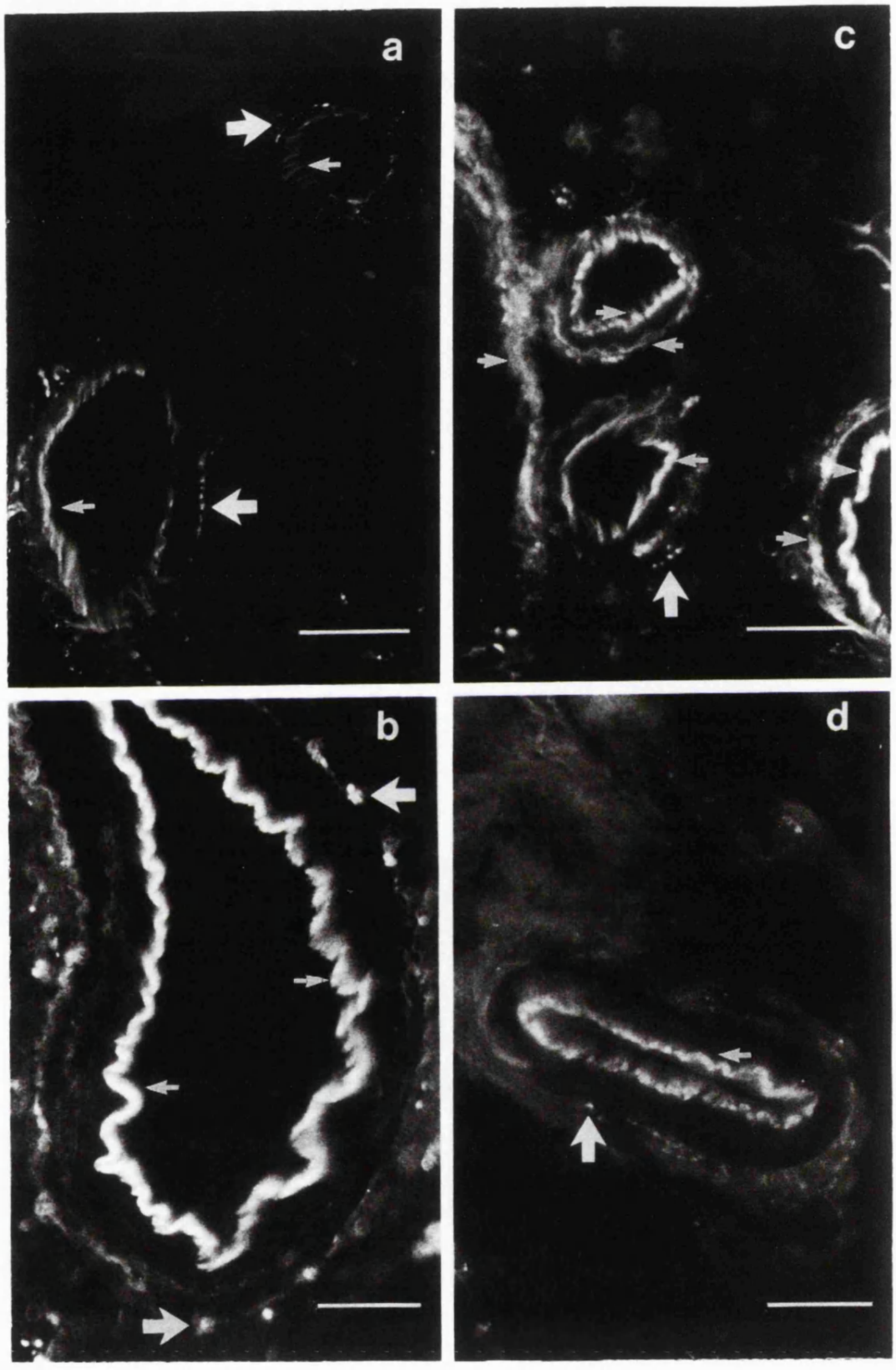

Figure 1

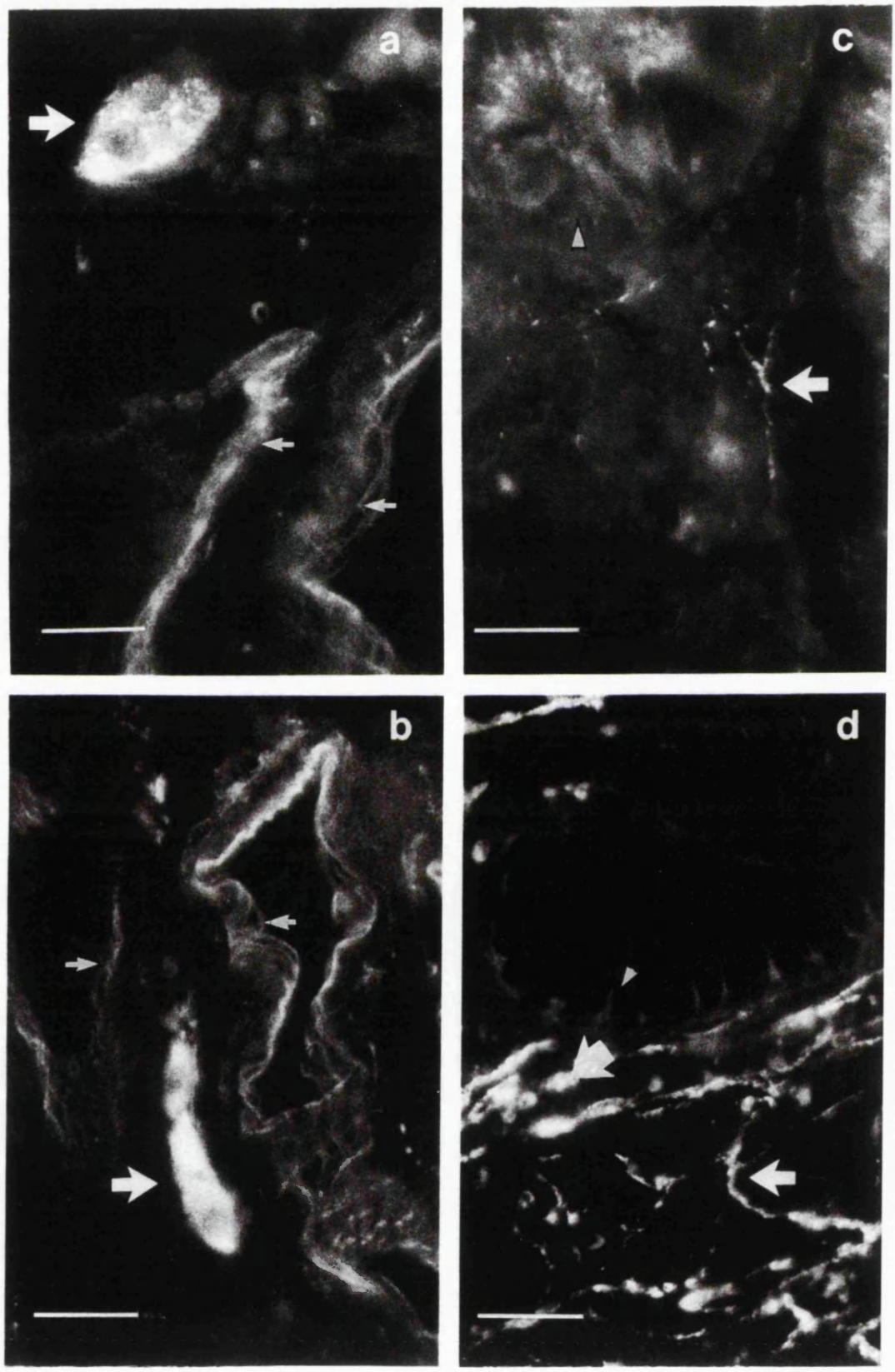

Figure 2

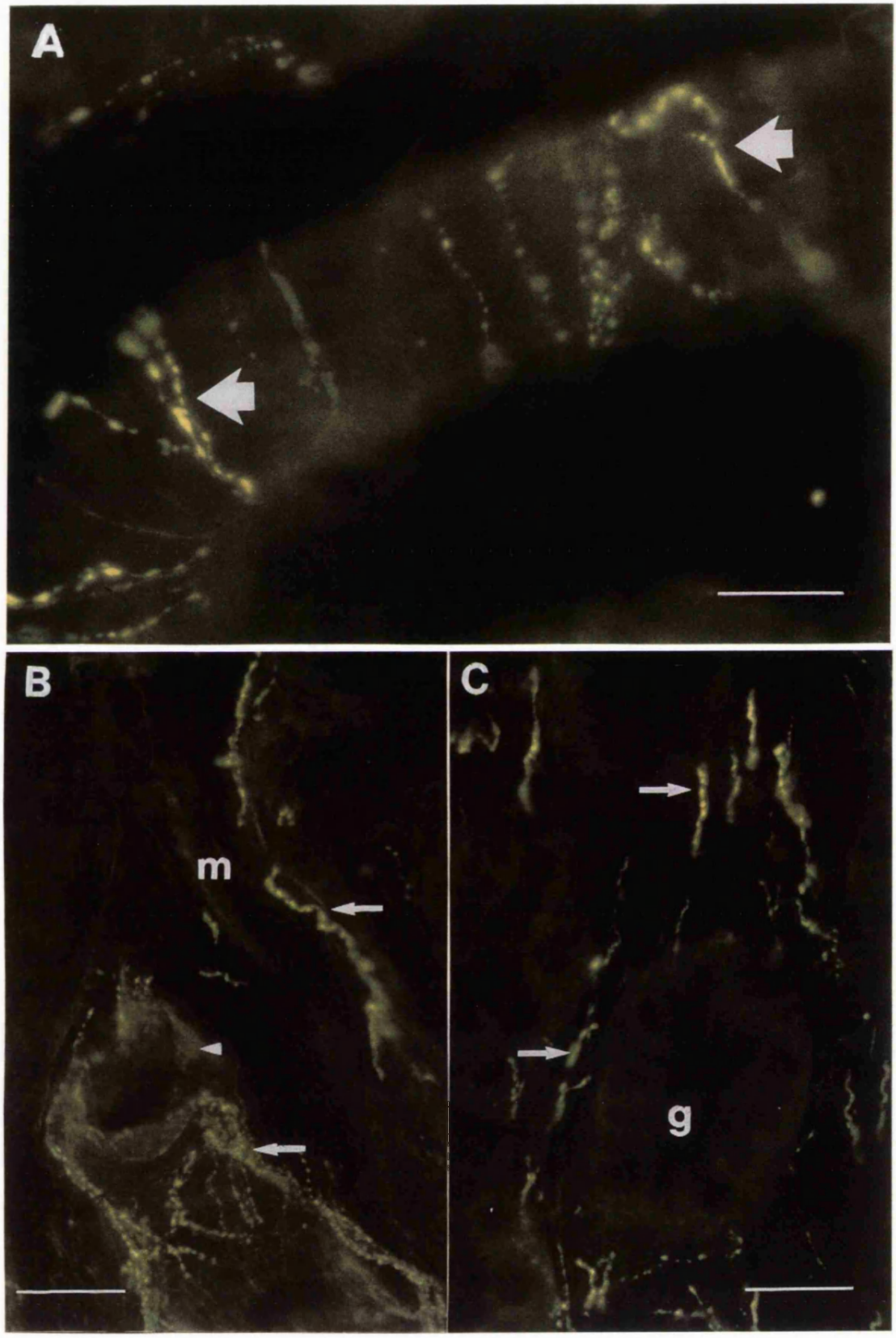

Figure 3

Chapter 5: Ultrastructure of the microvasculature of colorectal tumours

5.1 Introduction

5.2 Results: Normal submucosal vessels
Malignant tumour vessels
Dysplastic tissue
Mucosa adjacent to cancers
Benign adenomas
Malignant polyp

5.3 Discussion

CHAPTER 5. ULTRASTRUCTURE OF THE MICROVASCULATURE OF COLORECTAL TUMOURS, AND THEIR VASCULAR SMOOTH MUSCLE PHENOTYPES

Abstract

In the normal, contractile smooth muscle cells of blood vessels regulate vascular tone, whilst perivascular nerves and the endothelium modulate the activity of the smooth muscles. In order to investigate the reason for differences in the vascular response in colorectal tumours from normal, we studied the ultrastructure of blood vessels within and around colorectal tumours in comparison to normal colon, with particular reference to the smooth muscle elements.

Methods

Samples were obtained from colorectal cancers, colorectal polyps and normal colon. Vessels in selected areas of mucosa, dysplastic and neoplastic epithelium, as well as submucosa were studied by transmission electron microscopy.

Results

The majority of vascular smooth muscle cells in normal mucosa and submucosa of controls as well as in the normal submucosa adjacent to colorectal tumours displayed the ultrastructural features of a contractile phenotype.

In normal submucosa adjacent to primary colorectal polyps and cancer, arterioles showed reduction or loss of innervation.

Within colorectal cancer, smooth muscle cells of blood vessels were either absent or formed an incomplete layer, and showed a secretory rather than a contractile phenotype. The endothelial cells displayed features of high activity. Perivascular nerves were lacking.

Within colorectal polyps smooth muscle cells were of mixed phenotypes. The more advanced polyps showed features akin to malignant tumours; the smooth muscle cells were of a predominantly secretory phenotype, there was a reduction in perivascular innervation around vessels and the endothelial cells showed a high degree of activity.

Conclusion

It is suggested that the presence of secretory smooth muscles and loss of perivascular nerves in polyps may be useful markers of transformation towards malignancy, and may explain the differences in vascular response of tumours compared to normal.

5.1 Introduction

The reasons for the differences observed in the response of tumour blood vessels to vasoactive agents compared to normal are not clear (Mattsson et al. 1982). Since a regular layer of smooth muscle cells has been demonstrated in some tumours other factors must be considered (Mattsson et al. 1982). Whilst there have been studies on the ultrastructure of blood vessels in colorectal tumours, none have focused on the phenotypic appearances of vascular smooth muscles.

The architectural arrangement of vessels of the colonic wall has been the subject of previous studies by electron scanning microscopy (Skinner et al. 1990, 1995). Mucosal capillary loops arise from submucosal arterioles at the basal, abluminal side of the mucosa in the Sprague-Dawley rat and in the guinea pig colon (Aharinejad et al. 1992). In the normal human colon a similar arrangement of mucosal capillaries is observed; these capillaries arise from arterioles that divide at the border of the submucosa with the mucosa at the level of the muscularis mucosa (Skinner et al. 1990, 1996).

