The Role of Endothelin

in Colorectal Cancer

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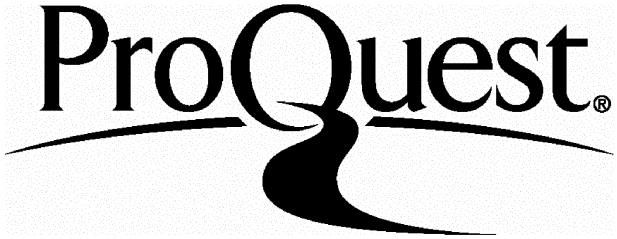

Abstract

***Background*:** The vasoactive peptide endothelin-1 (ET-1) acts via two receptors, ET_A and ET_B. ET-1 is overexpressed by human colorectal cancer tissue. The aim was to elucidate whether 1) there is differential expression of ET_A and ET_B receptors in colorectal cancer and 2) ET-1 is a mitogenic factor for colorectal cancer.

***Method*:** The distribution of receptors in normal colon and in tissue from patients with colorectal cancer was determined by immunohistochemistry and by autoradiography. *In vitro* experiments with colorectal cancer cell lines were carried out to determine the presence of ET_A and ET_B in these cells using autoradiography, the effect of exogenous ET-1 on their growth and if ET_A and ET_B antagonists could modulate the ET-1 induced growth.

***Results*:** ET_A is overexpressed in colorectal cancer as compared to normal colon while ET_B is underexpressed. The density of expression of ET_A in colorectal cancer significantly exceeded that of ET_B. Both receptors were expressed by epithelial, and endothelial cells in normal colon and colorectal cancer. Neuronal normal colonic tissue also expressed both receptors. ET-1 acted as a growth stimulator for colorectal cancer cells and this effect was inhibited by ET_A, but not by ET_B, antagonists.

Conclusion: ET_A receptors are overexpressed in colorectal cancer. ET-1 can stimulate the proliferation of colorectal cancer cell lines via the ET_A, but not the ET_B, receptor.

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Acknowledgements

Perfect as the wings of a bird may be (and they are far from perfect), they will never enable the bird to fly if unsupported by the air. This research project and thesis flew on the lift of a gale.

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Abbreviations

APC	Adenomatous polyposis coli
Big ET-1	Big endothelin-1
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor(s)
ErbB (2, 3, 4),	Epidermal growth factor receptor (two, three, four)
ET_A	Endothelin A receptor
ET_B	Endothelin B receptor
ET_C	Endothelin C receptor
ECE	Endothelin converting enzyme
EDE	Endothelin degradation enzyme
ET-1, ET-2, ET-3	Endothelin one, Endothelin two, Endothelin three
Fak	Focal adhesion kinase
FAP	Familial Adenomatous Polyposis
FGF (1, 2, 3, 4)	Fibroblast growth factor (one, two, three, four)
5-FU	5-Fluorouracil
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
IGF (I) (II)	Insulin like growth factor (one) (two)
IGFBP	Insulin like growth factor binding protein(s)
MAP kinase	Mitogen activated protein kinase
MSI	Microsatellite instability

PDGF	Platelet derived growth factor
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PKC	Protein kinase C
SMAD	Smart mothers against decapentaplegia
TGFα	Transforming growth factor alpha
TGFβ	Transforming growth factor beta
VEGF (A, B, C)	Vascular endothelial growth factor (A, B, C)

1 Introduction

1.1 Colorectal Cancer

1.1.1 Introduction

Colorectal cancer is the third commonest cancer in men, after lung and prostate cancer, with an incidence of 158700 per million, and in women, after breast and lung cancer, with an incidence 153600 per million UK (CRC cancer stats 1998). It is also the third commonest cause of death from cancers, with an annual mortality of more than 17, 000 in the UK (CRC cancer stats 1999).

1.1.2 Aetiology

Colorectal cancer develops by the accumulation of gene mutations, which ultimately provide a selective growth advantage. At least 2 pathways of tumour initiation and promotion have been identified for colorectal cancer based on insights into molecular events underlying 2 inherited syndromes: Familial Adenomatous Polyposis (FAP) and Hereditary Non-Polyposis Colorectal Cancer (HNPCC). The majority of colorectal cancers are sporadic, 80-85%, and are most prevalent in the 60-70 age group, unlike hereditary FAP and HNPCC cancers that present at a younger age. (Lynch et al 2000, Ilyas et al 1996). However, the same type of mutations have been identified in both sporadic and hereditary cancers, with approximately 85% of sporadic cancers exhibiting mutations which are usually found in the FAP pathway and 15% of sporadic cancers being more similar to the HNPCC pathway. Fearon and Vogelstein in 1990 were the first to construct a model of step-wise progression of colorectal carcinogenesis to link genetic mutations to oncogenic progression and a modified version is shown in Figure 1.1.

The predisposing mutation linked to FAP is in the adenomatous polyposis coli gene (APC). APC is a tumour suppressor gene, whose protein product complexes with β-catenin and targets it for degradation. The product of the mutated gene cannot do so, and this leads to accumulation of β-catenin, which in turn up-regulates genes critical for proliferation and transformation of colorectal epithelial cells. APC mutations are identified very early in colorectal tumourigenesis (Powell et al 1992, Kinzler et al 1991). The two other major mutations identified in this path are; Ras mutations that arise during the adenomatous phase and p53 mutations which coincide with transition to malignancy. Ras is a molecular switch/GTPase which drives proliferation and its mutated form remains in the switched-on form, while p53 is a tumour suppressor which when mutated or deleted cannot drive apoptosis (Chung et al 2000).

The predisposing mutations linked to HNPCC affect the 5 mismatch repair genes whose products form a complex proofreading system capable of correcting base pair mismatches during DNA replication (Kim et al 1994). Because of the uncorrected errors, a number of genes end up mutated. Particularly susceptible are genes that contain short repetitive sequences, i.e., microsatellite (MS) repeats. One such gene, with MS repeats in coding exons, is the class II receptor for TGFβ. Inactivation of this receptor, removes the potent inhibitory effect of TGFβ on colonic epithelial cell growth (Markowitz et al 1995, 2000). Therefore, such tumours are characterised by microsatellite instability (MSI), unlike tumours conforming to the FAP scenario which often exhibit chromosomal instability.

The relationship between the MSI phenotype and mutations of APC, ras or p53 remains unclear. However, it is currently believed that APC inactivation is probably the

initiating event of colorectal oncogenesis, regardless of the pathway followed. Other gene mutations and epigenetic events (such as hypo- and hyper- methylation) are also associated with colorectal tumourigenesis, but are not described here (Chung et al 2000).

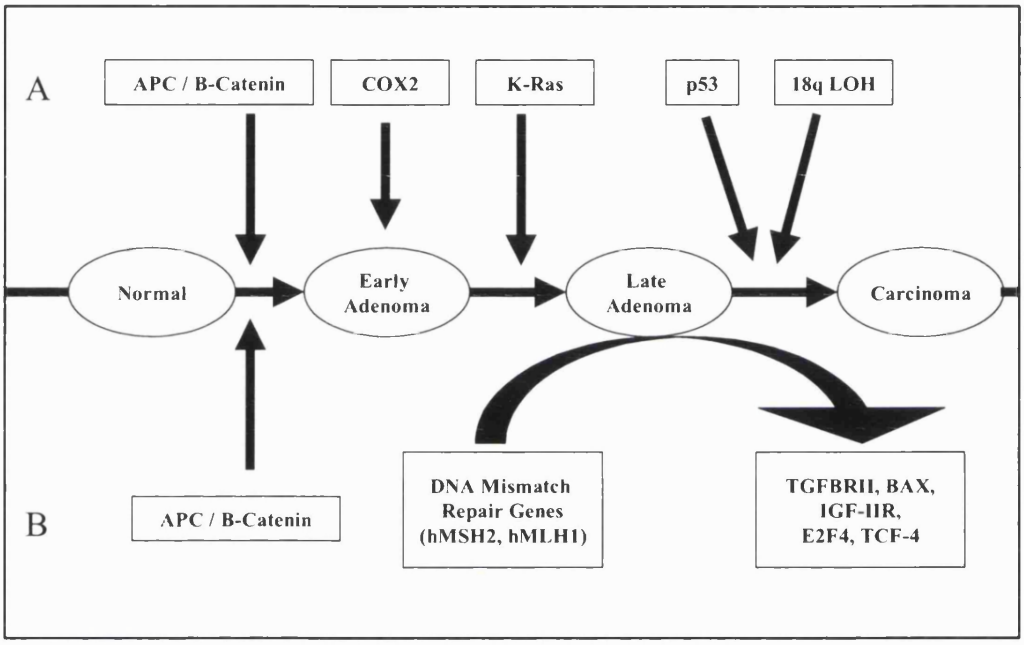


Figure 1.1 *Overall scheme of key genetic events in colorectal tumourigenesis. Two major pathways exist: Eighty-five percent of tumours follow genetic events illustrated in A (modified Vogelgram), which result in the classical adenoma-carcinoma progression. The remaining 15% of tumours are characterized by microsatellite instability and conform to path B. COX2 = Cyclo-oxygenase-2; LOH = loss of heterozygosity; BAX = apoptosis regulator molecule; IGF-IIR = Insulin like growth factor receptor class II; E2F = cell cycle regulated transcription factor; TCF-4 = T-cell factor 4, transcription factor. (Diagram modified from Chung 2000).*

Epidemiological studies have suggested that diets low in unsaturated fat (Stoneham et al 2000), high in red meat and alcohol and low in fibre are a risk factor (Gratin et al 2000), as is cigarette smoking. These risk factors are based on epidemiological studies (Waterhouse et al 1976, Bingham 1997). Other factors that may predispose to colorectal cancer include bile salts (Nagengast 1995, Toucchi et al 1996), and their metabolites (Alberts 1996), which may act as apoptotic factors in colorectal cancer (Grewal et al 1996), hence, suggesting a potential link between cholecystectomies and colorectal cancer (Rahman et al 1996). Although diet is believed to contribute to the pathogenesis of colorectal cancer, its precise aetiological significance is still undetermined.

Several diseases can also predispose to colorectal cancer. Those posing the highest risk are the inflammatory bowel diseases, including ulcerative colitis, (Langholz et al 1992, Heimann et al 1992, Levine et al 1991, Lennard-Jones et al 1990), radiation colitis (Tamai et al 1999, Minami et al 1998, Morita et al 1998) and schistosomiasis in the Far East (Chen et al 1981). If Crohn's disease occurs in the colon, this too can be a pre-disposing factor dependent on the number of years the disease has been present and its severity. (Yamazaki et al 1991, Sigel et al 1999, Savoca et al 1990, Stahl et al 1992, Kirk et al 1999). There is no link between diverticulitis and cancer.

1.1.3 Pathogenesis

Colorectal cancer arises in the majority of cases from dysplastic adenomas, with the earliest lesion termed the aberrant crypt focus (microadenoma). Evidence for the progression from microadenoma to adenoma to cancer has been found from studies in rats and cows (McLellan et al 1991, 1988, Pretlow et al 1992). The presence of

microadenomas has been demonstrated in humans (Pretlow et al 1991, Roncucci et al 1991, Tierney et al 1990) and post-mortem studies suggest a 35% prevalence of these lesions in asymptomatic people (Johannsen et al 1989, Jass et al 1992, Coode et al 1985). Epidemiological studies in humans have indicated a possible progression time of 10 to 15 years (Muto et al 1975, Winawar et al 1993). The incidence of colorectal cancer is reduced in patients who have undergone polypectomies (Winawar et al 1993), providing further evidence that the former constitute a sequential step in cancer development. The genetic events underlying colorectal tumourogenesis are described in the previous section.

1.1.4 Staging of colorectal cancer

The purpose of staging is to provide accurate information relevant to prognosis. The standard method of staging patients is based primarily on the pathological characteristics of the resected specimen. Duke's system originally described for rectal tumours is the accepted classification in the U.K. (Dukes 1930). Dukes' classification is based on the ABC system where:

Stage A: Confined to the bowel wall with no lymph node involvement.

Stage B: Extension through the bowel wall without lymph node involvement.

Stage C: Lymph node involvement. If the apical lymph node does not have tumour involvement then the stage is C_1, if it contains tumour then the stage is C_2. The apical lymph node is the one furthest or most distal from the tumour in the area of tumour drainage.

Since the original Dukes' description it has become customary to add Duke's D for those patients with distant metastasis (often to the liver for colorectal cancers).

The TNM classification system based on American Joint Committee on Cancer and the Union International Contre le Cancer AJCC/UICC is used where:

Stage 1 is N0, T1-T2.

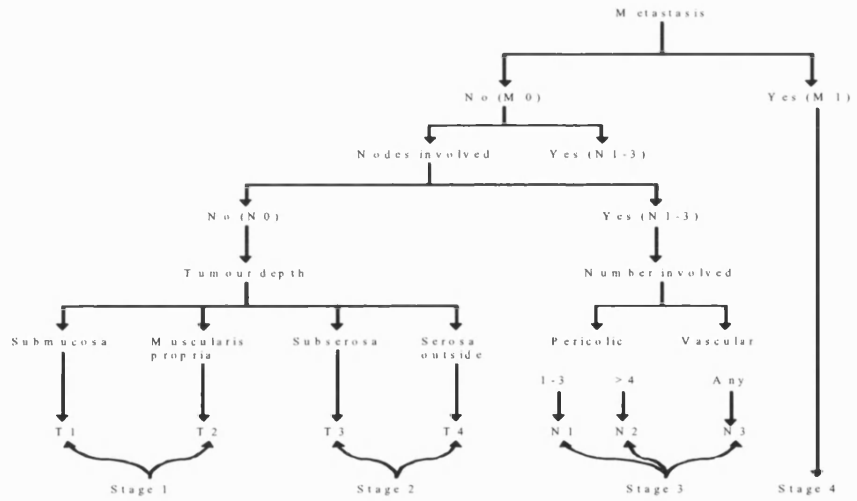
Stage 2 is N0, T3-T4.

Stage 3 is N1-N2-N3.

Stage 4 is M1.

(Figure 1.2)

At the time of diagnosis, 35% of patients have localised disease, i.e. stage 1, 38% have regional disease, i.e. stage 2, and 22% metastatic disease, i.e. stage 3 or 4 (Ries et al 2000).



1.1.5 Figure 1.2 *UICC TNM classification after Hermanek (1989)*

1.1.5 Treatment

1.1.5.1 Surgery

The mainstay of colorectal cancer treatment remains surgical. However approximately 40% of patients with a potentially respectable primary colorectal cancer have incurable disease as a result of the presence of metastases (Allum et al 1994).

The stage of colorectal cancer invasion at the time of presentation is the most influential prognostic factor. Five years after surgery, 94% survival has been reported in patients in stage 1 carcinoma, 70% in stage 2 carcinoma, and 9% in stage 3 and 4 cancers (Ries Lag et al 2000).

The most common site for metastases is the liver and resection remains the only curative treatment for colorectal liver metastases. Surgery is indicated as treatment of choice if there is a maximum of three tumour foci confined to one lobe of the liver, a satisfactory excision margin can be achieved (>1cm), and there is no extra-hepatic disease. Only 5-10% of patients fulfil these criteria and not all undergo operations (Taylor 1996). Studies show 5 year survival rates of approximately 25%. Recurrences (either within the liver or extrahepatically) appear in 65-80% of patients, and usually present within a year of resection (Geoghegan et al 1999).

1.1.5.2 Adjuvant therapy

Adjuvant therapies are either palliative or curative. The main stay of adjuvant therapy is in the form of chemotherapy, radiotherapy and newer forms such as immunotherapy, alcohol ablation, chemoembolisation, cryosurgery, laser and radio frequency ablation of liver metastases. These therapies remain experimental.

Chemotherapy

The most commonly used form of adjuvant chemotherapy is 5-fluorouracil (5-FU) in combination with levamisole and/or leucovorine. Newer chemotherapy agents are under research and include irinotecan, raltitrexed and oxaliplatin.

No chemotherapy is offered for stage 1 colorectal cancer. Adjuvant chemotherapy for stage 2 colon cancer does not give a significant improvement in survival compared to untreated patients (International multicentered pooled analysis), with only a 7% increase at 5 years (Mamounas et al 1999). This may be due to incorrect staging, for example if micro metastases were not recognised, and therefore sub-selection may improve survival (Liefers et al 1998).

There is a general consensus that adjuvant chemotherapy is recommended for patients with stage 3 colorectal cancers. The combination of 5-FU/levamisole in stage 3 colorectal cancer results in an increase in the 5 year disease-free survival of up to 40% (Marsoni 2001, Scheithauer et al 1998 and Moertel et al 1995) and a 5 year overall survival of up to 50%.

Stage 4 colorectal cancer treatment is palliative and is usually with systemic 5-FU with minimal survival benefits (Moertel 1994). Loco-regional infusion of 5-FU with or without leucovorin improves survival marginally (Poon et al 1991, Buroker et al 1994 and meta-analysis group 1998).

New drug combinations that show promise include irinotecan (Topoisomerase 1 inhibitor, CPT-11) with 5-FU, with better response rates but no proven survival

advantage (Iveson et al 1999, Drengler et al 1999). Of the other infusion modalities, the only one to improve 2-year disease-free survival is 5-FU/leucovorine with floxuridine (Kemeney et al 2000).

More recent treatments that have some benefit include oral fluoropyramidines, such as UFT (a combination of uracil & tegafur) and Capecitabine, which are prodrugs of 5-FU. (Nakagoe et al 2000, Kusuonoki et al 2000). Another approach utilizes ethynyleuracil which ultimately inhibits the degradation of 5-FU, and, therefore when given in combination with 5FU, ethynyleuracil improves 5-FU's therapeutic activity.

Newer approaches being investigated are based on immunotherapy and vaccines (Koda et al 2001, Safa et al 2001).

Radiotherapy

The use of radiotherapy is mainly confined to the anorectal cancers and confers a definite palliative advantage (Aleman et al 1995). There has been evidence to show a definite survival advantage for both preoperative and postoperative treatment with radiotherapy in anorectal adenocarcinomas prompting its routine use for stage 2 and 3 anorectal cancers. (Horgan et al 2000, Farouk et al 1997 and Lim et al 1999).

1.1.6 Growth factors and colorectal cancer

Growth factors may contribute to colorectal cancer progression, acting either as mitogens for epithelial cancer cells or as angiogenic factors. Mitogenic growth factors include TGFβ (transforming growth factor beta), TGFα (transforming growth factor

alpha), EGF (epidermal growth factor) and IGF (Insulin like growth factor). Angiogenic growth factors include VEGF (vascular endothelial growth factor), FGF (fibroblast growth factor) and PDGF (platelet derived growth factor).