It has been demonstrated by scanning electron microscopy in a rat model and in studies on human tissue, that benign and primary malignant colonic tumours have tumour vessels which are continuous with pre-existing

vessels of the mucosa, submucosa and muscularis propria (Skinner et al. 1990). The tumour vessels differ in structure showing elongation, increase in diameter and density, loss of the hexagonal pattern present around the normal mucosa, and a lack of organisation (Skinner et al. 1995, Sun et al. 1992).

Further studies have revealed that changes are present at the cellular level. Ultrastructural studies of tumour vasculature in rat liver metastases have revealed a lack of smooth muscle cells in blood vessels within the tumours (Ashraf et al. 1997). In human liver metastases blood vessels are endowed with proliferative smooth muscle cells in human liver metastases (Ashraf et al. 1996).

Other structural changes have been described in human liver tumours. At the electron microscope level, hypoplasia of arteriolar smooth muscle cells in hepatocellular carcinoma has been demonstrated. In addition there is decreased thickness of tumour vessel media and to a lesser degree in the media of host arteries supplying hepatocellular carcinoma (Suzuki et al. 1987).

In primary colorectal cancer, light microscopic studies have revealed a loss of innervation around blood vessels within the tumours and a reduction in perivascular innervation in submucosa adjacent to the tumours (Chamary et al. 2000a). In benign colorectal tumours perivascular

nerves are in general preserved whilst a reduction in these nerves was found in polyps with a malignant component (Chamary et al. 1999).

In the present study we examine the ultrastructure of the blood vessels supplying the mucosa and the submucosa in benign and malignant primary colorectal tumours in comparison with normal colon, with particular reference to their perivascular innervation, and the phenotypic appearance of the smooth muscles.

5.2 Results

For controls, vessels in the lamina propria at various levels of the mucosal layer and within the submucosa are described first. In diseased tissue vessels within tumour stroma, and within both mucosa and submucosa adjacent to tumours are next described, first in malignancy then in polyps.

Smooth muscles around blood vessels analysed at transmission microscopy were classified as contractile and synthetic (proliferative) according to their ultra-structural features, in keeping with the definitions given by Chamley-Campbell et al. (Chamley-Campbell et al. 1979). Contractile smooth muscle cells were characterised by an elongated shape, an abundance of plasmalemmal dense bands, myofilaments and cytoplasmic dense bodies. These cells contained only some Golgi apparatus and ribosomal endoplasmic reticulum (RER).

By contrast, synthetic phenotypes were characterised by shorter cells (hence nuclei were seen more often in cross section), with an abundance of Golgi apparatus and RER. Small amounts of myofilaments, dense bands and cytoplasmic dense bodies were present. As in contractile phenotypes there were calveoli and a continuous basement membrane.

Vessels in normal tissue at least 5 cms from cancer

In the lamina propria close to the muscularis mucosa, the vessels were typically endowed with a layer of perivascular cells, although this was sometimes incomplete. The smooth muscles were of contractile phenotype, and were arranged in a circular fashion around the lumen. The endothelium was of normal structure with villous like cytoplasmic projections into the lumen of the vessel, and in places it was in direct apposition to the smooth muscles. Cytoplasmic organelles were visible at high magnification. The connective tissue of the lamina propria contained a large number of nerves.

In the interglandular space the ultrastructural features of the blood vessels were similar to those of the lamina propria near the muscularis mucosa. Tight junctions were present in the endothelial layer. Nerves were found in close proximity to the blood vessels.

At the level of the capillaries, venules were thin walled, and were surrounded by pericytes forming a discontinuous layer. The basement membrane was lacking on the endothelial aspect, fenestrations were present, and characteristic Weibel-Palade bodies were observed (Weibel and Palade 1964). There were fewer organelles compared to the other vessels such as veins.

Submucosal vessels were larger in diameter compared to mucosal vessels. The arterioles possessed an external layer of fibroblasts, typical of arterioles in this tissue layer. The smooth muscle cells were of contractile phenotype and formed a complete layer around the blood vessel (fig. 1A,B). The basement membrane was well defined, often several layers thick. Smaller arterioles displayed a multilayer basal lamina similar to that seen in capillaries. The endothelium was of normal structure. Perivascular nerves were visible around the vessels (fig 1A). There was a close relationship between perivascular nerves and smooth muscle cells.

Veins of large diameter at the level of the submucosa displayed a complete layer of perivascular cells, with the characteristics of smooth muscle cells. The innervation of veins was less dense than arterioles.

SUMMARY OF STRUCTURE OF VESSELS IN CONTROLS

LAMINA PROPRIA	SUBMUCOSA
Endothelium: Arteriole All vessels, Capillary Monolayer Venule Vein	Endothelium: Monolayer Vein: Many W-P bodies.
Basal lamina: ALL vessel types: Continuous but fenestrated in capillaries.	Basal Lamina: Arteriole: Thick, with ring elastic lamina. Capillary: Multilayered
Smooth muscle: Complete layer around arterioles (Mucosa-submucosa junction).	Smooth muscles: Many cells thick in arterioles.
Pericytes: Few perivascular cells around arterioles and capillaries.	Pericytes: Forms continuous layer with smooth muscles in arterioles. In submucosa fibroblast type cells form an external layer

Malignant tumours vessels

In the outer layers of the malignant tumours the stroma contained an increased density of capillary like vessels with features of newly formed vessels. The smooth muscle layer showed poor organisation with a fragmented appearance and loss of contractile phenotype. Smooth muscle cells of arterioles were of varying phenotypes. Most cells were immature with varying degrees of secretory activity and integrity of the smooth muscle coat, whilst some showed mature features and contractile smooth muscle phenotypes. A longitudinal arrangement of smooth muscle cells in relation to the lumen was characteristic of arterioles within malignant tumours (fig 2).

The endothelium was synthetically active and formed a complete layer. There were no perivascular nerves (fig 3 B). Within a submucosal tumour mass, neovasculature was present; there were no perivascular nerves, and the perivascular cells were active.

Close to dysplastic epithelium, new vessels were found. In addition there were a number of large vessels. These contained smooth muscle fragments of largely secretory smooth muscle cells. No perivascular nerves were present.

Vessels in dysplastic tissue adjacent to malignant tumour

Within the area of dysplastic epithelium at the edge of the malignant growth, some of the larger vessels including the arterioles were of normal structure but the level of innervation was low even in these vessels. The endothelium showed evidence of increased synthetic activity.

At the interface of tumour and normal submucosa, arterioles were of contractile phenotype. Perivascular nerves were absent, whilst paravascular nerves were sometimes evident (fig 3A). In contrast arterioles in the adjacent muscular layers free of tumour were innervated in some samples.

Vessels in normal tissue adjacent to malignant tumour

In the normal mucosa adjacent to the malignant tumour, where new vessels were present as well as pre-existing mature ones, the arterioles were of normal structure. Close to dysplastic epithelium, the normal mucosa displayed pre-existing and new vasculature. The smooth muscle cells of pre-existing arterioles were of contractile phenotype. The endothelium showed moderate features of synthetic activity. The endothelial lining of the new vessels on the other hand was incomplete, the endothelial cells showed features of increased synthetic activity, and their basement membrane was lacking in

fenestra. Further away from the areas of dysplasia the endothelium in both smaller and the larger vessels displayed normal appearances. The level of innervation of the tissue appeared decreased

In the normal submucosa adjacent to malignant tumours the vessels were like normal control vessels. The vascular smooth muscles were mature and were of contractile phenotypes (fig 1B). The level of perivascular innervation was in general decreased.

Summary of perivascular innervation and smooth muscle phenotypes in and around cancers

The innervation of arterioles in the stroma of malignant tumours sampled in their peripheral part was lacking and in the dysplastic epithelium close to the tumours innervation was decreased. The submucosal arterioles had decreased perivascular innervation.

The smooth muscles of the blood vessels were of both secretory and contractile phenotypes in areas of dysplastic epithelium. By contrast arterioles in the normal submucosa adjacent to malignant tumours were of the same morphology as controls with smooth muscle cells of a predominantly contractile phenotype.

Within cancers the vascular smooth muscle cells were oriented in a longitudinal direction as opposed to normal

controls where they were arranged transversely in relation to the vessel lumen.

Vessels in benign polyps

The submucosa of benign polyps consisted of loose connective tissue. Arterioles were present in the submucosa. The muscularis mucosa was of irregular thickness and sometimes absent. Dysplastic epithelium was sometimes present close to the muscularis mucosa. In the lamina propria blood vessels were of larger calibre than normally seen at these sites particularly those close to the epithelium and the base of the glands.

In the stalk of a polyp, the structure of the arterioles appeared normal. The smooth muscles were of contractile phenotype, thin fibroblast cells encompassed the vessels in a similar fashion to normal controls, and the vessels were innervated (fig 4). Infrequently the vessels had smooth muscle cells of a less contractile phenotype displaying a low density of myofilaments and clear regions in the cytoplasm (fig 5).

However, the endothelial cells showed an increase in villar projections. Their basal lamina was single layered, there was a lack of internal elastic lamina, the space between the basal lamina and the perivascular cells

was increased, and cytoplasmic vesicles and cell bodies were numerous.

Many nerves were in close proximity to the vessels present but overall there appeared to be a decrease in the density of innervation, although nerves were present throughout the polyp.

In the body of polyps some large vessels were observed, many of these vessels with a thickened wall. The phenotypes of smooth muscles were variable but usually showed features of contractility with a high filament content. Mixed features were present in some arterioles which displayed a thick muscle wall (2-3 cell thick) and a high filament content (contractile), whilst showing characteristic secretory features with extended Golgi apparatus and dilated rough endoplasmic reticulum (secretory). Arterioles often lacked elastic material. Cells within veins showed marked secretory activity, particularly the endothelium.

A high level of innervation was present in the body of polyps. However these nerves were not always closely associated with the vessels.

Vessels in polyp with carcinoma

Tissue close to the malignant focus was examined in a polyp with an adenocarcinoma. Close to the invasive glandular tissue the arterioles had contractile smooth

muscles but the muscle coat was incomplete, with features of increased synthetic activity. Innervation was lacking.

In an area within the muscularis externa adjacent to this, but not invaded by malignant tissue, the arterioles were of contractile phenotype and the internal elastic lamina was present. These arterioles were innervated.

Summary of polyp vessel structure by site and type

In the lamina propria of benign polyps, blood vessels were of larger diameter compared to normal, the internal elastic lamina was lacking in some vessels.

The vessels of the submucosa of benign polyps were of normal structure. In some areas however there was a loss of internal elastic lamina in the arterioles and the vessel wall appeared thicker.

In a polyp with a focus of carcinoma, in tissue close to the cancer, the vascular smooth muscle layer was incomplete; the smooth muscle cells showed evidence of increased secretory activity.

Whilst the innervation of submucosal vessels was absent in polyp tissue associated with malignancy, it was reduced in benign polyps.

5.3 Discussion

Whilst there have been studies on the ultrastructure of blood vessels in primary colorectal tumours, none have focused on the phenotypic appearances of vascular smooth muscles. Within tumours in general, a lack of smooth muscle elements in blood vessels within tumours has been documented; these vessels were mostly capillary type structures and were characterised by dilatation, abnormal shapes and architecture (Bossi et al. 1995).

It has been suggested that the lack of smooth muscle cells in tumour vessels is responsible for the differences observed in the response of tumour blood vessels to vasoactive agents compared to normal. However, since a regular layer of smooth muscle cells has been demonstrated in some tumours other factors must be considered (Mattsson et al. 1982).