Normally, growth factors act by binding to receptors that in turn initiate a cascade of intracellular signal transduction pathways leading to nuclear events and causing either inhibitory or propagatory growth effects. If the feedback mechanisms that regulate growth factors and their cascades escape normal physiological control, the resulting imbalance may contribute to cancer growth. Escape from control mechanisms happens at four levels: Firstly, the amount of growth factor available may alter, i.e. the amount produced, the amount used or degraded, or the amount available which is subtly controlled by binding proteins. Secondly, the number or biochemistry of receptors available for binding may change. Thirdly, the downstream intracellular signals may be potentiated or attenuated, e.g. by amplification, or positive and negative cross-talk. Changes in intracellular pathway signals remain the most complex and least understood. Fourthly, the availability of target genes within the nucleus may be altered, e.g., by mutation. Some of the changes outlined above have been identified within the context of the adenoma carcinoma pathway or the MSI pathway (Figure 1.1).

A review of the evidence for the involvement of mitogenic and angiogenic growth factors in colorectal cancer follows.

1.1.6.1 Transforming growth factors beta

TGFβ's (Transforming growth factors beta) are potent inhibitory growth factors, with three isoforms identified in humans: TGFβ1, TGFβ2, and TGFβ3. The growth factor

attracts and binds transmembrane receptor II, TGFβRII, which in turn attracts TGFβRI receptor I. The ligand-receptor complex phosphorylates (activates) downstream molecules (SMADs) which in turn signal the nuclear transcription of amongst others inhibitors of cyclins and cyclin dependent kinases and therefore stop the cell cycle and inhibit proliferation. (Figure1.3)

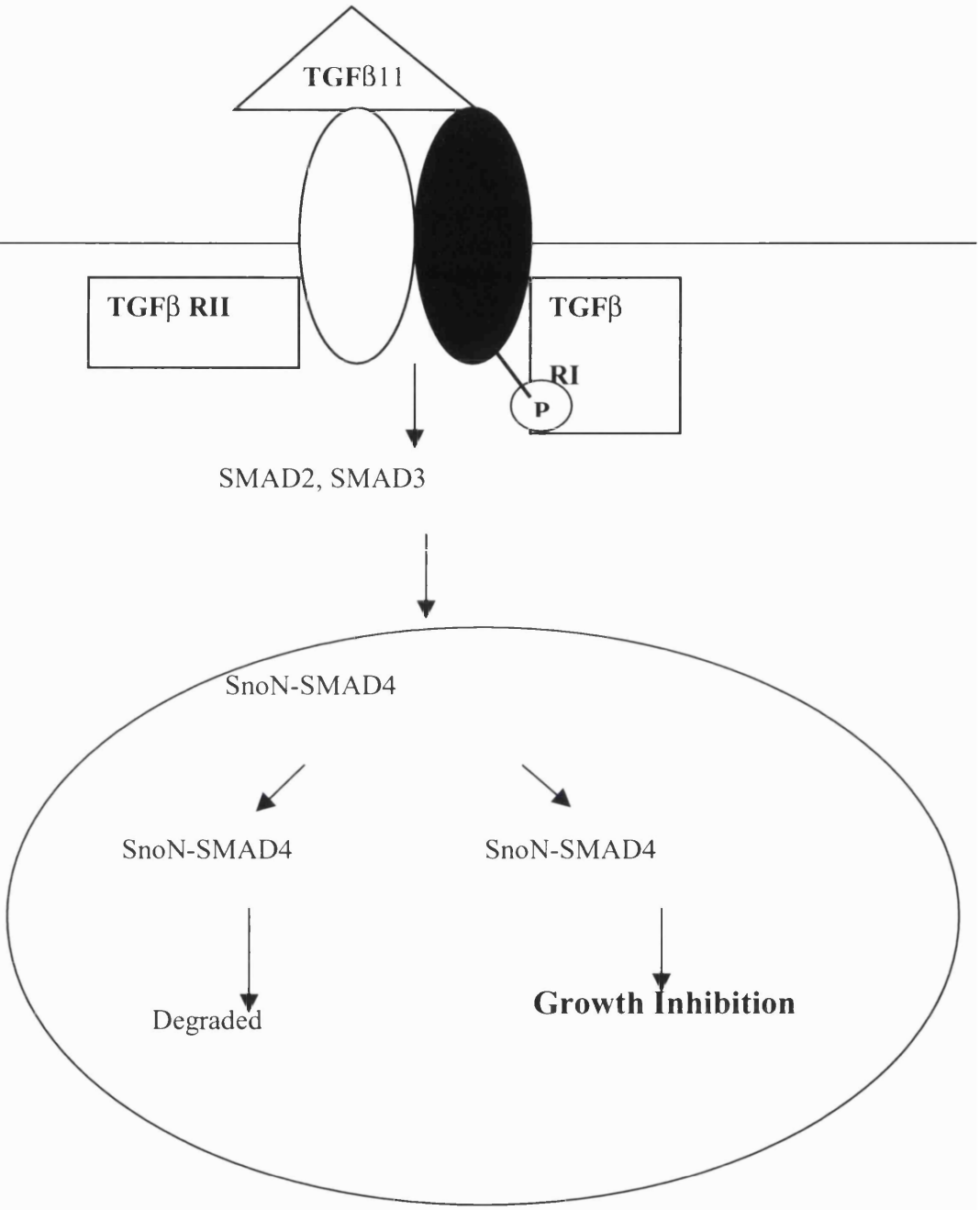


Figure 1.3 *Simplified signal transduction pathway for TGFβ. Growth factor binds to TGFβRII which is constitutively autophosphorylating. TGFβRI recognizes and joins bound ligand-R II and is in turn phosphorylated. All downstream molecules to be phosphorylated are SMADs (smart mothers against decapentaplegia) and some are transcriptional activators, capable of entering the nucleus and binding/activating DNA transcription of genes whose products inhibit the cell cycle.*

Manning et al in 1991 was one of the first to show that the growth of three non-tumorogenic human colorectal adenoma cell lines was inhibited by the addition of exogenous TGFβ, while no such inhibition was found in five tumorogenic colorectal cancer cell lines despite high concentrations of exogenous TGFβ. The conversion of one adenoma cell line to a tumorogenic cell line was accompanied by loss of response to TGFβ, suggesting loss of sensitivity to TGFβ (i.e. loss of growth inhibition) occurs relatively late in colorectal carcinogenesis.

Based on the above findings, a number of researchers looked for a progressive loss of TGFβ with disease progression. For example, Avery and colleagues in 1993 investigated TGFβ expression in colonic tissues. Positive TGFβ staining was found in 52 of 58 (90%) normal colons, 8 of 10 (80%) adenomas and 46 of 48 (96%) of carcinomas, suggesting no real difference between patient groups. TGFβ plasma levels and TGFβ mRNA were significantly increased in 22 patients with colorectal cancer compared to controls and furthermore there was correlation between both TGFβ parameters and Duke's staging, i.e. disease progression (Tsushima et al 1996). These and other similar findings show that TGFβ itself is not downregulated or under expressed in colorectal cancer. Any possible loss of growth inhibition may be due to alteration of the receptors.

There are two major ways in which that the receptors can become responsible for the loss of inhibitory action of TGFβ: a) Biochemical/functional abnormality of the receptor and b) down regulation of the number of receptors.

Inactivating mutations of the TGFβRII gene were first reported in colorectal cancer cell lines with high rates of microsatellite instability by Markowitz et al (1995) and Wang et al (1995). Furthermore, reversal of the transformed phenotype of cancer cells was achieved by transfection of functional TGFβRII (Markowitz and Roberts 1996), confirming that the receptors should be termed tumour suppressor gene products. Studies in human colorectal cancer tissue gave similar results. Souza and co-workers (1996) showed 13 of 16 (81%) colorectal cancers had TGFβRII mutations as compared to 2 of 44 (4%) normal colonic tissues and further work by a number of groups showed that approximately 90% of human colorectal cancers arising via the microsatellite instability (MSI) pathway have inactivations in the TGFβRII receptor (Souza 2000). Gene mutations are first detected in advanced MSI adenomas and are therefore late events in carcinogenesis.

The down regulation of TGFβRII receptors was explored by Sheng et al (1999), who showed that colorectal cancer cells exposed to TGFβ initially reduced their growth, but after a week, the remaining cells adapted by reducing TGFβRII levels by 95%. The down regulation of TGFβRII was also reported in human tissue and followed the adenoma carcinoma progression. Matsushita et al (1999) showed that TGFβ receptor mRNA was expressed in 100% of normal colorectal and adenoma epithelia but only in 36% of colorectal cancers, at much reduced levels. Eskinazi et al (1998) had shown that there is no dysfunction of the type II TGF-beta receptors in sporadic colorectal cancers. These findings are in stark contrast to hereditary non polyposis coli cancers (HNPCC), where approximately 77% of patients had insertion or deletion of the TGFβ II receptor. This brings the two types of receptor action loss into clinical realms

whereby sporadic tumours exhibit down regulation and MSI tumours exhibit mutations of the receptors.

The above findings bring together the apparently contradictory findings of unchanged or increased TGFβ production and down regulation of receptors. TGFβ is produced/released as an inhibitory molecule from the microenvironment of the cancer as an attempt at tumour inhibition. However, in the case of MSI cancers with inactive receptors, the raised levels are ineffective, while in sporadic cancers an overabundance of growth factor results in down regulation of its receptors and renders cancers less sensitive to growth inhibition

1.1.6.2 Insulin Growth Factor (IGF)

Unlike TGFβ, whose actions inhibit growth, the rest of the growth factors found to be involved in colorectal cancer have a mitogenic effect. Insulin like growth factor (IGF) is one such peptide. Two IGF isoforms were discovered, I and II, which act via two receptors, RI and RII. Escape from normal physiological function has been shown to occur mainly at the first level, i.e. the amount of circulating IGF available for binding to the receptors, and not at the intracellular or intra nuclear downstream stages. Some of the relevant evidence is presented below.

Durrant et al (1991) and Lahm et al (1992) made the link between IGF and mitogenesis by showing that both IGFI and IGFII cause up to 3 fold increase in the growth of colorectal cancer cell lines. In colorectal cancer tissues, IGFII gene expression was shown to be 800 times that of adjacent colon. However no such difference was found for IGFI gene expression (Lambert et al 1991). This link between IGF and colorectal cancer was further substantiated, by showing that tissue staining for IGFII correlated

with prognosis (Kwomoto et al 1998). Furthermore, Renehans et al (2000) demonstrated that patients with colorectal cancer had raised plasma levels of IGFII and that the levels dropped to normal after resection.

The possible mechanism for control of the free circulating IGF is related to IGF binding proteins (IGFBP) of which there are three, I, II and III. Binding proteins bind to these ligands, in this case IGF, and make them unavailable for binding to their receptors and therefore unavailable for biological activity. IGFBPIII, which is a major determinant of circulating levels of IGF, is inversely related to the risk of colorectal cancer. This is consistent with the proposition that IGFs are promoters of growth and that IGF levels are inversely proportional to binding proteins levels (Pollak et al 1999). Coinciding with these findings was the discovery of various gene location abnormalities in IGFBPIII in colorectal cancers (Zou et al 1998). This would suggest that reduced amounts of the binding protein are produced, as demonstrated by Pollak and his colleagues, or that the affinity for IGF of the altered protein is diminished. On the other hand, IGFBPII was shown to be elevated in the plasma of patients with colorectal cancer and also dropped after resection of the tumour. This is contrary to the above scenario. Possible explanations were either that IGFBPII was a mitogenic agent itself or, as suggested by Sheehan et al., a byproduct of tumour whose full biological function is not elucidated.

There was no significant difference in IGFRI receptor expression between colorectal cancers and normal colon and no relation between IGFRI and survival (Adenis et al 1995, Bhatavdkar et al 1995). However, Perer et al (2000) found that inhibition of the IGFRI receptor by antibodies in the presence of other chemotherapeutic agents, such as

5FU, results in up to a 10 fold increase in colorectal cancer cell numbers compared to no increase in the presence of 5FU alone. Souza et al (1999) has shown that the IGFRII gene is a tumour suppressor gene in which stimulation of the gene produces a decrease in growth and an increase in apoptosis in colorectal cancer cells.

IGF has a role in the adenoma carcinoma progression theory in colorectal cancer. A mouse model with APC gene which was genetically engineered to be a high producer of IGFII was shown to have a ten fold increase in the number and a diameter of colorectal adenomas when compared to mice that did not have these defects (Hassan et al 2000). Also patients with colorectal adenomas have raised levels of IGFII (Renehan et al 2001).

In summary IGF is a growth promoter for colorectal cancer whose main stay of physiological control is that of its plasma concentration with some changes at the receptor level.

1.1.6.3 Epidermal growth factor family

Epidermal growth factor (EGF) and EGF related peptides include transforming growth factor alpha (TGFα), cripto, amphiregulin and heparin binding EGF. They act as ligands for EGF receptors (EGFR). EGFR is a member of the erbB family of tyrosine kinase membrane receptors, which include EGFR ($erbB_1$), $erbB_2$ $erbB_3$ and $erbB_4$.

In dealing with the EGF family, attention will be given to EGF and TGFα only. As these are the most common and the ones for which most information related to colorectal cancer is available. They will be discussed at the level of bioavailability, i.e.

the amount of growth factor present and available for binding to its receptor; therefore, eliciting an effect. The receptors will be dealt with collectively, later. The signal transduction pathways or the nuclear pathways of the receptors will not be discussed as changes in these pathways do not appear to play a major role in colorectal cancer growth and progression.

The growth effects of EGF on colonic cancer cells were first shown by Scheithauer et al (1987); they found that, in experiments to improve conditions for colonic cancer cell proliferation, EGF more than doubled plating efficiency (i.e., the size the colony reached over time). Thomas (1994, 1995) found a 102% increase in the number of colorectal cancer cells by the addition of EGF.

TGFα has a mitogenic effect as well as a morphogenic effect on colorectal cancer cells. TGFα caused an increase in numbers of EGFR expressing colorectal cancer cells (Durrant et al 1991) as well as enhancing the crypt-like differentiation when grown in three dimensional collagen gels (Liu et al 1994). Solic et al (1995) demonstrated variable effects of TGFα on two colorectal cancer cells with similar levels of EGFRs. The exogenous addition of TGFα to one cell line caused an increase in cell number and to the other a change in the architecture of the colorectal cancer cell colonies. This study suggested the defining factor in the mitogenic and/or morphogenic action of TGFα is at the receptor level and below.

EGF was expressed by 7 of 19 (36.8%) colorectal cancers as compared to 1 of 12 (8.3%) normal colons. TGFα was present in 16/19 (84.2%) of colorectal cancers as compared to no staining in normal colons (Tanaka et al 1991). Younis et al (1996)

demonstrated a survival advantage in patients expressing TGFα in less than 25% of their tumour cells, compared to high expressors.

Tanaka et al (1992) followed the above findings with a demonstration of differential expression of EGF and TGFα in the adenoma carcinoma progression process. They showed using immunohistochemistry, that EGF was detected in 14/32 (43.8%) invasive cancers, 12/27 (44.4%) carcinomas *in situ* and 12/58 (20.7%) adenomas. The staining intensity for EGF was not related to histological grade. They also demonstrated similar findings for TGFα, with positive staining in 26/32 (81.3%) invasive cancers, 12/27 (44.4%) carcinoma *in situ* and 12/58 (20.7%) adenomas. Furthermore there was increased intensity of TGFα staining with increased grade of malignancy. *In vitro* experiments suggest EGF can protect adenoma cells against apoptosis for a limited period (Hague et al 1997).

Plasma levels of EGF and TGFα were not raised in patients with colorectal cancer (Klijn et al 1990). However, in contrast, Moskai et al (1995) showed a significant increase of TGFα in the sera of patients with colorectal cancer as compared to the random population and the levels of TGFα decreased after resection (Shim et al 1998).

The implication of these findings is that the bioavailability of both, EGF and TGFα, changes in colorectal cancer, and that other growth promoter effects may happen at the receptor level or further downstream. TGFα probably plays a bigger role in colorectal cancer mitogenesis than EGF. Therefore EGFR, $erbB_2$, $erbB_3$ and $erbB_4$ will be discussed in terms of their role in proliferation of colorectal cancer cell lines, their

differential expression in colorectal cancers and any possible roles they may have in the adenoma carcinoma pathways.

Studies of EGFR expression report variable findings for differences between normal colon and colorectal cancer. Moorghen et al (1990) demonstrated the same expression for normal colon from both adjacent to the tumour and more distant tissue and for adenocarcinoma and, similarly, Koretz et al (1990) found no correlation between EGFR and Dukes' stage. Conversely, an increase in EGFR in colorectal cancer compared to normal tissue was shown by Messa et al (1994) and, using immunohistochemistry to observe the presence of EGFR, Steele et al (1990) examined the relationship to survival and observed that although EGFR occurred in all colorectal tumours there were more receptors in Dukes' C tumours than in Duke's A and B and higher grade tumours had stronger staining. They concluded that EGFR levels relate to poorer prognostic factors.

There are only two reports of $erbB_2$ status in colorectal cancer. Saik and colleagues (1995) demonstrated, using immunohistochemistry, that $erbB_2$ was present in 13 of 45 (28.9%) of colorectal tumour compared to none in normal colon. They also found that 7 of 13 (53.8%) carcinomas with lymph node involvement expressed $erbB_2$, which is significantly more than those without metastasis. Immunostaining for products of the $erbB_2$ gene, (Shirai et al 1995) demonstrated higher expression in patients with flat colorectal cancers compared to those with adenomatous components to their cancer. They suggested that $erbB_2$ may be involved in the development of *de novo* cancers rather than those that follow the adenoma-carcinoma sequence. $ErbB_3$ and $erbB_4$ have not been examined in colorectal tissue.

1.1.6.4 Angiogenic factors

Neovascularisation is a critical requirement for tumour growth and metastasis formation. Numerous angiogenic factors that regulate this step have been identified including Vascular Endothelial Growth Factors (VEGF A, VEGF B, and VEGF C). Fibroblast Growth Factors (FGF1 (acidic FGF), FGF2 (basic FGF), FGF 3, FGF 4) and Platelet Derived Growth Factor (PDGF). These are the factors that are currently thought to be the most potent angiogenic factors, especially the VEGFs. Other, less potent, factors can also stimulate angiogenesis, including TNF alpha, TGF alpha, TGF beta, Interleukin 8, Angiogenin and Platelet Activating Factor. Only VEGF appears to be of importance in colorectal cancer and will be further discussed (Harris et al 1997, Folkman et al 1995).