Tumours may differ from normal not only with respect to the degree to which their blood vessels possess smooth muscle cells but also with respect to the types of smooth muscles. Two phenotypes of vascular smooth muscles have been described (Chamley-Campbell et al. 1979). Mature smooth muscles have a predominantly contractile function, whilst immature smooth muscles which are present at the early stages of development or during regeneration are endowed with the ability to migrate and proliferate.

Mature smooth muscles are elongated cells with a central nucleus and are characterised by an abundance of myofilaments. Secretory (or synthetic) smooth muscle cells, are characterised by an abundance of intracellular organelles such as endoplasmic reticulum and Golgi apparatus. The expression of myoproteins also differs in contractile compared to secretory smooth muscles. Desmin decreases and vimentin increases in the secretory immature phenotypes of smooth muscles (Kacem et al. 1997).

In an experimental model of rat sarcoma the microvascular bed studied by transmission electron microscopy contained vessels with contractile muscle (Mattsson et al 1982). However the vasculature within liver metastases was shown to lack smooth muscle cells (Ashraf et al. 1997), whilst blood vessels in human liver metastases were endowed with proliferative rather than contractile smooth muscle cells (Ashraf et al. 1996). Other sructural changes have been described in the human liver; regression changes in the media of host arteries supplying hepatocellular carcinoma were present at electron microscopic level (Suzuki et al. 1987). In benign primary colorectal tumours, blood vessels have been documented to possess normal structural characteristics, although specific studies of muscle phenotypes have not been reported (Wang and Campiche 1982).

In our study various areas of benign and malignant colorectal tumours and normal adjacent tissue were examined. Whilst in the submucosa adjacent to cancers most of the arterioles have similar characteristics to normal controls, we describe a number of ultrastructural differences between tumour arterioles both within the tumour and in mucosal tissue adjacent to the tumours.

In particular, secretory smooth muscle phenotypes are present in malignant tumours where the muscular layer is often lacking and incomplete, whilst in polyps vascular structures are more akin to normal are present. In advanced polyps however, that is in those polpys with features of malignant change, an increasingly more secretory phenotype is displayed by the smooth muscle cells. Therefore the degree to which colorectal tumours affect vessel structure would appear to depend on the degree of malignancy and the effect of neoplastic cells on surrounding tissue.

The orientation of smooth muscle cells also has a bearing on their function. Whilst in the normal smooth muscle cells were circularly arranged, and thus capable of causing constriction of the blood vessel, in the malignant tumours their longitudinal arrangement, as demonstrated in our study, would impair effective vasoconstriction.

In this study of ultrastructure, we show that blood vessels within colorectal cancers display a lack of perivascular innervation whilst those of normal tissue and of benign lesions are innervated. The present study thus confirms that most of the arterioles in the submucosa adjacent to the cancers with contractile smooth muscles have abnormal innervation (Chamary et al. 2000), suggesting that nerve loss has occurred around the pre-existing arterioles.

The mechanism by which tumours influence blood vessel phenotypes or their innervation is not yet clear. However when blood vessels were denervated in experimental studies changes in the smooth muscles occurred, suggesting trophic neural influences on vascular smooth muscles (Kacem et al. 1997). The lack of perivascular nerves within tumours is associated with absence or change in the vascular smooth muscle (Ashraf et al. 1996, 1997).

Progressive and parallel changes in innervation and vessel ultrastructure were demonstrated in the present study, suggesting that the loss of perivascular nerves precedes changes in vascular smooth muscle cells.
The colorectal cancer cells themselves may be directly responsible both for nerve loss and structural changes in blood vessels, probably through the release of tumour factors. Further evidence that the cellular elements of

tumours may affect innervation is also provided by our light microscopy study on colonic adenomas; the submucosal arterioles of adenomatous polyps, precursors of colorectal cancer, show decreased perivascular sympathetic innervation (Chamary et al. 1999).

A predominance of smooth muscle cell phenotype in the arterioles supplying colonic polyps and a loss of perivascular innervation are potential markers of malignant progression, and may become useful for diagnostic purposes.

LEGENDS TO FIGURE 1

1A. Arteriole of the normal submucosa in control samples at least 5 cms from the tumour specimen, in transverse section. Smooth muscle cells (smc) are arranged in a circular manner and are contractile. Note the tight junctions between the endothelial cells (ec). Collagen fibres are present between the endothelium and smooth muscle cells. A layer of fibroblasts (f) is typical of arterioles of the submucosa. Note the presence of perivascular innervation (arrow). Calibration bar: 2 micrometers
L=lumen.

1 B. Arteriole of the normal submucosa adjacent to colorectal cancer, within 3 mm of the tumour edge, showing structural features similar to control submucosal arterioles. Note the microvilli in the endothelial cells (ec), the complete layer of smooth muscles (smc). Small nerves are present (arrows). Calibration bar: 5 micrometers.
L=lumen.

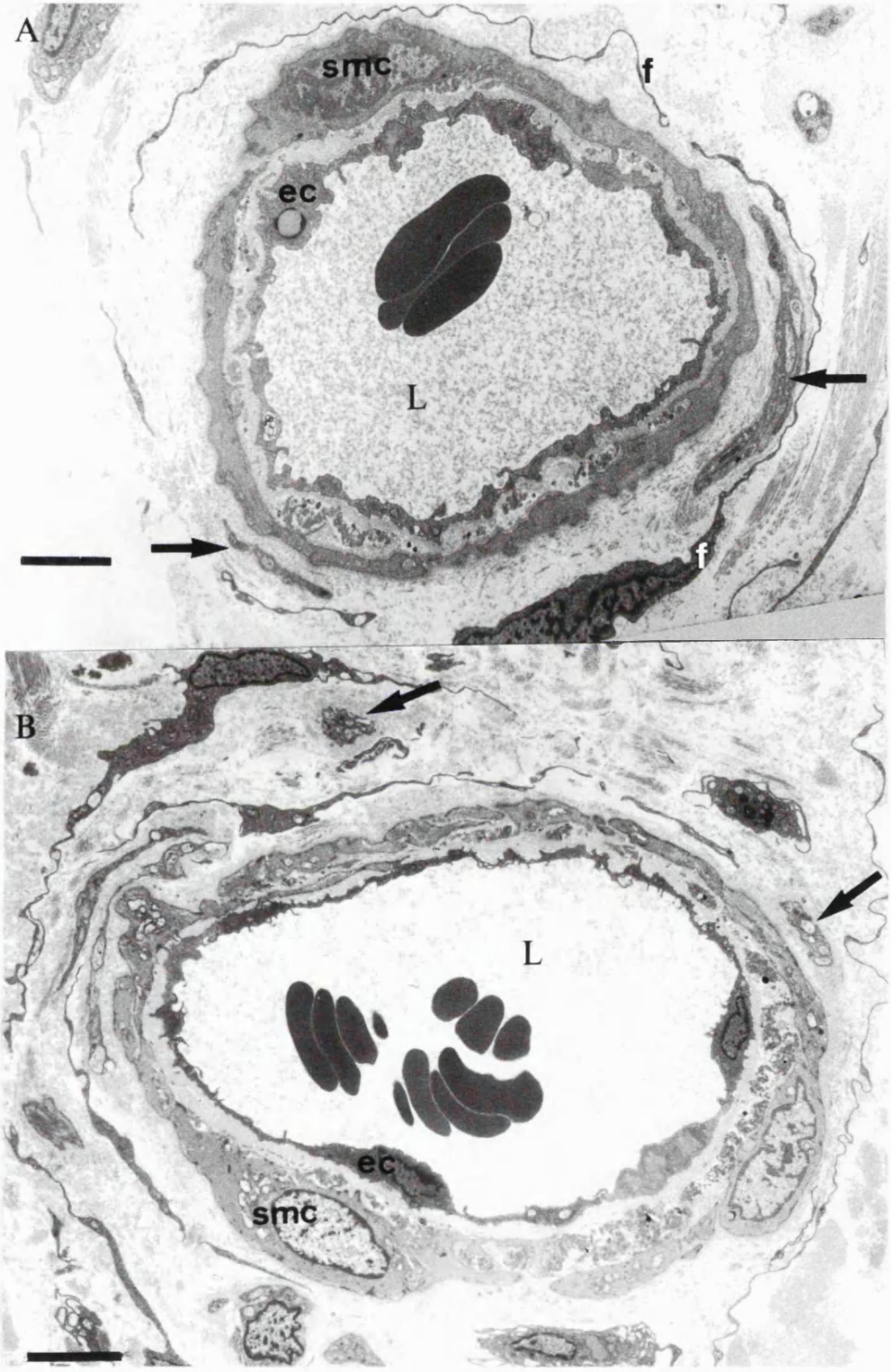

Figure 1

LEGEND TO FIGURE 2

Structure of smooth muscle cell (smc) of an arteriole within a colorectal cancer, shown in longitudinal section. The muscle cell is oriented in a longitudinal rather than a circular direction. There is a prominence of intracellular organelles (arrow) and a lack of myofilaments indicating a secretory phenotype. No nerves are present. ec= endothelial cell. L=lumen. Calibration bar: 2 micrometers.