1.1.6.5 Vascular endothelial growth factors

There are three VEGF peptides termed A, B, C. These act via three VEGF receptors. Furthermore each of the VEGFs has up to four isoforms that bind the receptors differentially and therefore exert different actions (Tokanaga et al 1998). However, in this thesis only the overall effects of VEGFs on colorectal cancer will be discussed, focusing in particular on VEGF production and distribution.

Colorectal cancer cell lines have been found to synthesise VEGF, for example, initially HT29 (Lobb et al 1985) and subsequently other lines (Ellis et al 1996). In colonic tissue, mRNA for VEGF occurs at a much higher level in colorectal cancer than in normal colon (Brown et al 1993) and is further increased in metastatic cancers compared to non-metastatic (Takahashi et al 1995, 1997 and 1998; Cheung et al 1998). Normal colon taken from immediately adjacent to a tumour showed higher expression of VEGF than tissue removed at a distance from the cancer (Nekata et al 1998) and

very high levels of VEGF were measured in ascites fluid from patients with very advanced colorectal cancer (Zebrowski et al 1999).

This pattern of higher levels of VEGF associated with more metastatic disease suggests that VEGF may be important for the invasion and metastasis of colorectal tumours. Further support for this came from experiments using a mouse model where, in a range of lesions mimicking the adenoma-carcinoma progression, high levels of VEGF were found in adenomas, the pre-malignant stage of the cancer, and even higher levels were found in adenocarcinomas, the malignant stage, compared to normal tissue (Wong et al 1999). Also the transfection of VEGF into the LoVo colorectal cancer line resulted both in a larger, more vascular tumour after subcutaneous inoculation and in higher capacity for establishing metastases after intraportal and intraperitoneal inoculation into nude mice, compared to the parent cell line (Kondo et al 2000).

1.2 Endothelin

The endothelin family consists of 3 related peptides, Endothelin-1 (ET-1), Endothelin-2 (ET-2) and Endothelin-3 (ET-3), that are closely related to the sarafotoxins, which are highly lethal peptides isolated from the venom of the burrowing asp (Attractaspis enggadensis) (Yanagisawa et al 1988, Kloog et al 1988). The endothelins and their receptors are widely distributed throughout human tissues. Originally they were identified as potent vasoconstrictors released by endothelial cells. They are now recognised as having multiple physiological and pathological actions and are thought to be involved in cancer.

1.2.1 Structure of endothelins

The endothelins are a family of 21 amino acid peptides, which are probably, expressed in all mammalian species (Inoue et al 1989, Saida et al 1989) as well as some non-mammalian species (Uemura et al 1991). The three endothelin isoforms have their amino acid chains cross-linked by two disulphide bridges and all have a carboxyl terminal tryptophan residue. (Figure 1.4)

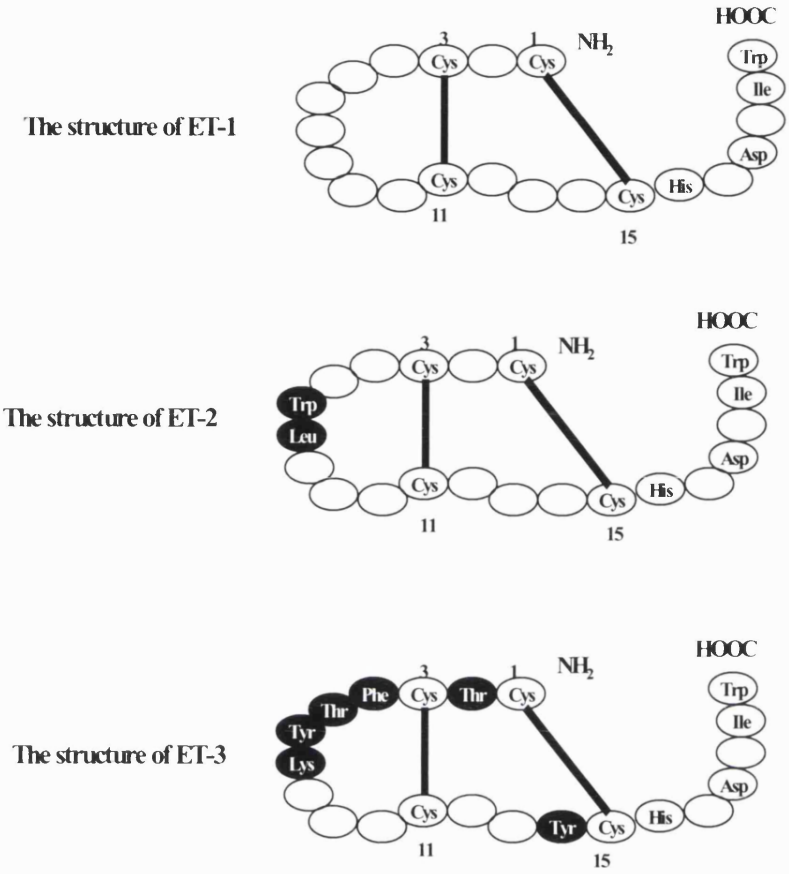


Figure 1.4 *Structure of the endothelins. The peptide chain is made up of 21 amino acids with two disulphide bridges represented in bold. The amino acid residues in ET-2 and ET-3, which differ from ET-1, are in bold. The C-terminal amino acids shown are those involved in binding.*

The genes for ET-1, 2, and 3 have been localised to chromosomes 6, 1 and 20 respectively (Bloch et al 1989, 1991, Arinami et al 1991). Human ET-1 is derived from a 212 amino acid precursor termed pre-pro-endothelin-1. This peptide undergoes a proteolytic cleavage to pro-endothelin, a 38 amino acid peptide normally called big

endothelin-1 (big ET-1) (Figure 1.5). The conversion of big ET-1 to ET-1 is an important regulatory event controlling the amount of ET-1 present and is catalysed by Endothelin Converting Enzyme (ECE) (Yanagisawa et al 1988). ECE is a metalloprotease that is inhibited by phosphoramidon but is insensitive to other closely related metalloprotease inhibitors. ECE is active at neutral pH (Ohnaka et al 1990, Opgenorth et al 1992).

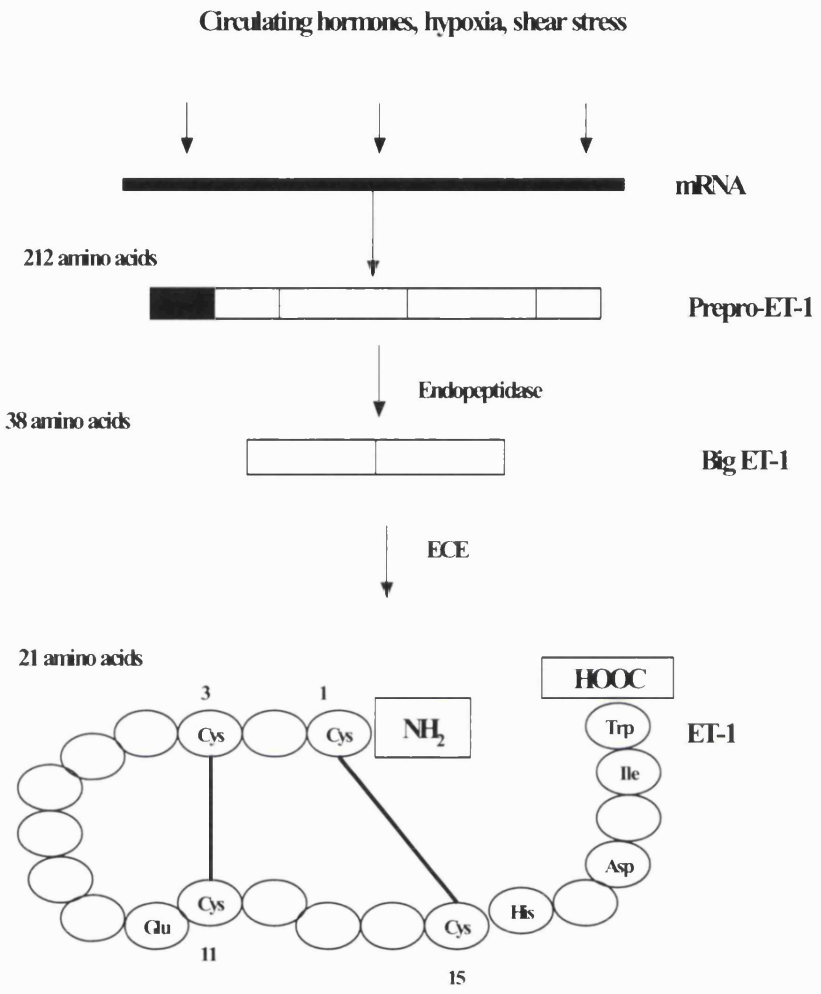


Figure 1.5 *The pathway of ET-1 synthesis. Pre-pro ET-1 is processed to pro ET-1 (big ET-1). Big ET-1 is further cleaved to ET-1 by ECE.*

1.2.2 Receptors

The endothelins act via two receptor subtypes ET_A and ET_B. The receptors contain seven segments; each made up of 20-27 amino acids. They belong to the G protein coupled receptor superfamily. The human ET_A receptor gene has been assigned to chromosome 4 and the ET_B receptor gene to chromosome 13. Both the ET_A and ET_B gene have a single copy in the human genome (Hosoda et al 1991, 1992). The affinity of the three isopeptides for the ET_A receptor is ET-1 > ET-2 > ET-3. In man the ET_A receptor has 1000 times greater affinity for ET-1 than ET-3 (Hosoda et al 1991). The ET_B receptor shows no selectivity for any of the ET isoforms (Sakamoto et al 1991, 1993).

The difference in binding affinity of ET-1 and ET-3 to ET_A led to the classification and characterisation of the receptor's physiological response. The vasoconstrictor response was produced by stimulation of the ET_A receptors on vascular smooth muscle cells and the vasodilator response by stimulation of the ET_B receptors on the endothelium. However subsequent experiments using isolated vascular preparations have provided evidence to indicate that renal and several arterial and venous circulations contain ET_B receptors, which produce vasoconstriction (Bigaude et al 1992, Clozel et al 1992, Cristol et al 1993). Further stimulation of the ET_B receptors on the vascular smooth muscle of the jugular and saphenous veins of rabbits also resulted in vasoconstriction (Moreland et al 1991, 1994, Summer et al 1992). As a result of the above evidence two ET_B receptor subtypes were thought to exist and this was confirmed by pharmacological studies (Warner et al 1993).

Finally the possibility of a third ET receptor was raised by Wren et al 1993 in which ET-3 was shown to mediate cell proliferation in wounded human umbilical vein endothelial cells. The addition of ET-1, ET-2 or selective agonists for ET_A and ET_B had no effect on this process. This suggested the presence of a third ET receptor, named ET_C. The ET_C receptor was demonstrated in Xenopus laevis (Karne et al 1993). However, no actual ET_C receptor has been found in humans.

Despite the pharmacological evidence to indicate the presence of endothelin receptor subtypes, biochemical or molecular evidence has remained elusive.

1.2.3 Signal transduction

Transmembrane signalling systems relay information from the exterior to the interior of a cell through a series of complex protein-protein interactions and second messenger cascades. Two types of signalling have been described: A) short term actions characterised by responses such as contraction or secretion, B) long term responses that are adaptive in nature such as cell growth or apoptosis (Okazawa et al 1998). These pathways and cascades are cytosolic and/or nuclear.

The initial step of ET-1 signalling is the docking of ET-1 on the G protein coupled receptor. The final biological effect depends on the availability of receptor subtypes, the state of the cell and the interactions between the target cells and its environment (Milligan 1993).

Ligand receptor binding leads to activation of G protein which in turn can switch on (phosphorylate) one of the following upstream initiators of different pathways:

phospholipase C (PLC), phospholipase D (PLD), phospholipase A_2 (PLA_2), adenylate cyclase (Mallat et al 1996), guanylate cyclase. Furthermore a Na^+-H^+ exchange mechanism to facilitate ion transfer is also activated. (Ambar et al 1993, Sokolovsky 1992, 1994, Aramori et al 1992, Simonson et al 1989, Vigne et al 1991). (Figure 1.9).

The PLC cascades appear to play a major role in mitogenesis. PLC activates protein kinase C (PKC) and also acts on the endoplasmic reticulum to release calcium within the cell. PKC in turn phosphorylates other intracellular proteins, which eventually turn on fos in the nucleus. The latter acts as a transcription factor for other genes involved in mitogenesis. However, extramitogenic stimuli seems to be generated from cross talk between the molecules described above and other cascades. To date (Figure 1.10), PLC/PKC/Ca^{2+} result in the phosphorylation of tyrosine kinases such as focal adhesion kinase (fak) and mitogen activated protein kinase (MAP kinase) (Liu et al 1999). This scenario is one possible explanation for synergism between growth factors and ET-1 (Smith et al 1998). These steps have been demonstrated in ovarian cancer cells, smooth muscle cells and fibroblasts (Bagnato et al 1997, Savill et al 1994, Fujitami et al 1995, Luttrell et al 1999, Schwartz et al 1999).

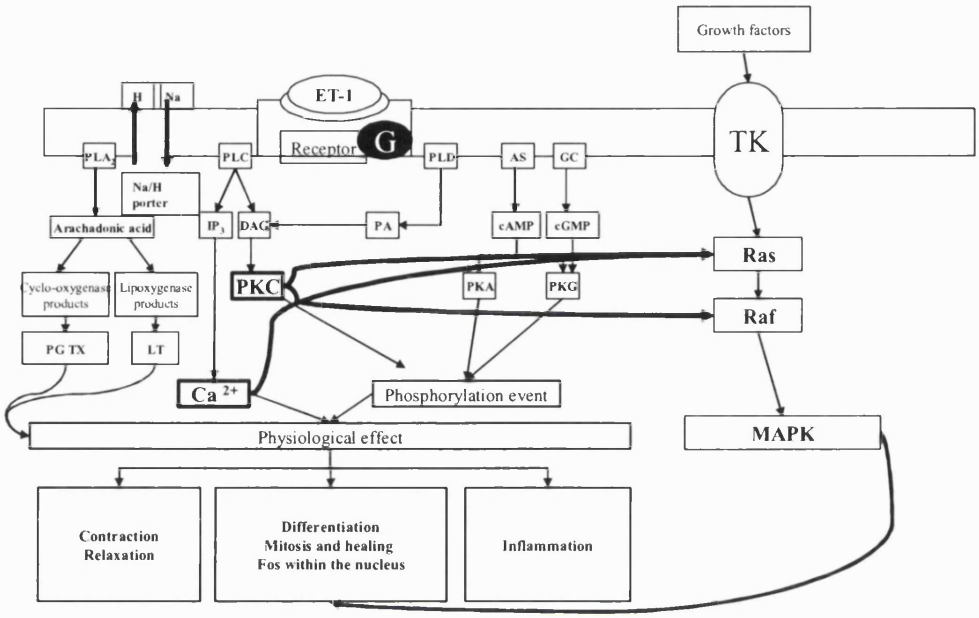


Figure 1.6 *Schematic representation of the signal transduction pathways associated with activation of the endothelin receptors. Upstream transduction molecules include: Phospholipase A_2 (PLA_2); Phospholipase C (PLC); Phospholipase D (PLD); Adenylate cyclase (AS) Guanylate cyclase (GC). Downstream molecules include: Phosphokinase A, G, C, (PKA, G, C) respectively; Prostaglandins (PG); Thromboxane (TX); Leukotriene (LT). Fos is one example of a nuclear transcription factor involved in mitogenesis. Cross talk between the PLC cascade and the tyrosine kinase cascade is shown by the bold arrows.*

1.2.4 Physiological function

The endothelins and their receptors are present throughout the whole body and have a homeostatic function in normal physiological processes. The physiological functions of the endothelins can be related to their effect on smooth muscle, particularly in blood vessels and the autoregulation of blood flow locally. Endothelins are also implicated in development, mitogenesis, inflammation and wound healing.

The mechanism of action of ET-1 on vascular smooth muscle is shown in Figure 1.7. ET-1 can bind to the ET_A and/or ET_{B2} receptor on smooth muscle cell causing vasoconstriction. Excess ET-1 is degraded by endothelin degradation enzyme (EDE). Experimentally, intravenous administration of ET-1 elicits an initial reduction in arterial pressure, which is followed by a prolonged increase in blood pressure (Lippton et al 1988, Wright et al 1988).

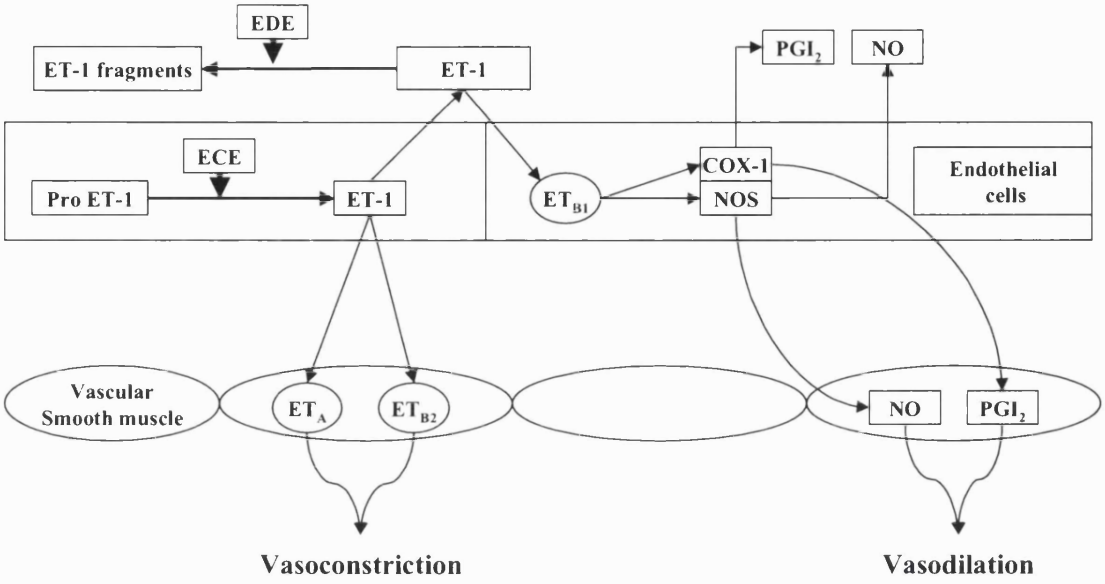


Figure 1.7 *ET-1 and other endothelium derived vaso-active factors and their interactions. In endothelial cells Endothelin Converting Enzyme (ECE) converts proET-1 to ET-1. ET-1 binds to ET_A and ET_{B2} receptor on the vascular smooth muscles causing vasoconstriction. ET-1 released in the lumen is degraded rapidly by Endothelin Degradation Enzyme (EDE). Circulating ET-1 can also bind to ET_{B1} receptor, which stimulates Nitric Oxide Synthetase (NOS) and Cyclo-oxygenase-1 (COX-1). The resultant nitric oxide (NO) and prostacyclin (PGI_2) acts on the underlying smooth muscle causing vasodilatation.*

Endothelin plays a vital role in the cardiovascular system both in the contraction of cardiac muscle (Yanagisawa et al 1988) and in the coronary perfusion of the heart (Haynes et al 1994). Contrary to expectation the endothelins do not control blood pressure on a minute-to-minute basis (Masaki 1993) but undoubtedly have a role in the overall maintenance of blood pressure through their effect on vascular tone and water balance (Wilkins et al 1993).

The production and release of endothelins has been shown to be sensitive to carbon dioxide levels and oxygen levels (Bell et al 1998). Therefore it follows that the endothelins play a role in normal respiratory function of blood perfusion through the autoregulatory mechanisms described above, the ventilation aspects of respiration at the level of the bronchioles through its effects on smooth muscle (Haynes et al 1993) and finally at the gaseous exchange levels with the involvement of the inflammatory mediators described earlier.

The endothelins are important in maintaining homeostasis of kidney function through their control of renal blood flow of both efferent and afferent blood vessels and the glomerular filtration rate, hence affecting the body's water and electrolyte balances. Additionally, there is evidence for a direct role of ET-1 on the tubules and mesangial cells of the kidney mainly by the ion pumps (Simonsen et al 1990, 1994).

The endothelins are thought to play an important role in liver function. Endothelin binding sites are found on endothelial cells of sinusoids, portal veins and central veins as well as on hepatic stellate cells (fat storing cells). Bile duct epithelium is able to

synthesise ET-1 (Mallat et al 1996). Endothelin acts on the liver to affect its blood flow, the metabolic function e.g. glycogenolysis (Gandhi et al 1990, Tran-Thi et al 1993, Serradeil-Le Gal et al 1991) and bile secretion in rats (Tanaka et al 1994) and humans (Fouassier et al 1998).

Endothelin has several effects on the gastrointestinal tract (GI), which will be discussed in section 1.2.8. The role of endothelin in the normal physiological function of the central nervous system remains unclear despite ample evidence for endothelin involvement in diseases resulting in the compromise of cerebral perfusion. This will be discussed further in the section below.

Finally and of most interest to this work is that endothelin plays a physiological role in cell growth and healing as a response to injury through inflammation, as a primary growth factor and to complete the cycle in cell death and apoptosis. The endothelins' physiological role in this area is described in the section on signal transduction pathways (1.2.3) and the section on the effect of endothelin on growth (1.2.6). Further evidence for the endothelins' role in growth and development comes from studies on the deletion of the ET-1 gene in knockout mice, which is lethal at birth due to respiratory failure and craniofascial abnormalities (Kurihara et al 1994). Knockout mice for the ET-3 gene or the ET_B receptor gene results in animals with aganglionic megacolon. (Hosoda et al 1994, Baynash et al 1994).

1.2.5 Role of endothelin in disease

Changes in the levels of endothelin immunoreactivity and receptor expression have been reported in several diseases, such as, hypertension, renal failure, myocardial

ischaemia, myocardial arrhythmia, asthma, atherosclerosis, Crohn's disease, ulcerative colitis (Murch et al 1992) and subarachanoid haemorrhages in addition to diseases involving abnormal vasculature, for example, eclampsia and Raynaud's disease. The evidence for the involvement of ET-1 in some of these diseases will be reviewed below (Hosoda et al 1992, Hori et al 1992, Elshourbagy et al 1993).

Patients with myocardial infarctions have raised plasma ET-1 levels (Lechleitner et al 1993, Tomoda 1993) that correlate with the degree of myocardial damage and prognosis (Yasuda et al 1990, Omland et al 1994). The use of ET_A receptor antagonists increased coronary blood flow and decreased myocardial damage indicating a primary role for ET-1 in myocardial infarctions (Grover et al 1993, 1992, Lee et al 1994, Nelson et al 1994). The ability of ET-1 to promote growth and stimulate migration of smooth muscle cells and fibroblasts has been implicated as a cause of atherosclerosis and restenosis following balloon angioplasty in coronary vessels (Ohlstein et al 1993). Further evidence for ET-1 as a factor in myocardial infarctions is provided by the administration of ET_A receptor antagonists in a rat model, which reduced neo-intimal thickening by up to 50%, (Douglas et al 1994) and blocked angiotensin induced cardiomyocyte hypertrophy in an *in vitro* model (Ito et al 1994).

ET-1 may be a factor in respiratory dysfunction as increased ET messenger RNA expression was found in the tissue of patients with asthma (Kraft et al 1994), adult respiratory distress syndrome and pulmonary hypertension (Filep et al 1992). Also an ET_A receptor selective antagonist reduced pulmonary hypertension and pulmonary artery medial wall thickening in the rat (Bonvallet et al 1993, 1994).

In the central nervous system, endothelium dependent relaxation is known to be impaired in cerebral arteries from patients suffering from subarachanoid haemorrhage (Faraci 1993). The use of the ET_A receptor antagonist, BQ123, reversed artery vasospasm by approximately 70% (Foley et al 1994) and significantly reduced neuronal death following global cerebral ischaemia (Feuerstein et al 1994, Nirei 1993) found that ET_A receptor antagonists depressed the early vasoconstriction following haemorrhage. These studies all implicate ET-1 in central nervous system disease.

There is considerable evidence suggesting ET-1 involvement in the pathogenesis of acute and chronic renal failure as well as cyclosporin toxicity. For example, administration of antibodies to ET-1 following ischaemic insult to the rat kidney improved renal blood flow and glomerular filtration rate (Sandok et al 1992). In an animal model of kidney disease treatment with high doses of the ET_A receptor antagonist, BQ123, improved creatinine clearance (Mino et al 1992), however low doses had no effect (Chan et al 1994, Gellaiet al 1994) confirmed this and found a remarkable decrease in mortality, which was attributed to an improvement in tubular function and the prevention of lethal increases in K^+. (Perico 1993) has proposed a hypothesis for chronic renal failure in which a role is attributed to ET-1. Here, renal insult gives haemodynamic changes, which release ET-1 from glomerular endothelial cells, causing an increase in the arteriolar resistance and glomerular hypoperfusion resulting in renal damage. Benigni et al (1991, 1993) have evidence supporting parts of this model and have shown that the ET_A receptor antagonist, BQ123, reduced the level of chronic renal failure in the rat with improved glomerular filtration rate, reduced proteinurea and better creatinine clearance compared to controls.

Endothelins influence the microcirculation of all liver cells (Gandhi et al 1996) and during injury can directly contribute to the transformation of stellate cells (fat storing cells) into myofibroblasts (Gabriel et al 1999). Via these mechanisms they are thought to have a role in liver cirrhosis (Gandhi et al 1996) and fatty liver changes. Despite the above evidence there are, as yet, no studies demonstrating modulation of liver disease by endothelin receptor agonists or antagonists.

The above are just a few examples of the diseases in which endothelin may play a role. This thesis is based on the growth inducing properties of endothelin, their role in cancer and specifically in colonic / colorectal cancers.

1.2.6 Effect of endothelin on growth

The suggestion that ET-1 could act as a mitogen and/or angiogen was inferred from studies, which demonstrated that ET-1 regulated DNA synthesis in various smooth muscle cells (Hirata et al 1989, Serradeil-Le Gal et al 1991). The mechanism was thought to occur by stimulation of c-fos and c-myc (Komuro et al 1988), via ET_A (Ohlstein et al 1992). ET-1 was also found to stimulate mitogenesis of fibroblasts, including fibroblasts from rat kidney (Yeh et al 1991), human breast (Schrey et al 1992), human skin (Kahaleh et al 1991) and Swiss 3T3 cells (Brown et al 1989, Takuwa et al 1989, Fabregat et al 1989, Kusuhara et al 1990). ET-1 induced DNA synthesis in a multitude of other cell types such as bovine brain capillary endothelial cells (Vigne et al 1990), rat astrocytic glial cells (MacCumber et al 1990), rat and murine osteoblastic cells (Schvartz et al 1992, Takuwa et al 1989) rat mesangial cells (Badr et al 1989, Simonson et al 1989, 1992), and human prostate secretory epithelial cells and fibromuscular stromal cells (Walden et al 1998, Saita et al 1998).

ET-2 can also stimulate DNA synthesis and is equipotent with ET-1 in stimulating synthesis in rat osteoblasts and human melanocytes, (Bohm et al 2001, Imokawa et al 2000). ET-3 is less active (Takuwa et al 1990, Yada et al 1991) (Table 1.1). Human big ET-1 is substantially less effective in promoting DNA synthesis than ET-1 (Yada et al 1991).

However, some of ET-1's mitogenicity may come from acting as a co-mitogen. *In vitro* experiments will contain other growth factors from serum or additives e.g. insulin, or from the endogenous secretion of growth factors by cells (Weissberg et al 1990). Evidence that ET-1 acts more powerfully as a co-mitogen is demonstrated using Swiss 3T3 mouse fibroblasts. The addition of ET-1 alone produced an increase in cell number up to 7 fold, whereas ET-1 with EGF produced a 35 fold increase in numbers, with TGFβ a 22 fold increase, and with PDGF a 28 fold increase (Brown et al 1989). Similar results were found using normal human breast fibroblasts (Schrey et al 1992) and human melanocytes (Yada et al 1991).

A further possible explanation for the apparent ET-1 induced growth may be attributed to the increased lifetime of cells by altering programmed cell death e.g. in human melanoma cells (Okazawa et al 1998). ET_B receptors were implicated in apoptosis in rat vascular smooth muscle cells, as shown by Cattaruzza et al (2000). Similar results were demonstrated by Ehrenreich et al (2000) in human rat and rabbit neurones. ET-1 also induced apoptosis in rat vascular endothelial cells (Suenobu et al 1998), which was mediated by the ET_B receptor (Shichiri et al 1998). The above demonstrates the possible homeostatic role of the endothelins on growth through multiple pathways,

where ET_A receptor pathways induce mitogenesis as described earlier and ET_B receptors mediate apoptosis.

On the contrary other studies have shown that ET-1 inhibits growth, particularly in liver stellate cells (Mallat et al 1995, 1996).

1.2.7 Effect of endothelin on cancer

Several cancer cell lines synthesize ET-1. Kusuhara et al (1990) detected ET-1 production by 13 of 42 human cancer cell lines, with high ET-1 production by mammary, pancreatic, and colonic carcinoma cell lines. Schrey et al (1992, 1995) and Yamashita et al (1993) showed that ET-1 is produced by breast cancer cell lines and that this production is stimulated by bombesin, glucocorticoids and interleukin 6. Other cancer cell lines that secrete ET-1 include; pancreatic (Oikawa et al 1994), gastric (Mathieu et al 1995), prostatic (Le Brun et al 1999), ovarian (Moraitis et al 1997), cervical, laryngeal (Shichiri et al 1991) and endometrial (Pekonen et al 1992), (Economos et al 1992).

Shichiri (1991a, b) has shown that ET-1 is an autocrine / paracrine growth factor for human cervical cancer cell lines (HeLa) and laryngeal cancer cell lines (HEP-2). Further evidence for ET-1 as a growth factor in cancer cells was supplied in human ovarian cancer cell lines by Bagnato et al (1995, 1997) and Moraitis et al (1997). They demonstrated a direct ET-1 induced growth by these cells and showed that ET-1 acts as an autocrine growth factor via ET_A. Specifically, ET-1 acted via protein kinase C and tyrosine kinase pathways, and caused an increase in intracytoplasmic Ca^{2+} and

thymidine uptake, both being inhibited by the ET_A antagonist BQ123 (Bagnato et al 1999).

The above are the only published studies that show a direct link between ET-1 and human cancer cell growth. However, other studies imply this relationship, for example: Le Brun et al (1999) found up-regulation of ET-1 in conjunction with other growth factors by human prostate cancer cells. Functional ET receptors have been localised in human melanoma cells (Yohn et al 1994). ET-1 regulated intracellular Ca^{2+} concentration and by inference influenced growth of human adrenocortical carcinoma cells (Rossi et al 1997, Patel et al 1995) demonstrated that breast cancer cells could process big ET-1 to ET-1, which in turn acted as a paracrine mitogenic factor for breast fibroblasts.

In tissue from patients with cancer, differential expression of ET-1 or its receptors has been shown. Increased ET-1 immunoreactivity was first described in breast cancer (Yamashita et al 1991, Patel et al 1995). Several other cancers were shown to have increased ET-1, including pulmonary tumours and their blood vessels (Giaid et al 1990, Zhao et al 1995), prostatic cancer (Nelson et al 1995, 1996), testicular cancer (Takeda et al 1994) hepatocellular carcinoma (Ishibashi et al 1993, Suzuki et al 1998). However, Ben-Baruch et al (1993) found reduced ET-1 binding in endometrial cancers as compared to uterine tissue from myomatous women. This suggested that ET-1 might act to increase blood flow to the tumour rather than as a direct mitogen for endometrial cancer.

High concentrations of ET-1 in the plasma of patients with cancer, for example hepatocellular carcinoma (Nakamuta et al 1993), breast cancer (Yamashita et al 1995), oesophageal and gastric cancers (Itoh et al 1996) have been reported.

Studies of endothelin receptors in cancer tissue are confined to comparison of the distribution of the receptor types with those in normal tissue and the ratio of ET_A to ET_B in tumour compared to normal tissue. In prostatic cancer, which perhaps is the most studied, (Nelson et al, 1996) down-regulation of ET_B receptors as compared to normal prostate without any such change in ET_A receptors ratio has been reported by Nelson et al (1997) using binding studies. They also demonstrated a concomitant down-regulation of mRNA for ET_B.

In tissue from patients with ovarian cancer an increase of ET_A receptors compared to normal tissue was demonstrated using reverse transcriptase PCR (Bagnato et al 1999).

1.2.8 Endothelin and the gastrointestinal tract

1.2.8.1 Endothelin and gut physiology

Endothelin-like immunoreactivity was detected throughout the gastrointestinal (GI) tract, from saliva in the mouth of humans (Lam et al 1991) to high levels in the fundus of the stomach, jejunum, ileum and colon in rats (Takahashi et al 1990) and in humans (Escrig et al 1992). Takahashi and colleagues also showed that ET-1 was present in the colonic mucosa as well as the smooth muscle layers in the rat. Inagaki et al (1991 a, b) demonstrated that all endothelins bound at specific sites within nerve bundles throughout the human colon and to most ganglia cells. Endothelin binding was also

detected in the mucosa and submucosa of colon but with reduced affinity compared to nerve and muscle binding. The results were similar in pigs (Hemsen et al 1991).

One possible physiological function of endothelins is the control of intestinal secretions of Na^+ and K^+ ions in humans (Brown et al 1991) and Cl^- and K^+ secretion in the rabbit descending colon (Roden et al 1992, Reddix et al 1998). In the rat, this ion secretion is brought about by stimulation of ion transport (Moummi et al 1992) through the cyclo-oxygenase pathway and activation of the enteric nerves (Kiyohara et al 1993). Further work by Hosokawa et al (1994, 1995) has shown that ET_A and ET_B receptors regulate ion transport and that the use of receptor antagonists alters the balance of receptors able to be activated and consequently ion secretion and transport.

Endothelins may regulate blood flow to the GI tract. ET-1 causes a reduction in the blood flow of the colonic vascular bed, both directly due to the vaso-constrictive effect of ET-1 acting on vascular smooth muscle (Usune et al 1991, Bitar et al 1992, Kitsukawa et al 1994, Okabi et al 1995) and nerve fibres (Wiklud et al 1991) and indirectly by inhibition of the vaso-active intestinal peptide (VIP) (Blank et al 1991). Endothelin receptor antagonists have been shown to both increase and decrease blood flow to the gastrointestinal tract (Lazaratos et al 1995, Gulati et al 1997).

The endothelins may be involved in the regulation of the immune response in the gut, as suggested by the presence of endothelin (ET-1, 2, 3 and big ET-1, 2, 3) immunoreactivity in mast cells (Liu et al 1998). ET-1 can also act via the ET_A receptor to induce leukocyte adhesion to the submucosal venules, which may increase the number of leukocytes able to intravasate to the submucosa (Boros et al 1998).

1.2.8.2 Endothelin involvement in gut pathology

The pathological sequelae of the dysfunction of the endothelin system in the gut are multiple and include inflammatory, developmental, functional and secretory abnormalities.

Inflammatory abnormalities related to the endothelin system have been shown by Slomiany et al (1999) in which a direct relation between ET-1 levels and gastric mucosal injury was demonstrated using a rat model. This injury was reduced by antacid treatment. The use of endothelin receptor antagonists reduced acute and chronic colitis in the rat (Gulluoglu et al 1999).

Developmental abnormalities in the colon only occur with ET-3 and its interaction with ET_B; mutations of the endothelin-B receptor gene produced megacolon in mice (Hosoda et al 1994, Baynash et al, 1994). *In vitro* work by Wu et al (1999) suggested that the ET-3/ET_B receptor interaction prevented the premature differentiation of neural crest derived precursors. This in effect controls the migration neural crest cells enabling the precursor population to persist for long enough to finish colonising the bowel. *In vivo* evidence has been obtained by Leibel et al (1999) who found high expression of ET-2 mRNA in mouse caecal mesenchymal cells during embryogenesis.

High plasma ET-1 levels correlated with long oro-caecal times in moderate pancreatitis and *vice versa* (Chen et al 1999). This suggested that ET-1 contributes to low gut motility. The hypothesis, i.e. that ET-1 plays a role in gut motility, was supported by work in an experimental rat model of paralytic ileus, where endothelin potentiated the actions of acetylcholine in the control group but not the experimental group (Tekin et al

1999). Also, ET-1 has an excitatory effect on the anal sphincter of the opossum via a protein kinase c and a Ca^{2+} pathway (Chakder et al 1999).