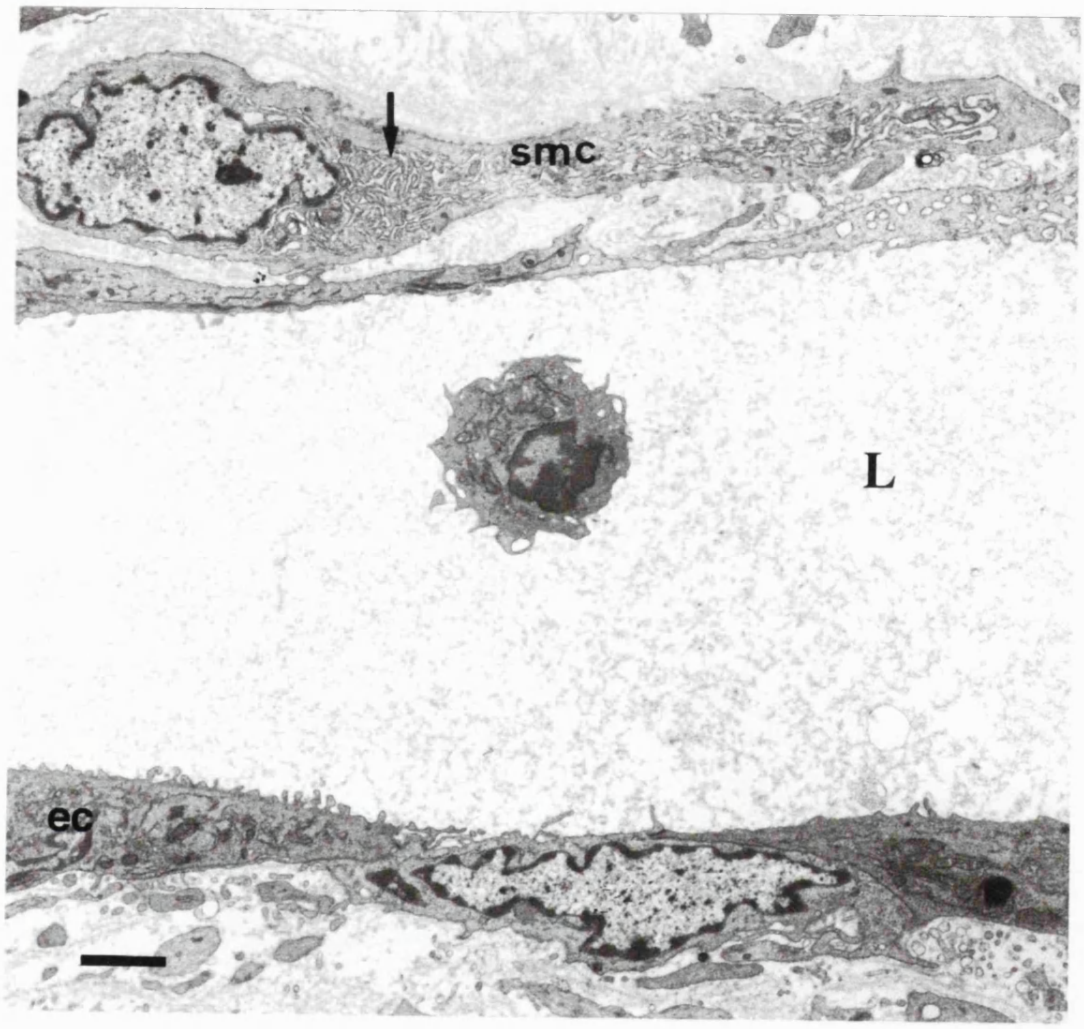

Figure 2

LEGENDS TO FIGURES 3 AND 4

3. Ultrastructure of arteriole within 100 microns of the tumour edge, (A) outside the tumour, (B) inside the tumour.

(A). In the tissue adjacent to the cancer the number of smooth muscle cells (smc) appear increased, the individual cells shorter than in controls, the cells displays myofilaments, which are less prominent than in controls. The endothelial cells (ec)appear thicker. Note the presence of a white cell adhering to the endothelium. No nerves are present.

B. Arterioles within the cancer show a fragmented appearance, there is a lack of nerves, and the smooth muscle cells (smc) display a less contractile phenotype.

Calibration bars (A & B): 5 micrometers.

4A & 4B Structure of submucosal arterioles of benign adenomatous polyps. The vessels have a disorganised appearance. There is an increase in the number of nuclei visible in the endothelial layer (ec) and the nuclear: cytoplasmic ratio is increased. Myofilaments are present in the smooth muscle cells (smc), the cells appear shorter. A few nerves are present in 4B (arrow).

L=lumen.

Calibration bars: A=5 micrometers. B=2 micrometers.