1.2.8.3 Endothelin and colorectal cancer

The production of ET-1 by colorectal cancer cell lines was shown by (Kusuhara et al 1990) and binding sites for ET-1 were demonstrated by (Inagaki et al 1992). Previous work originating from this department demonstrated that ET-1 was present in higher amounts in colorectal cancer tissues than in normal colon (Asham et al 1997, Shankar et al 1998) and that patients with colorectal cancer or liver metastases had higher plasma levels of ET-1 than control patients. Asham et al (2001) has also shown ET_A antagonism reduces colon cancer mass in a rat model *in vivo*. However, the effect of ET-1 on colorectal cancer development and/or progression remains to be resolved, that is, does ET-1 act as a mitogen as has been shown for several cancer cell lines? (Bagnato et al 1995, 1997, Moraitis et al 1997, Shichiri et al 1991 a, b) or is it influencing apoptosis as found by Eberl et al (2000) in rat colon carcinoma cell lines *in vitro*?

The aim of the thesis is to determine if ET-1 has a direct link to colorectal cancer growth. This will be investigated by: -

1) Examining if there is quantitative and/or qualitative differences in ET receptors in colorectal cancer tissue compared to normal colon.
2) Determining if ET-1 can stimulate growth of colorectal cancer cell lines.
3) Assessing if ET receptor antagonists inhibit the growth of colorectal cell lines.

2 Localisation of Endothelin Receptors using Immunohistochemistry

2.1 Introduction

The increase in endothelin-1 demonstrated in tissue from patients with colorectal cancer and the higher levels of endothelin-1 found in the plasma of these patients suggest that this peptide may act in colorectal cancer. To exert an effect on the cells endothelin would have to bind to its receptor so activating signal transduction pathways as described in section 1.2.3. Therefore the first step was to determine if endothelin receptors are present in colorectal cancer, which type of receptor occurs and the distribution within the tissue. Antibodies to ET_A and ET_B were used to examine their presence and distribution in normal colonic and colorectal cancer tissue from patients with colorectal cancer.

2.2 Materials and Methods

2.2.1 Materials

2.2.1.1 Chemicals:

These were from Merck, Poole, Dorset, U.K. and of at least AnalaR grade unless stated otherwise.

2.2.1.2 Tissues:

All tissue was from patients undergoing a surgical resection for colorectal cancer. Fresh-frozen - normal colon, both adjacent to the tumour and distant (at least 10cm) from it, and tumour was collected, divided into $1cm^2$ pieces, snap frozen and stored in liquid nitrogen. Archival material that had been formalin fixed and wax embedded was also used. From all samples 5µm sections were cut to stain for endothelin receptors.

2.2.1.3 Antibodies:

The primary antibodies were all polyclonal and two were used for each receptor. Sheep anti-endothelin A receptor (anti-ET_A) antibodies (Alexis Corporation, Bingham, Notts, U.K. and Research Diagnostics, Flanders, New Jersey, U.S.A.) and sheep anti-endothelin B receptor (anti-ET_B) antibodies (Alexis Corporation, Bingham, Notts, U.K. and Research Diagnostics, Flanders, New Jersey, U.S.A.).

Secondary antibodies: For the 3-layer avidin biotin method, a kit containing a rabbit anti-sheep IgG biotin-labelled antibody and the components to prepare the avidin biotin complex was used (Vector Laboratories, Peterborough, Cambs., U.K.). For the two layer techniques either a rabbit anti-sheep IgG labelled with horseradish peroxidase (HRP) at 1 in 50 in 5% normal rabbit serum or one labelled with fluorescein isothiocyanate (FITC) at 1:100 in 5% normal rabbit serum were employed (both from Southern Biologicals, Birmingham, Alabama, U.S.A.)

2.2.2 Method development

A small pilot study was carried out to determine the optimum conditions for staining for the endothelin antibodies, investigating the following:

1. The concentration of the ET_A and ET_B antibodies was tested at 1:20, 1:50, 1:100, 1:150, 1:200, 1:400 and 1:800. The optimum dilution for frozen sections was 1:100 and for paraffin sections was 1:150.
2. A variety of fixatives for the frozen sections were examined; acetone at 4°C, acetone and chloroform (1:1), acetone and methanol (1:1), 4% paraformaldehyde, and 10% neutral buffered formalin. Best results were obtained with formalin fixation.

3. Three different immunohistochemical staining techniques were employed, a two-layer with a FITC labelled secondary antibody, a two layer with an HRP labelled secondary antibody and a three layer avidin biotin system. The three-layer system produced the most consistent results and was therefore employed and is described below (section 2.2.3).

2.2.3 Three layer avidin biotin technique

Sections from frozen tissue were fixed in formalin for 30 minutes and then washed in phosphate buffered saline (PBS) three times. Paraffin embedded sections were dewaxed in xylene, rehydrated in reducing concentrations of alcohol from 100% to 70% and washed in PBS. The slides were then treated identically.

The tissue sections were permeabilised, for 30 minutes, with 0.1% Saponin (Sigma, Poole, Dorset, U.K.) in PBS, to facilitate entry of the antibody to its cytoplasmic epitope. Endogenous peroxidase activity was prevented by incubating the slides with 0.3% hydrogen peroxide in methanol for 30 minutes, followed by washing with tap water for 3 minutes, distilled water for 5 minutes and then PBS for 5 minutes. Non-specific binding to the primary antibody was blocked by incubating the sections with 10% normal rabbit serum (Sigma, Poole, Dorset, U.K.) for 10 minutes. Excess serum was removed from the slides and then the sections were incubated overnight at 4°C with anti-ET_A or anti-ET_B antibodies prepared in 1% normal rabbit serum in PBS. After washing three times with PBS the sections were incubated with secondary antibody at 1:100 in 5% normal rabbit serum at room temperature for 45 minutes in a humid atmosphere. Following a further three washes in PBS the avidin biotin complex, prepared according to the manufacturers instructions, was applied to the sections and they were incubated for 45 minutes at room temperature and then washed

three times in PBS. To be able to visualise the endothelin receptors the sections were incubated with the chromagen, diaminobenzidine (0.25mg/ml) in Tris buffer pH 7.5 containing 0.1% hydrogen peroxide for 12 minutes. After washing in tap water the nuclei were counter-stained with Harris haematoxylin for 10 seconds, the slides 'blued' in tap water, dehydrated with 70% followed by 100% alcohol and xylene, and then mounted using DePX.

The slides were viewed by light microscopy (Olympus, London, U.K.) and the distribution and intensity of the staining was assessed by three observers independently. There appeared to be a distinctive pattern of staining of the epithelial cells and therefore a subjective scoring system was devised in which 0=no staining, 1=1-33% cells stained, 2=34-66% epithelial cells stained, 3=67-100% epithelial cells stained.

2.3 Results

2.3.1 Introduction

Sections of normal colon from adjacent to a tumour (n=35) and from at least 10cm from a tumour (n=10) and of colorectal tumours (n=45) were stained for endothelin receptors, A and B using an indirect immunoperoxidase technique. The tumours were staged as 18 Dukes' A, 13 Dukes' B and 14 Dukes' C and most (32) were moderately differentiated with 7 well differentiated and 6 poorly differentiated (Appendix 6.1).

2.3.2 ET_A staining

2.3.2.1 Distant normal colon:

There was very little staining for ET_A in the distant normal colon with no staining of epithelial cells (Table 2.1) or blood vessels and in just two patients (20%) ET_A was seen in the stroma (Table 2.2).

2.3.2.2 Adjacent normal colon:

The distribution of ET_A in normal colon immediately adjacent to colorectal tumours was similar to that observed in the tumours. The majority (91%) of the patients had staining for ET_A in the epithelial cells with approximately a third having the majority of the epithelial cells stained (Table 2.1). The number of epithelial cells expressing ET_A was reduced with distance from the tumour. No staining for ET_A was observed in the blood vessel walls in the adjacent normal colon and in only 6 patients (17%) was it present in the stroma (Table 2.2)

2.3.2.3 Tumours:

ET_A was seen in the epithelial cells of all sections of tumour (Figure 2.2) and in one-third of cases more than 67% of the cells was stained (Table 2.1). For poorly differentiated tumours it was not always possible to estimate the percentage of epithelial cells stained. The presence of ET_A could also be seen associated with blood vessels in 14 (31%) patients, usually in the muscle layers but occasionally within endothelial cells. In twenty of the patients (45%) ET_A was also present in the stroma (Table 2.2). There was no correlation between the staining for ET_A and either the stage or the grade of the tumour.

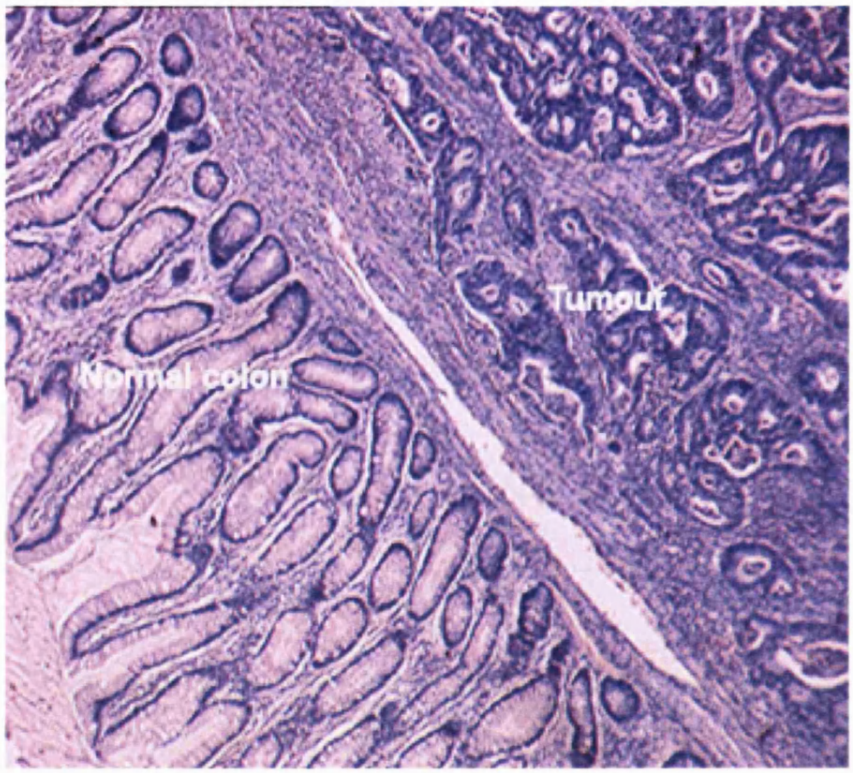


Figure 2.1 *Sections across the junction between normal colon and moderately differentiated colonic adenocarcinoma. Control for figures 2.2–2.4 in which the primary antibodies have been omitted. The nuclei have been counterstained with haematoxylin. Original magnification X100.*

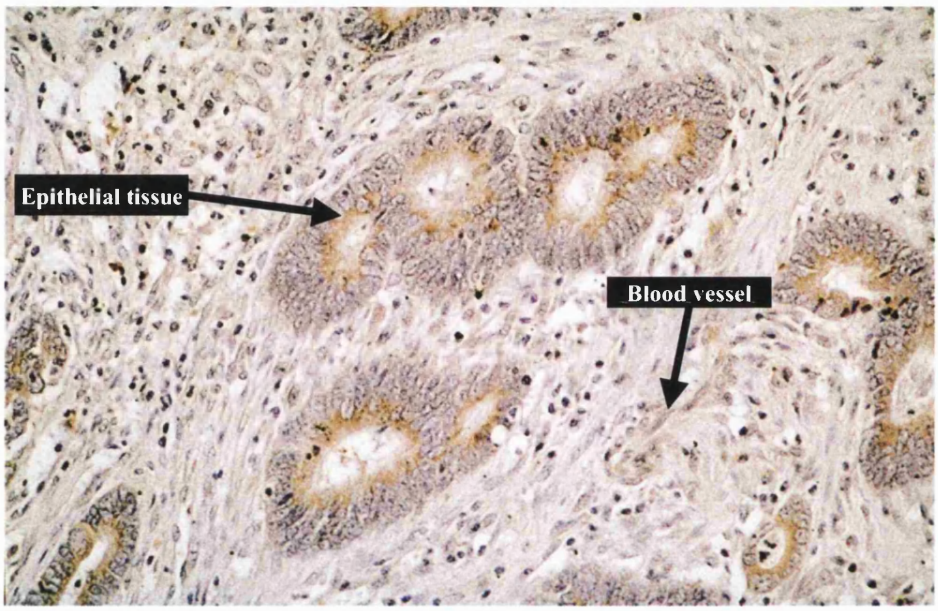


Figure 2.2 *Staining of a moderately differentiated tumour with antibody to ET_A and visualised with 3-step avidin-biotin and DAB technique. ET_A, seen as brown particles, is present in the majority of epithelial cells (score 3) and can also be observed in some blood vessels. Original magnification x500.*

Table 2.1 *Staining of epithelial cells for ET_A in normal and malignant colon*

Score (0-3)	Normal colon distant from tumour	Normal colon adjacent to tumour	Tumour
Number of samples	10	35	45
% Stained	0	91.5	100
Score 0	0	3 (8.5%)	0
Score 1	0	7 (20%)	8 (18%)
Score 2	0	15 (43%)	23 (51%)
Score 3	0	10 (28.5%)	14 (31%)

Sections of normal colon taken from either adjacent to the tumour or at least 10cm from it and sections of tumour were stained using an indirect immunoperoxidase technique for ET_A. The staining of the epithelial cells was assessed as 0=no staining, 1 =1-33% cells stained, 2=34-66% epithelial cells stained, 3=67-100% epithelial cells stained. The sections were viewed by 3 observers independently.

Table 2.2 *Summary of ET_A staining*

Area of tissue	Normal colon distant (n=10)	Normal colon adjacent (n=35)	Tumour (n=45)
Epithelial cells	0	91%	100%
Blood vessels	0	0	31%
Stroma	20%	17%	45%

Percentage of patients stained for ET_A in each region of the colon

2.3.3 ET_B staining

2.3.3.1 Distant normal colon:

Staining for ET_B occurred in only 30% of patients in the epithelial cells and in only a third of these were the majority of cells stained. In three patients (30%) ET_B was observed in the blood vessels, both in the endothelial cells and in the vessel walls (Table 2.4). Staining in the stroma was observed in just one patient (10%).

2.2.1.1 Adjacent normal colon:

There was far more staining for ET_B in the adjacent normal colon than in the distant normal colon. ET_B occurred in all patients in their epithelial cells but not all cells were stained (Table 2.3, Figure 2.3). The receptor was present in the blood vessels of 36% patients and stromal staining occurred in 27% of patients.

2.2.1.2 Tumours:

ET_B was present in epithelial cells, in blood vessels and in the stroma in colorectal cancers (figure 2.4,Tables 2.3 and 2.4). All patients contained some staining for epithelial cells though only in 22% were more than two-thirds of the cells stained (Table 2.3). In only 16 patients (36%) was ET_B present in the blood vessels occurring in endothelial cells and in the vessel walls. Stromal staining for ET_B was present in 12 patients (27%) (Table 2.4). There was no correlation between the staining for ET_B and either the stage or the grade of the tumour.

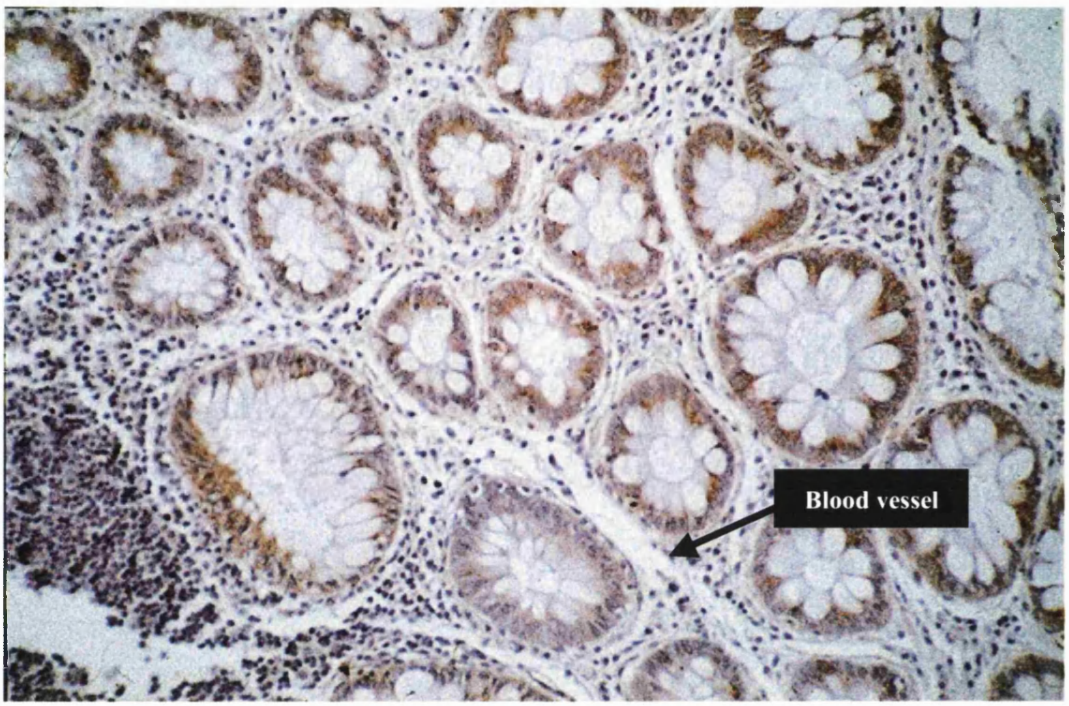


Figure 2.3 *Normal colon stained for ET_B using 3-step avidin-biotin technique. The brown staining represents ET_B staining in the epithelial cells and in blood vessels. Original magnification x100.*

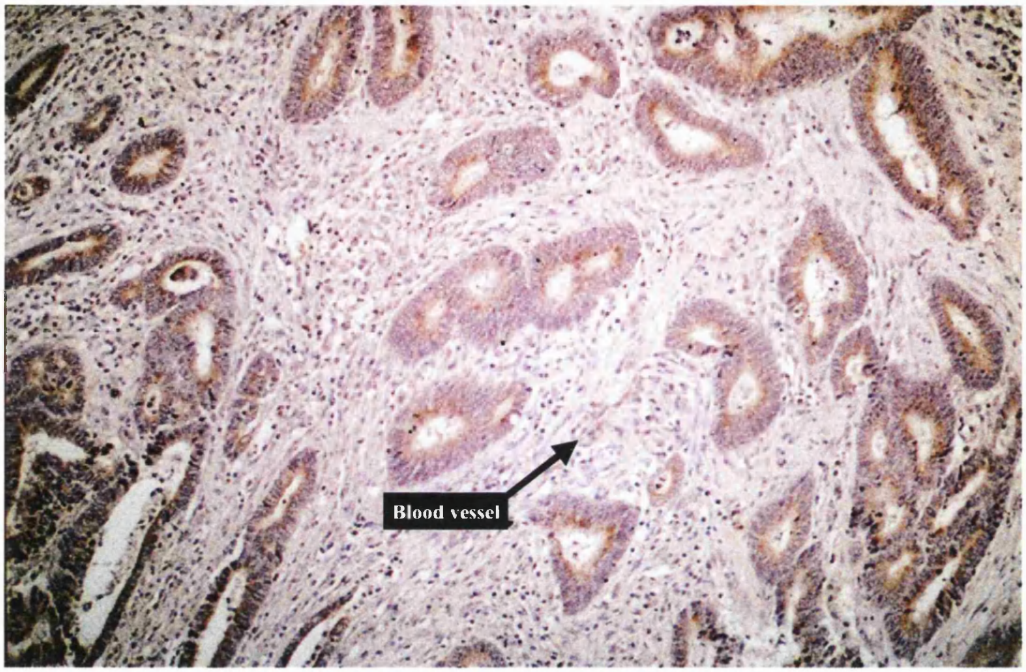


Figure 2.4 *Staining of a moderately differentiated tumour with antibody to ET_B and visualised with 3-step avidin-biotin and DAB technique. ET_B, seen as brown particles, is present in the majority of epithelial cells (score 3) and can also be observed in some blood vessels. Original magnification x40.*

Table 2.3 *Staining of epithelial cells in normal and malignant colon for ET_B*

Score (0-3)	Normal colon distant from tumour	Normal colon adjacent to tumour	Tumour
Number of samples	10	35	45
% Stained	30	100	100
Score 0	7 (70%)	0	0
Score 1	1 (10%)	6 (17%)	12 (27%)
Score 2	1 (10%)	24 (69%)	23 (51%)
Score 3	1 (10%)	5 (14%)	10 (22%)

Sections of normal colon taken from either adjacent to the tumour or at least 10cm from it and sections of tumour were stained using an indirect immunoperoxidase technique for ET_B. The staining of the epithelial cells was assessed as 0=no staining, 1 =1-33% cells stained, 2=34-66% epithelial cells stained, 3=67-100% epithelial cells stained. The sections were viewed by 3 observers independently.

Table 2.4 *Summary of ET_B staining*

Area of tissue	Normal colon distant (n=10)	Normal colon adjacent (n=35)	Tumour (n=45)
Epithelial cells	30%	100%	100%
Blood vessels	30%	36%	36%
Stroma	10%	27%	27%

Percentage of patients stained for ET_B in each region of the colon

2.4 Summary

The aim of this section was to investigate the distribution of ET_A and ET_B receptors in normal colon and in colorectal cancers by immunohistochemistry.

To determine the optimum conditions for staining a pilot study was carried out. From this study the avidin-biotin 3-layer technique produced optimum staining, that is a low background with clear staining for the receptors, when the tissue was fixed in formalin, incubated overnight with the primary antibody at 4°C and visualised with DAB/H_2O_2.

The staining was assessed to determine the distribution of the receptors. As striking differences were observed in the percentage of epithelial cells stained both between tumour and normal tissue and within a tissue group a subjective estimation of the number of cells stained was made. All sections were viewed by three people and there were no noteworthy differences in their evaluation of the staining.

There was a large increase in the expression of ET_A in tumours compared to distant normal tissue, as staining for ET_A in this normal tissue was restricted to the stroma of only 20% of patients. In contrast, in tumour samples, epithelial cells stained positively for ET_A and staining was also present in blood vessels and the stroma, though not in every case. Interestingly, microscopically normal tissue from adjacent to the tumour demonstrated a pattern intermediate to distant normal colon and tumour; it showed the same pattern of staining as for normal tissue in the stroma and blood vessels, but -

similarly to the tumour - the epithelial cells expressed ET_A, suggesting that factors released by the tumour affect the adjacent epithelial cells.

ET_B staining was also increased in colorectal cancer but only in the epithelial cells, which increased three-fold compared to distant normal tissue. Like ET_A, the levels of ET_B were similar in the adjacent normal tissue to the tumour. There was no difference in the ET_B staining of blood vessels or stroma between any of the three groups of tissue.

In summary, ET_A and ET_B profiles are markedly different in tumour tissues compared to normal colon. It remains to be seen if this is part of a mechanism that contributes to tumour growth.

3 Autoradiographic Quantitative Analysis of Endothelin Receptors and Double Localization Techniques

3.1 Introduction

To assess the presence of ET_A and ET_B in sections of normal colon and samples of colorectal cancer autoradiography was carried out. This technique enables the binding affinities of the two receptors to be assessed and by combining high resolution autoradiography with immunohistochemistry the cell types expressing the receptors can be identified.

3.2 Materials and Methods

3.2.1 Tissue collection and preparation

Cancer and normal colon specimens (at least 10cm away from the tumour) were obtained at routine resections from 9 patients with colorectal cancer (appendix 6.1, Table 6.3). The tissues were divided into approximately $1cm^3$ samples and stored frozen in liquid nitrogen. The samples were taken out and serial cryostat sections 10μm thick were cut and mounted on gelatin coated slides. The slides were stored at $-70^{o}C$ for up to 7 days.

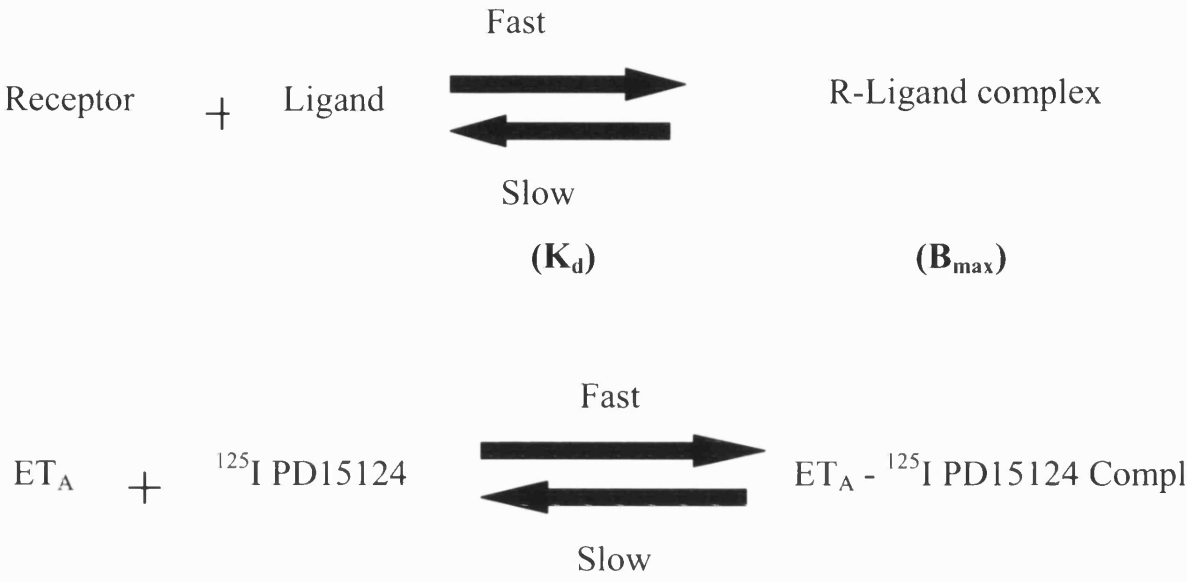
All tumours were diagnosed histopathologically as moderately differentiated adenocarcinomas. When frozen sections were cut, 6 of the cancer specimens also incorporated adjacent normal colon, easily distinguishable morphologically on staining with haematoxylin and eosin (H&E).

3.2.2 Endothelin A and B receptor (ET_A and ET_B) binding

The sections were equilibrated to room temperature for 20 minutes and then incubated in 50mM Tris buffer, pH7.4, for 15 minutes at room temperature to allow endogenous levels of ET-1 bound to the receptors to decrease by dissociation.

Sections were incubated for 2 hours at room temperature in Tris buffer containing 5mM $MgCl_2$, 1% bovine serum albumin and 100,000IU/ml aprotinin in the presence of 150pM ET_A selective ligand [^{125}I]-PD151242 (an antagonist) or 150pM ET_B selective ligand [^{125}I]-BQ3020 (an agonist) (each 2000Ci/mmol specific activity, from Nycomed Amersham, Little Chalfont, Bucks, UK). The sections were washed twice in Tris buffer (as above) at 4°C for 10 minutes each time and dried in a stream of cold air. Sections were post-fixed in paraformaldehyde vapour at 80°C for 2 hours. Non-specific binding was determined by incubating adjacent sections in the presence of 1μM unlabelled ET-1.

For characterisation of receptor subtype binding, competitive inhibition analysis was carried out by incubating 100nM unlabelled ET-1 with increasing concentrations from 25pM to 1nM of ET_A selective ligand [^{125}I]-PD151242 or ET_B selective ligand [^{125}I]-BQ3020 to obtain the apparent dissociation constant (K_d), that is the value at which the forward and backward reactions are at equilibrium, and the maximum binding capacity (B_{max}), that is the concentration of the ligand required to saturate 100% of the receptors according to the following equation:



Equation 3.1 *The general reversible equations for ligands used in autoradiography.*

Analysis was carried out using Inplot curve fitting software (Graphpad, San Diego, CA, USA).

This procedure enabled the comparative analysis of amounts of ET_A and ET_B receptors in colorectal cancer, adjacent normal colon to the cancer and distant normal colon.

3.2.3 Image generation

Two types of autoradiographic image analysis were employed. Firstly, macroscopic autoradiography, which investigated gross regional variations of radioactive ligand uptake within a section, and secondly, high resolution (microscopic) autoradiography, which examined uptake of the radioactive ligands at the cellular level. The latter technique was carried out with ET_A and ET_B individually and then, to identify the cells

binding ET-1, a combination of autoradiography using ET-1 with immunohistochemistry for cell markers was employed.

3.2.3.1 Macroscopic autoradiography

Autoradiographs were generated by opposing sections to Hyperfilm-^{3}H (Nycomed Amersham) for 2 to 4 days at 4°C. Films were developed in undiluted D19 (Kodak, Hemel Hempstead, Herts. UK) for 5 minutes at room temperature and fixed with IF23 fixer (Ilford, Mobberley, Cheshire, UK) diluted with 1:4 in tap water for 5 minutes at room temperature and rinsed in running tap water. The films were then dried.

3.2.3.2 Microscopic autoradiography

To obtain a higher degree of anatomical resolution of the binding sites, sections were also prepared by the 'dipping technique'. After post-fixation, sections were removed from the paraformaldehyde vapours and allowed to cool for several hours until residual vapours had dispersed. The sections were then defatted in ascending concentrations of alcohol (70% to 100%) followed by xylene for 1 hour. The sections were rinsed in distilled water, warmed to 40°C, dipped in liquid emulsion K-5 (Ilford) at 42°C, exposed for 7 to 10 days at 4°C and developed and fixed as for macroscopic autoradiography. To visualise the cell nuclei slides were counterstained with haematoxylin.

The high temperature in some sections caused some loss of the radioactive ligand. Therefore high resolution autoradiography was also carried out using cut coverslips dipped in liquid emulsion; left to dry then applied against prepared sections. The coverslips were secured using DePX as mountant. The slides were developed as above.

3.2.4 Identification of cell types expressing ET receptors

To identify the cell types that express endothelin receptors, high resolution autoradiographs using ET-1 were combined with immunohistochemical techniques using antibodies to specific cell types.

Frozen sections of distant normal colon, adjacent normal colon and colorectal tumours were allowed to equilibrate at room temperature for 45 minutes and then fixed with acetone for 15 minutes at 4°C. The slides were then washed three times in Tris buffered saline (TBS 50mM Tris in saline pH7.4), each wash was for 5 minutes at room temperature. This same washing procedure was employed for all subsequent washes. The sections were treated with 0.5% H_2O_2 in methanol for 10 minutes to block endogenous peroxidase activity, washed and non-specific antibody binding was blocked by incubating the sections with 20% sheep serum in TBS for 20 minutes at room temperature. Excess serum was tapped off the slides and the sections incubated for one hour at room temperature with primary antibody diluted in TBS containing 1% normal rabbit serum. The antibodies used were: NF200 a marker of nerves at 1/200 (donated by Dr. M. Dashwood, Department of Biochemistry and Molecular Pathology, Royal Free & University College London School of Medicine, London, UK); PECAM, an endothelial cell marker at 1/50 (donated by Dr. M. Dashwood) and ASO, a fibroblast marker, at 1/100 (Dianova, Hamburg, Germany), control slides were included in which the primary antibody was replaced with TBS. After washing, sections were incubated with biotinylated sheep anti-mouse diluted 1/500 in TBS containing 2% normal sheep serum for 30 minutes at room temperature. The avidin-biotin complex was formed by incubating avidin:biotin:buffer (1:1:100) for 30 minutes before applying to the sections for 30 minutes. After washing, the sections were

incubated with diaminobenzidine (DAB 250μg/ml) in Tris pH7.4 containing 0.1% H_2O_2 for 10 minutes at room temperature. The slides were washed under running tap water for 5 minutes and then microautoradiography with ET-1 was carried out.

3.2.5 Image analysis

Images, obtained from macroautoradiography, were digitised using a Hewlett Packard scanner and were analysed by reading grey densities using the histograms in Adobe Photoshop 5 (Epson, Hemel Hempstead, Hertfordshire, UK). To check that this software package did not lead to distortion of the results, the images were also analysed using Corel Draw 7 (Kodak, Hemel Hempstead, Herts, UK) and the same readings were obtained.

The histogram system works by dividing each cm^2 into 255 points. Each point is given a comparative figure from 0 to 250 scale (where black is zero and white is 250) and the mean and standard deviation calculated.

3.3 Results

3.3.1 Characterisation of ET_A and ET_B receptors

To enable a comparison to be made between ET_A and ET_B receptors by autoradiography the ligands for the two receptors have to bind comparably that is to bind at just saturation levels so no binding sites are left undetected. To achieve this dissociation experiments were carried out using increasing concentrations of ligands to calculate the K_d maximum (association constant) for the receptors. For ET_A the K_{dmax} was 0.125nM and for ET_B it was 0.068nM (Figure 3.1).

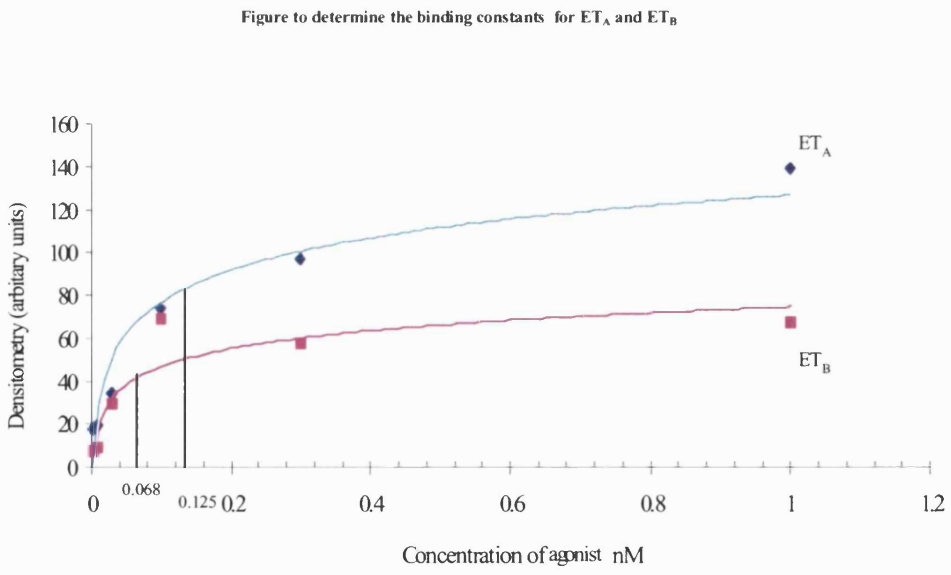


Figure 3.1 *The association curves for ET_A and ET_B to determine the minimum concentration of the agonists required for comparable binding, K_d=0.125nM for ET_A and K_d=0.068nM for ET_B.*

3.3.2 ET_A receptors

There was an approximately 60% increase ($p<0.0001$) in ET_A receptor antagonist in colorectal tumour compared to adjacent and distant normal colon (Table 3.1, Figures 3.2 and 3.3). The uptake in normal colon was the same for tissue from adjacent and distant normal colon from the tumour.

Table 3.1 *Up-take of ligands to ET_A and ET_B*

	ET_A uptake mean ± s.d. (arbitrary units)	**ET_B uptake mean ± s.d. (arbitrary units)**
Distant normal colon	129.19 ± 15.66	207.01 ± 35.46
Adjacent normal colon	128.68 ± 15.15	206.58 ± 35.35
Tumour	205.88 ± 27.02	122.30 ± 24.5

Summary table of receptor densitometry reflecting uptake of ligands for ET_A and ET_B in tumour, adjacent and distant normal colon. There were nine patients in each group and three sections were assessed for each tissue from each sample.