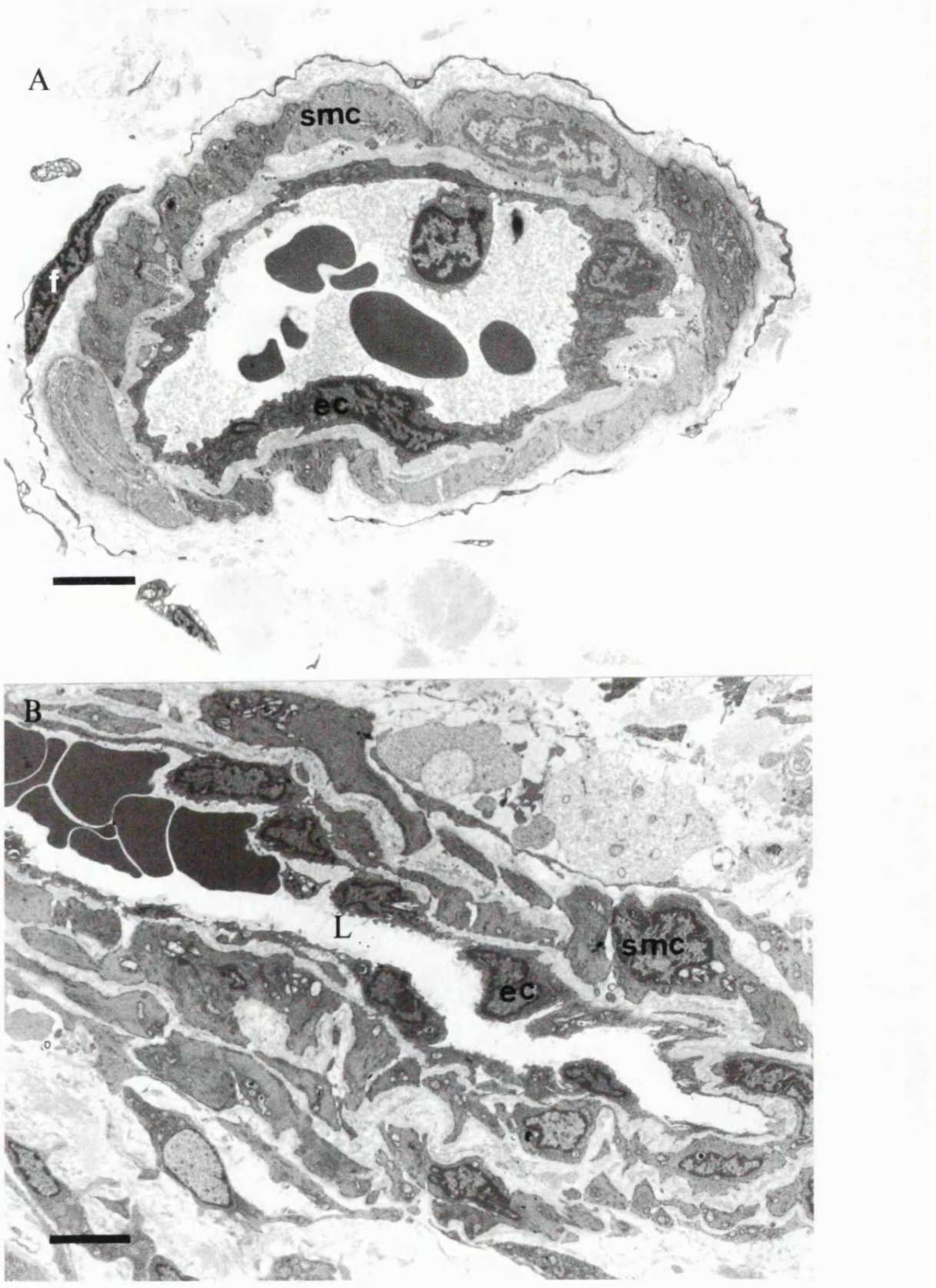

Figure 3

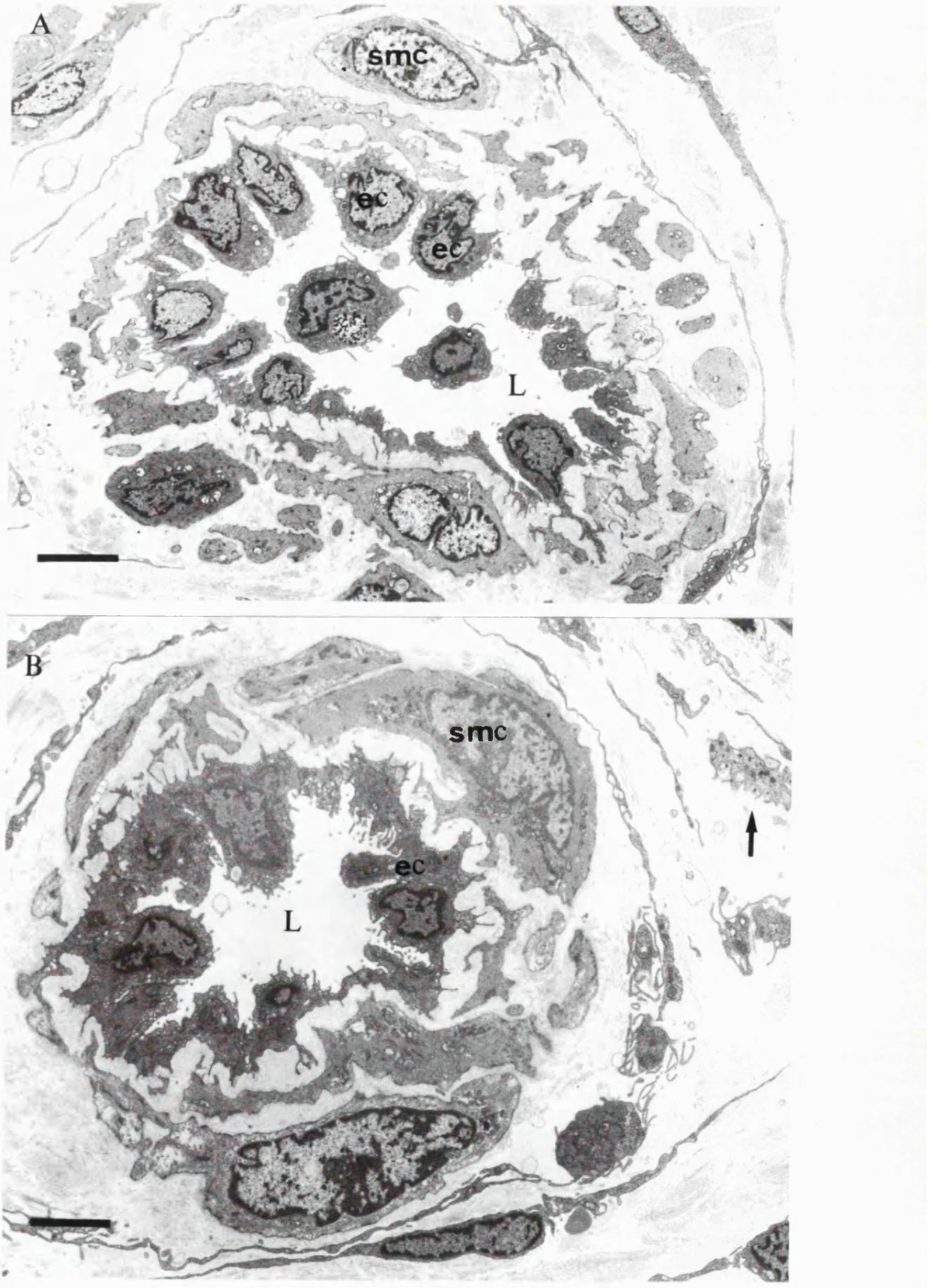

Figure 4

LEGENDS TO FIGURE 5

Smooth muscle structure of submucosal arterioles of polyps, illustrating secretory phenotypes. The features displayed in 5B are found in a minority of arterioles of benign polyps but in the majority of arterioles in a polyp with a focus of carcinoma.

A. The smooth muscle cells (smc) show distinct features of secretory activity with an abundance of intracellular organelles. Myofilaments are present, giving a mixed phenotypic appearance. L=lumen, ec=endothelial cell.

B. More advanced changes are present with clear loss of myofilaments within the smooth muscle cells (smc). L=lumen.

Calibration bars: A & B=1 micrometer.

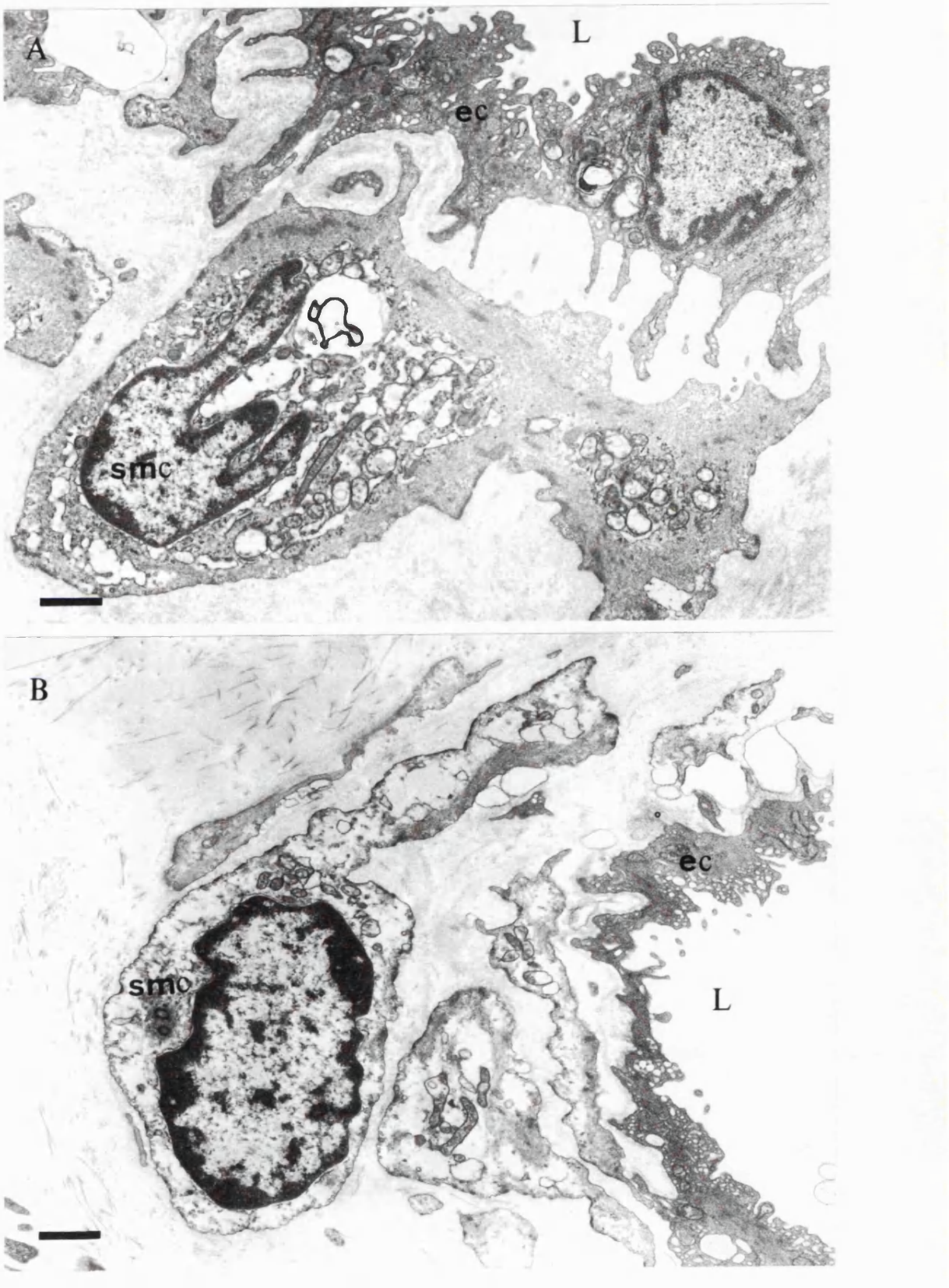

Figure 5

Chapter 6: General Discussion

6.1 Innervation of tumour vessels

6.2 Smooth muscle cell phenotypes

6.3 Hypotheses

6.4 Future applications

CHAPTER 6. GENERAL DISCUSSION

6.1 Innervation of tumour vessel

An absence of nerves within malignant tumours was first described by Krylova (Krylova 1969). Since then several reasons have been advanced for the absence of innervation in cancers. These include the rapid proliferation of blood vessels, thus outstripping perivascular nerve growth, and the production of inhibitory factors by tumour blood vessels (Ashraf et al. 1996). A deficiency of nerve growth factors, and an increase in pressure within tumours have also been considered to be possible reasons for preventing nerve growth.