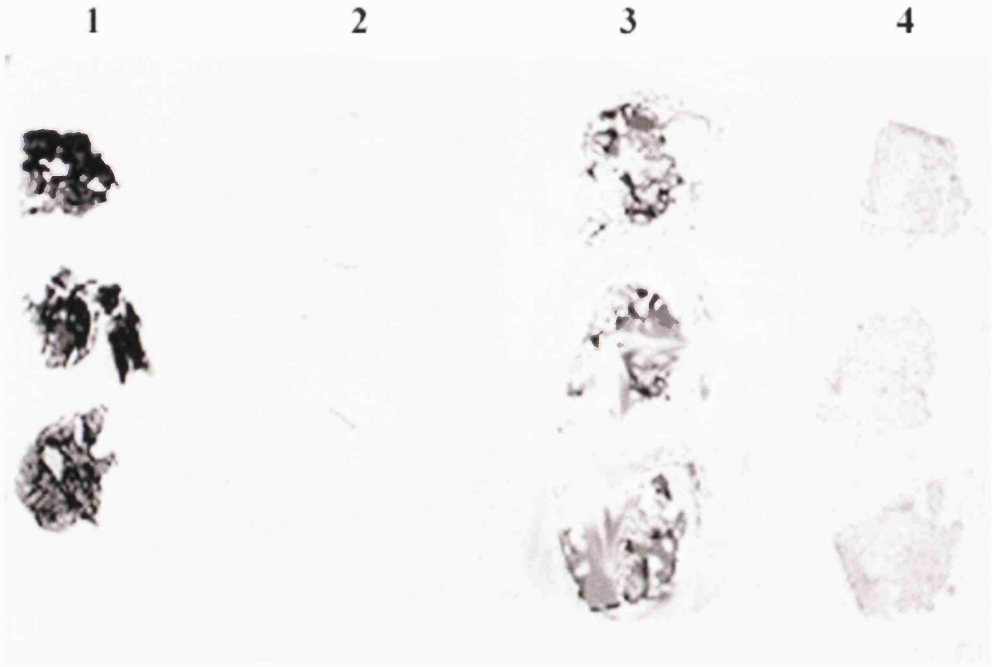


Figure 3.2 *Gross autoradiography demonstrating the specificity of the up-take of ligands in a section across the junction between normal colon and a moderately differentiated tumour. Non-specific up-take of the ligands is minimal.*

Lane1: ET_A , *Lane 2: Non-specific control for ET_A ,*

Lane 3: ET_B , *Lane 4: Non-specific control for ET_B ,*

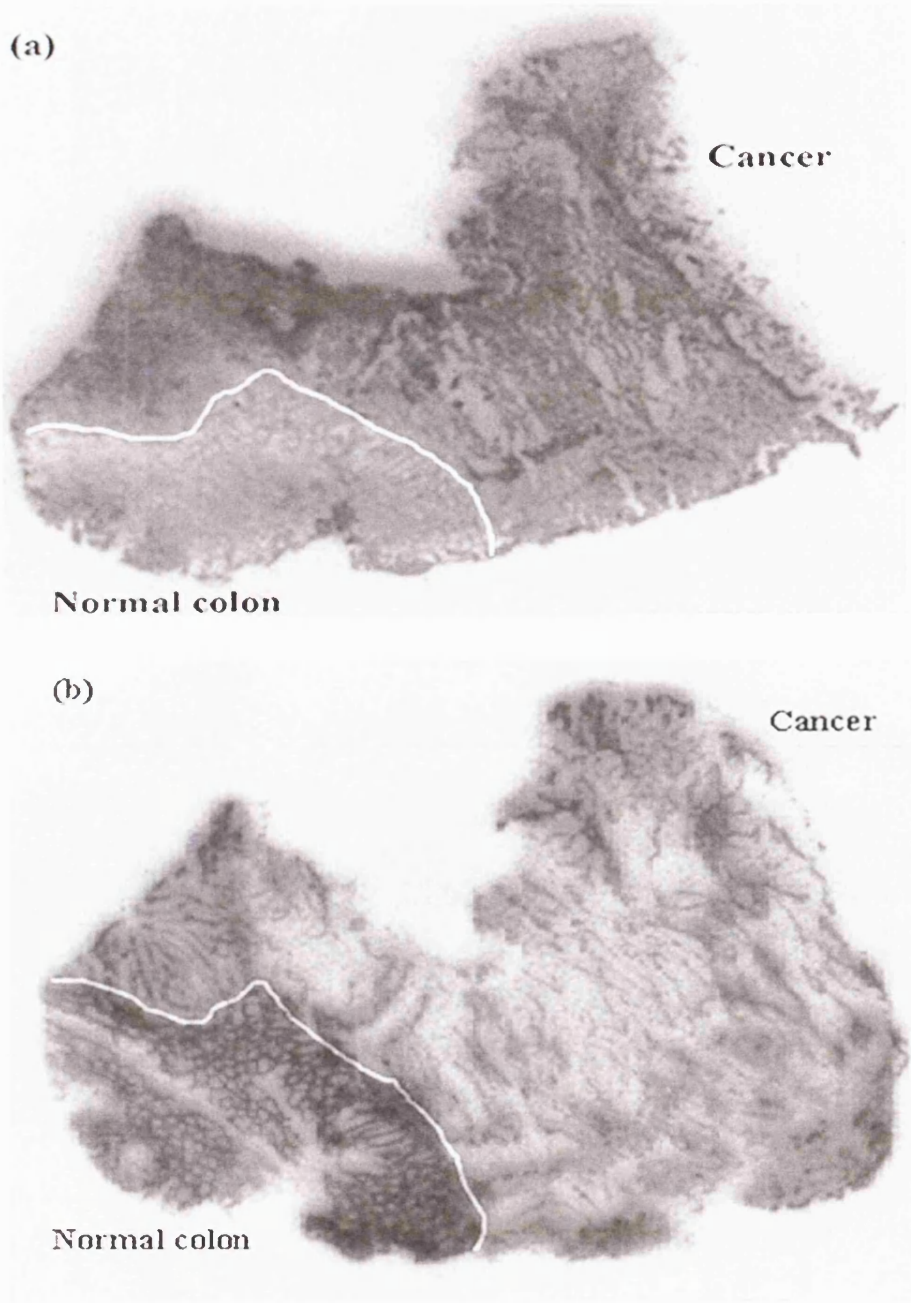


Figure 3.3 *Autoradiograph of section from the junction between normal colon and moderately Differentiated adenocarcinoma in which receptor binding for* ET_A *(a) and* ET_B *(b) has been demonstrated (seen as black granules of silver nitrate).* ET_A *is present in greater levels on the cancer than the normal colon, whereas* ET_B *occurs at a higher level on normal colon than cancer.*

3.3.3 ET_B receptors

For ET_B, there was a much greater uptake of receptor ligands by normal colon both adjacent to and distant from the tumour than from the tumour itself (Table 3.1, Figures 3.2. and 3.3). This 70% difference was significant $p<0.0001$(paired t-test). There was no difference in the ET_B uptake between adjacent and distant normal colon.

3.3.4 Comparison between ET_A and ET_B receptor uptake

There was a significantly higher 68% ($p=0.0001$) uptake of ligands by ET_A receptors as compared to ET_B in tumours. Whereas for normal colon there was a significantly higher uptake of ET_B, 60% for adjacent normal colon and 62% for distant normal colon compared to ET_A ($p=0.00015$, $p=0.00013$ respectively).

3.3.5 Microautoradiography

Using antagonists and agonists to ET_A and ET_B showed that ET_A appeared to be predominately in the epithelial cells of the tumour with some up-take by the stroma (figure 3.4 a). For ET_B the microautoradiography suggested a predominant uptake by the epithelial cells of the normal colon (figure 3.4 b).

To confirm the stromal distribution of the cell types that can bind ET-1, autoradiography using labelled ET-1 was carried out simultaneously with immunohistochemistry using antibodies to specific cell types. This demonstrated that ET-1 was taken up by blood vessels in both normal (figure 3.6) and tumour (figure 3.7). Also there was good uptake of ET-1 by nerves (figure 3.8). Unfortunately the

fibroblast antibody did not give conclusive staining and it was therefore not possible to determine if these cells could bind ET-1.

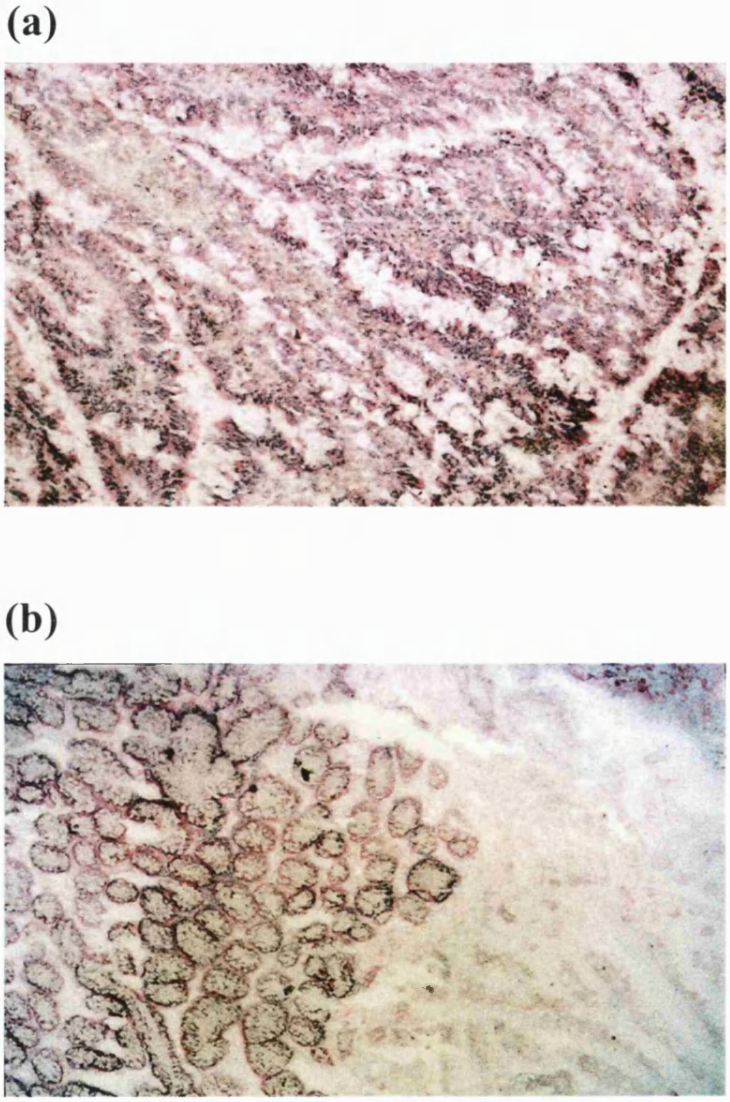
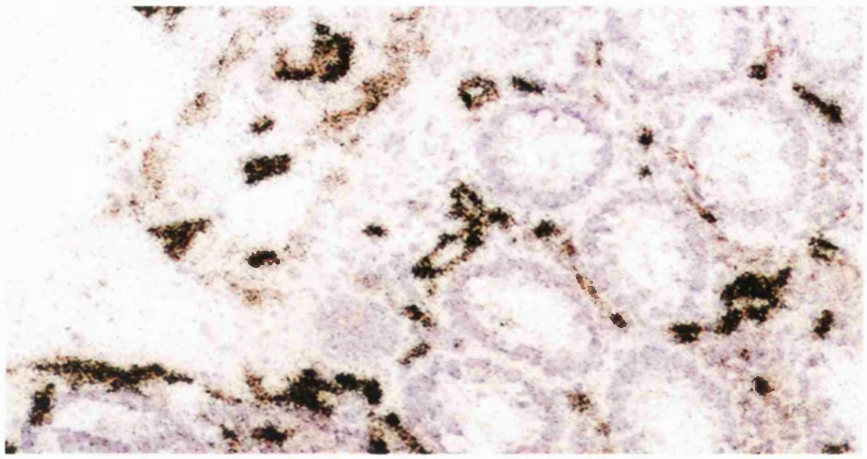


Figure 3.4 *Microautoradiograph of figure 3.3 reflecting increased ET_A receptor uptake in moderately differentiated adenocarcinoma (a) and increased ET_B receptor uptake in normal colon (b). The receptors are reflected by the black silver nitrate grains and are in the epithelium with a significant amount in the stroma. Original magnification X 8.*

(a)



(b)

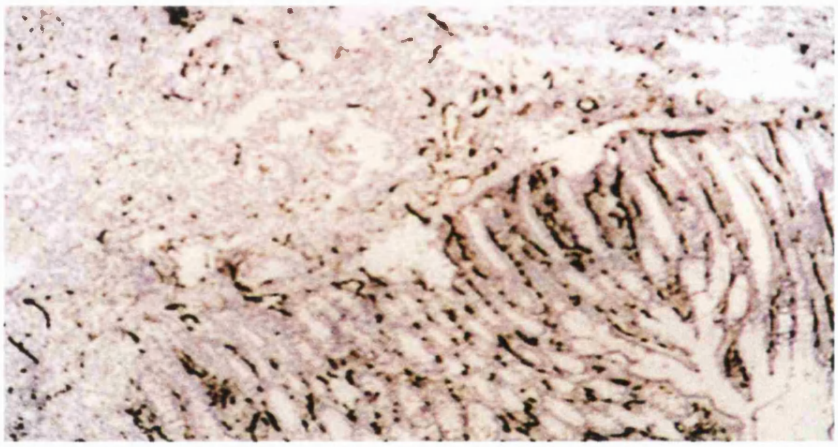


Figure 3.5 Section of normal colon (a) and from the junction of normal colon and a moderately differentiated adenocarcinoma (b) that have been stained with an antibody to endothelial cells using an indirect immunoperoxidase technique (stained brown) followed by microautoradiography for ET-1 (black silver nitrate granules), see methodical details in section 3.2. There are some areas of co-localisation of brown and black granules demonstrating that endothelial cells are able to bind ET-1.

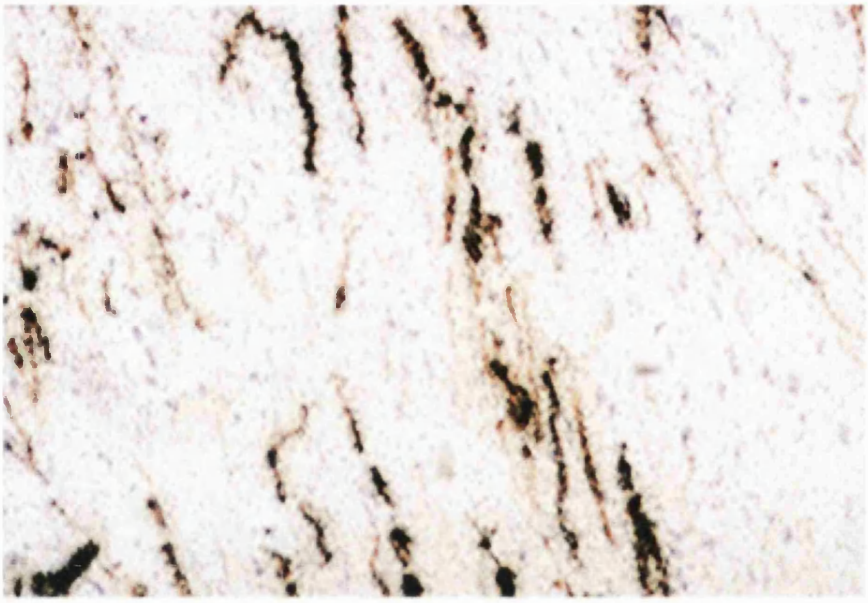


Figure 3.6 *Section of a moderately differentiated adenocarcinoma stained with an antibody to nerves (see section 3.2.4 for details) followed by microautoradiography for ET-1. The presence of nerves is indicated by the brown staining and ET-1 is seen as the black silver nitrate grain. There are areas in which the brown staining co-localises with the black grains demonstrating that the nerves in the colon are able to bind ET-1.*

3.4 Summary

The previous chapter has shown that ET_A and ET_B receptors are expressed in colorectal cancer. These results were based on observational analysis of immunohistochemistry. The next step was to try and use a method that was more sensitive, allowed mapping of receptors grossly and at the cellular level in a dynamic way and could be analysed objectively. We used autoradiography, a method for studying receptors first established by Young and Kuhar in 1979 and modified in 1980 by using reversibly bound ligands. Autoradiography is easily amenable to semi-quantitation. Also, it provides a dynamic way of looking at the receptors in an environment that is closer to the *in vivo* situation. Furthermore, microautoradiography allows identification of the type of cells that express these receptors.

This method allowed a quantitative dynamic measurement of these receptors using pharmacokinetics, i.e., (1) the ligands used were high affinity reversibly bound ligands. (2) The speed of the forward reaction was fast enough to be practical (2 hours), (3) while the process of dissociation, or backward reaction was slow enough to be measured (10-14 days). (4) The Bmax value (concentration of radioligand required to saturate 100% of the receptors) was reasonably low, therefore could be calculated from ligand concentrations within safe radioactive limits (5) The dissociation constant (K_d) value (i.e., the value at which the forward and backward reactions are at equilibrium) was small enough for the ligand concentrations used to be able to exceed it and reach close to Bmax.

The radioisotope used was I^{125} that has a half-life of 56 days. The ligands also were very specific for the receptors; I^{125}PD151242 is ET_A selective, and I^{125}BQ3020 is ET_B selective. Both ligands have been used extensively, a Medline search since 1994 found 29 publications using the ET_A ligand and 53 for ET_B.

At the beginning of the experiments the K_d values and Bmax approximations were extrapolated for ET_A and ET_B ligands. The K_d values measured 0.068 and 0.125 nM respectively. For Bmax, the concentration used was 0.25nM, which is close to the Bmax value for both receptors. This ensured maximum binding capacity at a similar level for both ET_A and ET_B receptors, therefore allowing comparison between the receptor uptake to take place.

This study showed, by gross autoradiography, a higher ET_A expression by cancers compared to normal colon, similar to that seen by immunohistochemistry. Densitometric semi-quantitation suggested slightly more than a 2-fold increase in ET_A uptake in tumour compared to normal. However, ET_B receptors were reduced in colonic cancer as compared to normal colon, a finding which is directly opposite to the results obtained by immunohistochemistry. ET_B uptake by tumours was about 70% that of normal colon, as calculated by densitometry. Densitometry values for normal distant colon and normal adjacent colon were very similar, suggesting a cut off effect at the tumour/normal boundary. When compared directly, there was a significantly higher uptake of ligands by ET_A receptors than ET_B receptors in tumour.

The discrepancy between autoradiographic and immunohistochemical results for ET_B is discussed in the overall discussion chapter.

Microautoradiography showed that ET_A was present predominately in the epithelial cells of the tumour with some up-take by the stroma. For ET_B there was a predominant uptake by the epithelial cells of the normal colon.

To confirm the stromal distribution of the cell types that can bind ET-1, autoradiography using ET-1 was carried out simultaneously with immuno-histochemistry for specific cell types. ET-1 was taken up by blood vessels in both normal and tumour. Also there was good uptake of ET-1 by nerves. Fibroblast staining was inconclusive.