In this thesis we express the view that extrinsic nerve loss occuring in tissue surrounding the bowel wall, may be the cause of the differences observed in both amount and the pattern of loss of perivascular nerves within and adjacent to colon and rectal cancers. The present study demonstrated for the first time a decrease of perivascular nerves in submucosal tissue adjacent to colorectal cancers. The loss of neurotransmitters was more marked in advanced cancers, and was more extensive for the sympathetic substances.

Such selective pattern of perivascular nerve loss has also been observed in experimental pig small bowel

autotransplantation (Shen et al. 1993). Similarly small bowel transplants removed at 22 and 8 months after transplantation in humans showed a loss of extrinsic adrenergic fibres. Perivascular fibres were absent in all intestinal layers of failed grafts. The distribution of intrinsic neurones was unchanged, although nerve density appeared decreased (Sugitani et al. 1994).

Following extrinsic denervation, reinnervation of submucosal arterioles may occur. Intrinsic SP-containing nerves appear at 50-90 days after denervation; these SP fibres originate from the myenteric plexus. An increase in VIP- immunoreactive nerves has also been noted after denervation (Jiang and Surprenant 1992).

SP reinnervation of the submucosal arterioles was not observed in our study probably because in vivo tumour factors continue to act upon the nerves, unlike the experimental situation where denervation is effected at specific times and reinnervation may occur later.

Thus the evidence from experimental studies and from transplantation support the hypothesis that extrinsic nerve loss occurs in the large bowel in colorectal cancer.

6.2 Smooth muscles phenotypes

The loss of contractile phenotype in smooth muscle cells of blood vessels described in this study also occurs after sympathectomy (Fronek and Turner 1980). These changes may not occur until 2 months have elapsed after sympathectomy (Dimitriadov et al. 1988). Smooth muscle cells in denervated arteries display an immature phenotype, with loss of thick myofilaments, an increased content of organelles involved in the synthesis of extracellular matrix (Azevado and Oswald 1986, Kacem et al. 1995, 1997).

Changes in smooth muscle cells are also observed in other chronic diseases. In hypertension intimal damage and infiltration by lipoproteins are associated with a change in smooth muscle phenotype from contractile to an immature proliferative type. Smooth muscle cells also lose their contractile phenotype in atherosclerosis and in primary culture (Kacem et al. 1995, 1997).

In tumours the degree and type of smooth muscle cells in the vessel wall is variable. A regular layer of smooth muscles was found in the wall of blood vessels in a subcutaneously implanted tumour in the rat (Mattsson et al. 1977). Whilst tumour vasculature within experimental liver metastases lacked smooth muscle cells (Ashraf et al.

1997), in the human a layer of proliferative smooth muscles was present (Ashraf et al. 1996).

In our study a change to secretory phenotypes was found in the majority of blood vessels in malignant tumours and in adenomas with carcinoma. In both instances there was clear evidence of nerve loss, suggesting that the change in phenotypic appearances may be secondary to loss of nerves. The changes in smooth muscles seem to occur in parallel with changes in innervation.

Other evidence supports the hypothesis that nerve loss may be the primary abnormality. The intimal layer of blood vessels is also affected by denervation (Loesch et al. 1992, Ralevic and Burnstock 1996, p.157). In colorectal cancers the expression of vasoactive substances in endothelium of these tumours is similar to that following denervation (Chamary et al., unpublished data).

6.3 Hypotheses

Tumour factors

The loss of perivascular innervation in the tissue adjacent to colorectal cancers, and at some distance away from the tumour cells suggests that the loss results from factors produced by tumours or from a host-tumour interaction. Tumour factors such as angiogenic factors may lead to changes in host tissue up to 5 mm away from the tumour itself (Folkman and Cotran 1976), making our hypothesis of tumour factor release to explain the loss of perivascular nerves a plausible one.

However the greater loss associated with more advanced tumours cannot be explained readily unless the anatomy of the colonic vessels and of the associated nerves as well as the method of spread of colorectal cancers are considered. More advanced cancers (Dukes B and C, 1932 classification) involve the perirectal and pericolonic tissues (Dukes B) and the mesenteric lymph nodes (Dukes C), the latter lie in close relationship to the blood vessels.

Tumour factors produced at these sites may thus cause more extensive damage to the perivascular nerves by affecting them more proximally since the vessels and their closely related perivascular nerves are distributed from the mesentery to the colon in a fan wise fashion (fig 1,

chapter 1). Furthermore the pattern of perivascular neurotransmitter loss found in our study is similar to that observed in vessels of the bowel wall following experimental and surgical denervation of mesenteric nerves (Sugitani et al. 1994).

Genetic changes

In this study changes innnervation of blood vessels were progressive in relation to their proximity to malignant tissues. The values obtained for polyps were intermediate between normal and cancers. Similarly progressive changes at ultrastructural level were noted in smooth muscle cell phenotypes.

The factors at play in the progression of polyps towards malignancy may provide an explanation. Specific genetic changes, occuring in sequence are implicated in the progression from normal mucosa through adenomas to carcinoma (Fearon and Vogelstein 1990). The ability of neoplastic cells to affect perivascular innervation or smooth muscle phenotypes may relate to these genetic changes.

6.4 Future Applications

Facilitating diagnosis and treatment.

Two areas of clinical practice can be explored in relation to the findings of this study. First preoperative assessment is increasingly important in the treatment of rectal cancer to select patients for radiotherapy. Second the diagnosis and treatment of recurrent disease following surgery is often difficult because recurrence may arise from residual cancer cells in the mesorectum following surgery (Heald et al. 1982).

In both cases cancer may not be detected in the mesorectum and adjacent tissues by current investigations. Malignant tumour within the perirectal tissue (Dukes B) and within the mesenteric nodes (Dukes C) may affect the perivascular innervation of the submucosal vessels. Further studies are required to assess whether immunohistochemical studies of biopsy of the rectal mucosal and submucosal arterioles can act as pointers to cancer recurrence.

At an earlier stage of colorectal cancer progression, polyp surveillance and excision is an important form of prevention of colorectal cancer. At the ultrastructural level the predominance of smooth muscle cells with an immature secretory phenotype particularly in submucosal vessels may be indicative of malignant transformation.

Furthermore a loss of VIP nerves has been noted in polyps that progress to malignancy (Chamary et al. 1999). This may be an early sign of transformation prior to growth of the polyp into a malignant tumour. If confirmed by a larger study these histological features of the mucosa and submucosa may provide additional criteria when evaluating the indications for radical excision of an adenoma.

Manipulation of blood flow:

The predominance of sympathetic nerve loss, which occurs in both benign and malignant colorectal tumours, appears to favour tumour growth by causing vasodilatation. Further studies reveal that there is an increase in the mean diameter of arterioles in tissue adjacent to both benign and malignant colorectal cancers compared to control tissue (Chamary et al., unpublished data), suggesting that vasodilatation does occur in submucosal arterioles adjacent to colorectal tumours. Since the submucosal arterioles are the last resistance vessels, an increase in blood flow to the tumours seems to occur. Manipulation of submucosal vessels feeding colorectal tumours may alter their progression.

Modulation of blood flow to colon cancers has been documented (Lubbe and Huhnt 1994). The presence of normal contractile smooth muscle phenotypes in the submucosal blood vessels of early polyp types may open the

way to pharmacological manipulation either locally or systemically. Preliminary studies have shown that these early polyps respond to vasoactive agents and that the vascular smooth muscles possess the ability to contract (Chamary et al., unpublished data).

Purinergic receptor agonists and antagonists may be of value. Benzyl ATP, which is an agonist selective for the P2X7 receptor and agents such as Coomassie brilliant blue or KM62, which selectively antagonise the receptor are potential candidates (G Burnstock, personal communication)

CONCLUSION

This thesis confirms the absence of perivascular nerves within colorectal cancers and presents for the first time evidence of perivascular nerve loss around original vessels in the tissue adjacent to colorectal tumours.

It is hypothesised that perivascular nerve loss may result from release of tumour factors from the neoplastic cells.

The predominance of sympathetic nerve loss observed would promote vasodilatation of arterioles of the submucosa in cancers and adenomas, hence promoting tumour growth.

The change to a secretory phenotype by smooth muscles of arterioles in colorectal cancers are similar to those observed in studies of denervation, suggesting a similar mechanism in colorectal cancers.

The changes observed in the submucosal arterioles and the innervation of colorectal tumours in this study could in future be exploited to facilitate diagnosis and treatment.

APPENDIX I

Details of patients from whom samples were taken, and of the site and histology of samples. Size of polyps is given in brackets only for samples where quantitative analysis was possible. Controls were taken at least 5 centimetres from tumour specimens. Diff=differentiated.

PATIENT	SEX AGE	PRESENTATION	HISTOLOGY	DUKES GRADE	SOURCE	LESION SITE (SIZE,cm)
1	M 63	Diarrhoea, Mucus.	Well to Moderately Differentiated Adeno-carcinoma	A	Open Surgery	Rectum
2	F 78	Bleeding	Control mucosa		Open Surgery	Rectum
3	M 64	Abdominal pain, Mass.	Well Differentiated Adenocarcinoma with areas of poor differentiation	C	Open Surgery	Splenic flexure

4	M 72	Altered bowel, bleed.	Well Diff Adeno-Carcinoma	B	Open Surgery	Rectum X2
5	F 56	Anaemia.	Well Diff Adeno-Carcinoma	C	Open Surgery	Caecum
6	M 59	Bleed, Tenesmus.	Well Diff Adeno-Carcinoma	A	Open Surgery	Rectum
7	F 65	Bleed, tenesmus,alte -red bowel.	Well to moderaately Differentiated Adenocarcinoma	B	Open Surgery	Rectum
8	F 55	Diarrhoea, Abdominal pain.	Well Diff Adenocarcinoma	B	Open Surgery	Splenic Flexure
9	F 60	Bleed, Tenesmus.	Well Diff Adenocarcinoma	B	Open Surgery	Rectum
10	F 65	Bleed, tenesmus, altered bowel.	Tubovillous Adenoma		Open Surgery	Descending Colon (1.5 cm).
11	M 53	Diarrhoea, Bleed.	Control mucosa		Open Surgery	Rectum
12	F 60	Bleed, mucus loss, diarhoea.	Tubular Adenoma		Open Surgery	Recto-sigmoid (3.0 cm)
13	F 55	Diarrhoea, Abdominal pain.	Tubular Adenoma		Open Surgery	Splenic Flexure (1.5 cm)

14	M 77	Diarrhoea, Bleed.	Tubovillous Adenoma		Open Surgery	Descending Colon(1.5 cm)
15	M 69	Diarrhoea, Tenesmus.	Well Diff Adeno-carcinoma	A	Open Surgery	Rectum
16	M 77	Diarrhoea, Bleed.	Well to moderately Differentiated Adenocarcinoma	B	Open Surgery	Rectum
17	F 67	Bleed, urgency.	Well Diff Adeno-Carcinoma	A	Open Surgery	Sigmoid Colon
18	F 83	Anaemia, general malaise.	Well to moderately Differentiated Adenocarcinoma	B	Open Surgery	Caecum
19	F 52	Discharge, Bleed.	Metaplastic polyp		Open Surgery	Transverse Colon (0.3 cm)
20	M 70	Bleed, Diarrhoea.	Well Diff Adenocarcinoma	C	Open Surgery	Rectum
21	F 74	Discharge, Bleed.	Villous Adenoma with severe dysplasia		Open Surgery	Rectum (4.5 cm)

22	M 72	Follow up Previous polypectomy.	Tubular Adenoma Adenoma		Colonoscopy Polypectomy Colonoscopy biopsy	Descending Colon (1.0 cm) Rectum (1.0 cm)
23	F 65	Rectal bleed.	Melanosis coli Tubovilous Adenoma		Colonoscopy	Rectum Biopsy (0.5 cm)
24	M 56	Diarrhoea, Pneumaturia.	Polyps, Meta- plastic(X3)		Open Surgery	Sigmoid Colon (0.5 cm) (0.5 cm) (0.5 cm)
25	F 65	Altered bowel, mass.	Control Mucosa		Open Surgery	Sigmoid Colon
26	F 65	Bleed.	Control Mucosa		Open Surgery	Sigmoid Colon
27	M 78	Mucus loss, diarrhoea.	Villous Adenoma		Open Surgery	Rectum (30cm)
28	F 58	Abdominal pains.	Normal		Colonoscopy Biopsy	
29	M 52	Follow up Acromegaly.	Metaplasia Mixed metaplasia		Colonoscopy Polypectomy	(0.7cm) Transverse colon (0.8cm)

APPENDIX II

Numbers of arterioles in 3 fields at 250 magnification in control submucosa (columns 1), and in the submucosa adjacent to and within 3 mms of cancers (columns 2) for each of the neurotransmitter substances. The numerator is the number of arterioles with perivascular nerve immunoreactivity and the denominator is the total number of arterioles. Pt=patient.

Pt	TH		NPY		VIP		SP		CGRP	
	1	2	1	2	1	2	1	2	1	2
01	5/5	0/5	8/8	4/5	2/4	0/3	6/10	2/5	2/17	0/10
02	13/13		10/13		6/6		7/10		0/6	
03	5/8	0/10	7/11	1/6	5/11	3/9	3/10	0/3	0/10	0/5
04	7/10	1/7	7/8	0/7	5/8	0/5	3/7	0/2	0/7	0/7
05	4/15	0/5	10/10	0/5	5/10	0/11	2/10	0/3	0/5	0/8
06	4/7	1/5	6/6	4/5	5/7	3/7	3/7	3/5	0/9	0/8
07	1/5	1/5	6/6	5/5	2/6	2/3	4/5	4/5	0/7	0/8

08	10/10	2/9	7/7	0/6	3/4	2/12	4/5	2/5	0/10	0/12
09	3/3	0/2	2/2	0/6	1/5	0/5	0/3	0/2	0/7	0/7
15	7/7	0/7	7/7	4/9	5/5	3/6	0/4	3/5	0/8	1/8
16	4/5	0/7	3/3	0/7	4/6	0/5	5/5	0/7	0/5	0/5
17	7/10	0/4	6/6	2/6	0/4	0/4	0/5	0/6	0/8	0/7
18	5/9	0/10	10/10	0/3	3/12	0/4	1/6	0/2	0/3	0/3
20	1/5	0/5	7/7	0/5	0/3	1/6	0/5	0/3	0/5	0/3

APPENDIX III Numbers of arterioles in 3 fields at 250 magnification in control submucosa (columns 1). Column 2 refers to values in polyps. The numerator is the number of arterioles with perivascular nerve immunoreactivity and the denominator is the total number of arterioles.

Pt	TH		NPY		VIP		SP		CGRP	
	1	2	1	2	1	2	1	2	1	2
10	1/5	1/4	6/6	3/5	2/6	3/4	4/5	3/8	0/7	5/5
11	7/7		8/8		3/6		0/6		0/7	
12	5/5	2/6	9/9	5/5	2/7	5/5	0/5	0/7	0/5	0/5
13	10/10	6/7	7/7	6/6	3/4	6/7	4/5	4/5	0/10	0/10
14	4/5	7/7	3/3	8/8	4/6	5/9	5/5	7/7	0/5	0/8
21		3/9		7/7		3/9		2/5		0/5
19		1/9		5/5		6/7		0/7		0/7
22 (1)		0/6		3/3		7/7				0/3
22 (2)		1/3		3/11		3/4		3/5		0/8
23		6/6		5/5		5/5		7/7		3/7

APPENDIX III (continued)

Pt	TH		NPY		VIP		SP		CGRP	
	1	2	1	2	1	2	1	2	1	2
24 (1)	3/6	3/4		3/4	1/7	4/5		1/7		0/4
24 (2)		0/6		7/8		4/5		4/6		0/7
24 (3)		1/4		7/9		7/10		3/6		0/10
26	11/11		8/10		6/7		0/7		0/5	
27		0/3		0/5		0/5		0/5		0/5
29 (1)		8/8		5/5		6/6		2/4		3/6
29 (2)		0/3		4/6		0/6		0/6		0/4
25	5/5				4/7		4/8		0/8	

APPENDIX IV. Case by case analysis of the most common neurotransmitters around arterioles of submucosa in controls versus normal submucosa adjacent to colorectal cancer. Pt = patient.

CONTROLS n=14		CANCERS n=15 (DUKES STAGE)	
Pt 1	NPY, TH	NPY	(A)
Pt 2	NPY	-	
Pt 3	NPY	VIP	(C)
Pt 4	NPY	TH	(B)
Pt 5	NPY	NONE	(C)
Pt 6	NPY	NPY	(A)
Pt 7	NPY	NPY	(B)
Pt 8	NPY, TH	TH	(B)
Pt 9	NPY, TH	NONE	(B)
Pt 15	NPY, TH, VIP	NPY	(A)
Pt 16	NPY, SP	NONE	(B)
Pt 17	NPY	NPY	(A)
Pt 18	NPY	NONE	(B)
Pt 21	NPY	VIP	(C)

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