Having demonstrated differential receptor profiles between tumour and normal, the question which needs to be addressed is the biological significance of ET-1 in colorectal cancer. The scenario investigated in the next chapter assumes that ET-1 may act as a growth factor for epithelial colorectal cancer cells, using an autocrine loop.

4 Cell Culture

4.1 Introduction

To investigate the effect of ET-1 on colorectal cancer epithelial cells, colorectal cancer cell lines were used. Initially the ability of the cell lines to secrete ET-1 was studied and from this investigation two cell lines, one that secreted high levels of ET-1 and one that produced moderate levels were selected to examine the addition of exogenous ET-1 to the cells and if ET_A and/or ET_B receptor antagonists had an effect on the ET-1 induced growth of the cells.

4.2 Materials and methods

4.2.1 Cell lines

The following cell lines were tested for their ability to produce ET-1: HT29, SW620, SW480, Lovo (all from European Collection of Cell Culture, Porton Down, Salisbury, Wiltshire, U.K.), LIM1215, SKCO1, SKCO17, (all kindly donated by Prof. M. O'Hare, Ludwig Institute of Cancer Research, London, U.K.). Two cell lines were chosen for further experiments in this thesis: HT29, a high ET-1 producer, and LIM1215, a moderate ET-1 producer.

4.2.2 Endothelin receptor agonists and antagonists

Development of the method for the *in vitro* system was carried out using the ET_A receptor antagonist A122271 and the mixed antagonist PD145 (Alexis, Bingham, Nottinghamshire, U.K.). For further cell culture studies the ET_A receptor antagonists

BQ123 and BQ610 and the ET_B receptor antagonist BQ788 were used (all from Alexis). ET_A / ET_B receptor agonists became commercially available only after the development part of this work had finished. ET-1 (Sigma, Poole, Dorset, U.K.) was used as the mixed agonist for ET_A and ET_B receptors.

4.2.3 Cell culture

Cells were routinely grown in flasks (Falcon plasticware, Becton-Dickinson, Marathon, London, U.K.) in Dulbecco's Modified Eagles Medium (DMEM) with phenol red pH indicator and F-12 medium (1:1) to foetal calf serum (10% FCS), L-glutamine (2mM), penicillin (100IU/ml), streptomycin (100mg/ml) (all from Imperial Labs, Basingstoke, Hants, U.K.) and were maintained at 37°C at 5%CO_2 in 95% air. The cells were passaged at a split ratio of between 1 in 3 and 1 in 5 at approximately 90% confluence every 3 to 7 days dependent on the cell type. Passaging was carried out by washing thoroughly in phosphate buffered saline (PBS, Oxoid, Basingstoke, Hants, UK), then incubating at 37°C in 1mg/ml trypsin (Sigma, Poole, Dorset, UK) in 0.02% EDTA in PBS (Imperial Laboratories, Basingstoke, Hants, UK) until the cells detached.

4.2.4 ET-1 production by colorectal cancer cells

To measure ET-1 production by each colorectal cancer cell line and to obtain relative amounts, media was collected from the growing cultures 24 or 48 hours after adding fresh media. Both media with and without FCS were used and all other additives were present. The media collected was immediately spun at 400g for 5 minutes and stored at –70°C until assay. To be able to compare ET-1 levels between the different cell types the number of cells in each flask was counted by trypsinizing, spinning and

resuspending the cell pellet in a known volume of media and counting on a haematocytometer and therefore ET-1 production could be expressed as per cell number. As a negative control, media containing appropriate additives but without cells was used and HT29 acted as a positive control since it is known to be a high producer of ET-1 (Kusuhara et al 1990). ET-1 levels in the media were measured using an ELISA kit (Nycomed Amersham, Little Chalfont, Bucks, UK) and read at 550nm on a plate reader (MRX Denley, Billinghurst, West Sussex, UK). The assay has a sensitivity of 1 to 32 fmol per well for a 96 well plate and specificity of 98% for ET-1, cross reactivity with ET-2 < 2% and ET-3 <0.01% (details provided by the manufacturer). In view of the fact that serum contains ET-1, albeit in small amounts, it was decided to carry out further studies using serum free media.

From the above, two cell lines were chosen; HT29, which is a high producer of ET-1 and LIM1215, a moderate producer, both grow readily and are easy to maintain. All subsequent cell culture experiments were carried out with these two cell lines only.

4.2.5 Endothelin receptor expression in colorectal cancer cells using autoradiographic techniques

Cells (HT29 and LIM1215) were grown as above then trypsinised and cytospins were prepared, by adding 200µl of a cell suspension containing 1 million cells/ml to cytospin slides and spinning at 300g for 4 minutes. The slides were incubated with 150 pM of ^{125}I ET-1 (specific activity 2000 Ci/mM, Nycomed Amersham) and non-specific binding was determined by incubation of alternate sections with 500nM unlabelled ET-1. Endothelin receptor subtypes were identified using 150 pM ^{125}I-PD15142 for ET_A or ^{125}I-BQ3020 for ET_B, specific activity 2000 Ci/mM for both

(Nycomed Amersham). The technique was described in detail in chapter 3 section 3.2.2 and 3.2.3.

4.2.6 Influence of ET-1 and endothelin receptor antagonists on growth

A pilot study was carried out to determine the range in which the methylene blue assay gave linear readings for cell growth over a 4 day period. 20,000 cells per well were chosen as an appropriate seeding density for both cell lines.

Twenty thousand cells per well were plated into 24-well plates and grown for 24 hours in fully supplemented medium. The cells were washed and incubated in serum free medium containing 10^{-7} to 10^{-12}M ET-1/well for 24 hours, 48 hours or 72 hours. At each time the cells were fixed in 10% formalin and the cell number measured using the methylene blue assay (see 4.2.7). The optimum concentrations of ET-1 from this experiment were used to investigate receptor antagonism.

This experiment demonstrated that the optimum concentration of ET-1 was 10^{-8}M for LIM1215 and 10^{-9}M for HT29 but only after 48 and 72 hours incubation. Therefore to investigate the receptor antagonists 100nM of the ET_A receptor antagonists BQ123 and BQ610 and ET_B receptor antagonist BQ788 were added to each well with or without ET-1 at 10^{-8}M for LIM1215 or 10^{-9}M for HT29 and after 48 and 72 hours the plates were fixed with 10% formalin and the cell number measured by the methylene blue assay.

All experiments were repeated at least eight times.

4.2.7 Methylene blue assay

4.2.7.1 Fixation of cells

To remove the media prior to fixing the cells comparisons between tipping (inverting) and vacuum aspirations of the media from individual wells were made for HT29 and LIM1215. There was no difference detected between the two techniques and the standard deviations were the same.

At the endpoint (48, 72 hours), the culture medium in each well was removed by inverting the plate gently. The wells were then filled with PBS and reinverted. The cells were fixed by adding 0.5 ml of 10% formalin to each well for 30 minutes. Time of fixing and type of fixative have been previously investigated (Oliver et al 1989), with the conclusion that formalin gives the most consistent results, and a fixation time between 15 and 45 minutes produced constant results. At this stage the plates were wrapped in aluminium foil and stored to be assayed as a batch.

4.2.7.2 Cell staining and readings of plates

The fixative was removed and 0.5ml filtered 1% methylene blue in 0.01M borate buffer pH 8.5 added to each well for 30 minutes. Excess dye was discarded and plates were washed gently by dipping into 0.01M borate buffer (pH 8.5) baths, this washing step was repeated six times by which time all the non-bound dye was removed. Consistency in washing is absolutely crucial for reproducibility and therefore six washes were used every time (Oliver et al 1989).

The dye was eluted by adding 1ml of 1:1 95% ethanol: 0.1 M HCl to each well. The plates were then shaken using a plate shaker (Model 804, Luckham, Billingshurst,

West Sussex, U.K.) at level 6 for 30 minutes. To be able to measure the absorbance the eluted dye had to be transferred to a 96-well plate, 100µl per 96-well was added, with 4 wells on 96-well plate from each well of the 24-well plate. The absorbance was read at 650nm (A_{650}) on a microplate reader (MRX Denley, Billinghurst, West Sussex, UK). A background reading was obtained by leaving four wells empty; the mean of these wells was used as the blank by the photometer and subtracted from every other reading.

4.3 Results

4.3.1 Production of ET-1 by human colorectal cancer cells

ET-1 was measured in conditioned media from the 6 colorectal cell lines, LIM1215, SW620, LOVO, SKCO1, SKCO17 and HT29 (Table 4.1). There was a wide variation in the amount of ET-1 measured at 24 and 48 hours by the colorectal cancer cell lines, the highest producer being HT29 (71.7fmol/ml/10^6 cells at 48 hours) and the lowest SW620 (8.4fmol/ml/10^6 cells at 24 hours) and SKCO17 (10.1fmol/ml/10^6cells at 48 hours). HT29 and LIM1215 were selected for further study. HT29 secreted the largest amount of ET-1of the cell lines investigated and therefore might be responsive to ET-1 and more specifically, HT29 might be a good model for demonstrating the effect of inhibiting the action of ET-1 through the use of antagonists to endothelin receptors ET_A and ET_B. LIM1215 secreted a moderate amount of ET-1 and potentially might be able to respond to further addition of ET-1 as well as responding to antagonists to the receptors. For further experiments only HT29 and LIM1215 were used.

Table 4.1 *ET-1 production by colorectal cancer cell lines*

Cells	ET-1 fmol/ml/10^6cells	
	24 hours	48hours
Control medium	0.6	0.6
SKCO1	9.7	11.4
SKCO17	9.5	10.1
HT29	41.7	71.7
LIM1215	21.3	22.6
LoVo	9.8	20.4
SW620	8.4	11.3

ET-1 was measured in the media of colorectal cancer cell after incubation of the cells for 24 or 48 hours using an ELISA assay. The concentration of ET-1 has been corrected for the amount in the basic media.

4.3.2 Endothelin receptor expression in HT29 and LIM1215

Binding for ET-1, ET_A antagonist (PD15142) and ET_B agonist (BQ3020) in HT29 and LIM1215 was demonstrated by autoradiography. Total binding was clearly in excess of non-specific binding for ET-1 for the receptor subtypes and ET-1 in these cells, suggesting the presence of these receptors on the cells (Figure 4.1)

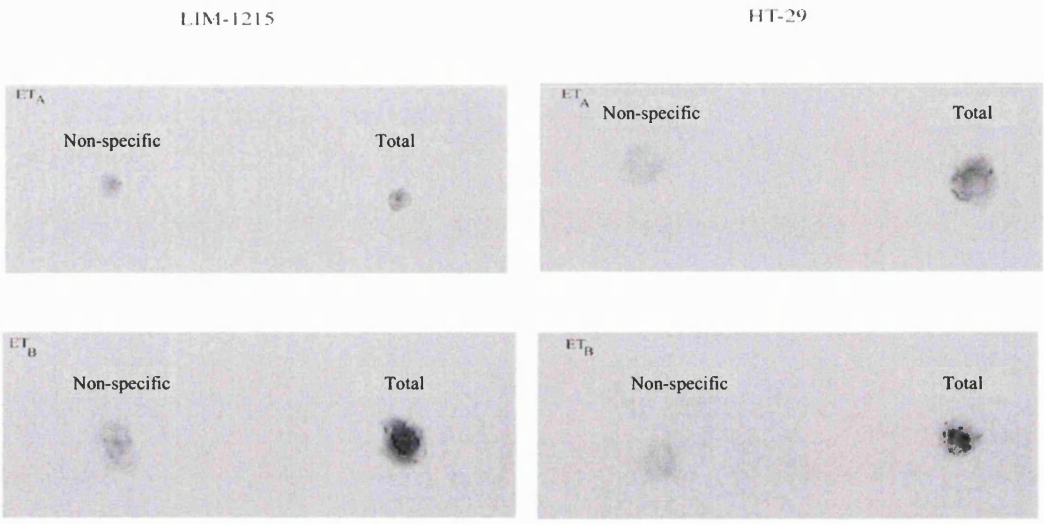


Figure 4.1 *Binding for ET_A and ET_B receptors on LIM1215 and HT29 cell cytospins were demonstrated by autoradiography. Slides were incubated with ET_A antagonist (^{125}I-PD15142) or ET_B agonist (^{125}I-BQ3020) for total binding. Non-specific binding was determined by incubation with excess unlabelled ligand. Total binding was clearly in excess of non-specific binding suggesting the presence of both receptors in these cell types.*

4.3.3 Effect of exogenous addition of ET-1 and response to ET_A and ET_B antagonists

4.3.3.1 Effect of ET-1 on HT-29 and LIM1215 growth

Addition of ET-1 at 10^{-7} to 10^{-12}M to LIM1215 and HT29 stimulated their growth in a dose-dependent manner (Table 4.2, Figures 4.2 and 4.3). For LIM1215 this was maximal at 10^{-8}M with a rise of 32.7% and 28.4% above controls at 48 and 72 hours respectively. In contrast the maximal number of cells for HT29 occurred with 10^{-9}M ET-1 when there was an increase of 13.4% and 15.7% above control numbers at 48 and 72 hours respectively.

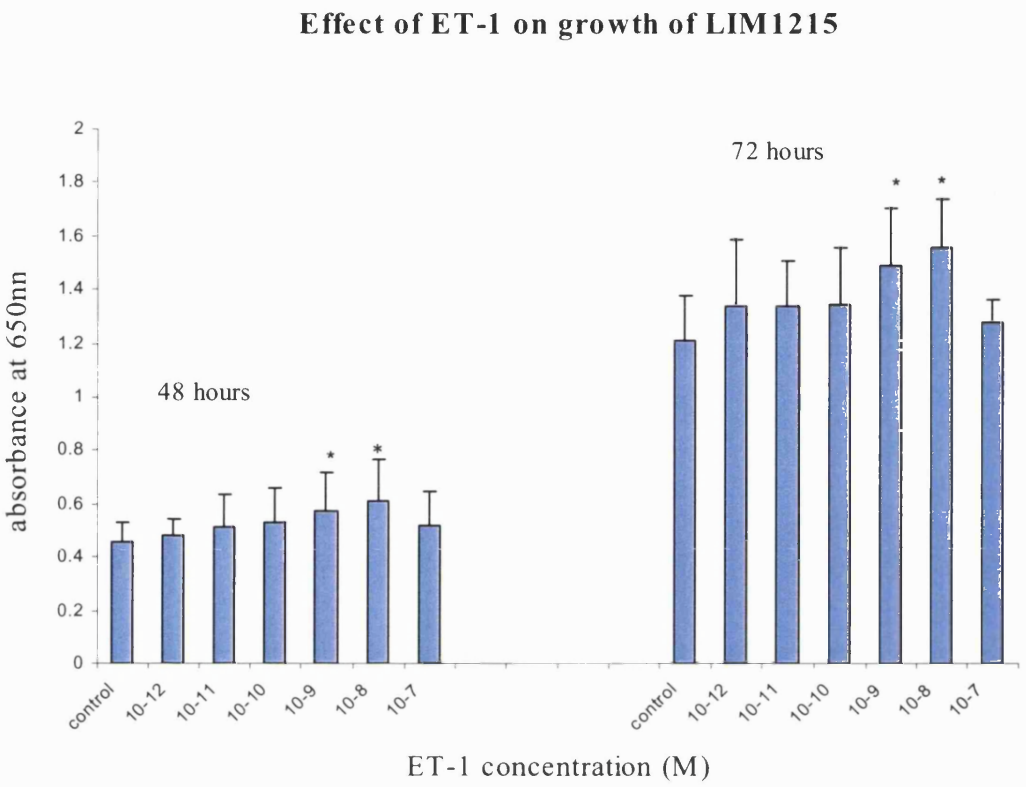


Figure 4.2 *LIM1215 cells were plated in serum-containing media and left to settle for 24h. The media was then removed and the cells fed with serum-free media containing 10^{-7} to 10^{-12}M ET-1. After 48h or 72h the cells were fixed with formalin and the number of cells determined by the methylene blue assay. A statistically significant increase in cell number occurred with ET-1 at 10^{-7} and 10^{-8}M ($p<0.05$ by Student's t-test).*

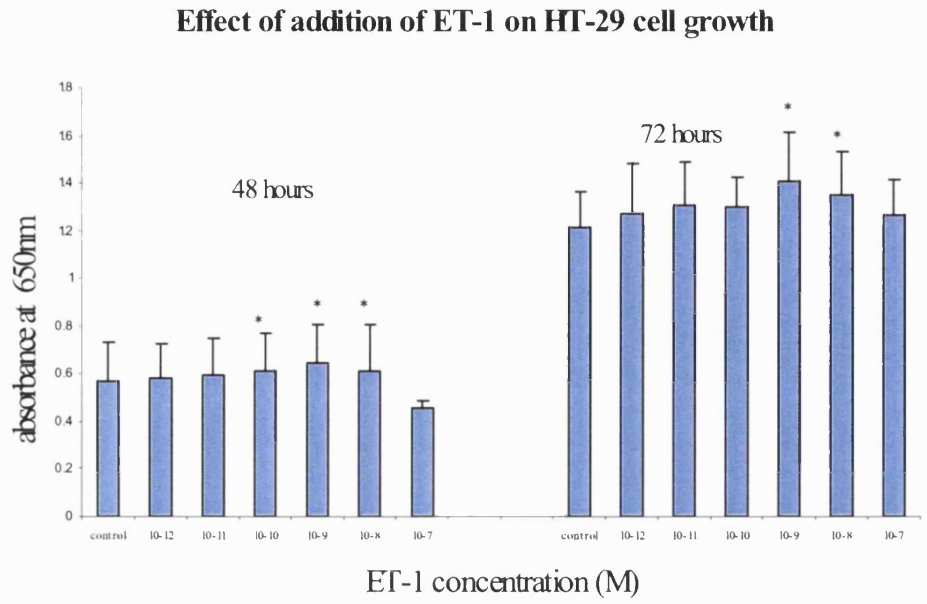


Figure 4.3 *HT29 cells were plated in serum-containing media for 24 hours and then the media was removed and the cells fed with serum-free media containing ET-1 at concentrations from 10^{-7} to 10^{-12} M. After 48h or 72h the cells were fixed with formalin and the number of cells determined by the methylene blue assay. A statistically significant increase in cell growth occurred with ET-1 concentrations 10^{-8} to 10^{-10} M (*$p<0.05$).*

Table 4.2 *Stimulation of growth of colorectal cancer cell lines by ET-1*

Cell Line	Controls	Endothelin-1 concentration (M)					
	0	10^{-12}	10^{-11}	10^{-10}	10^{-9}	10^{-8}	10^{-7}
LIM1215 48h	0.459 (0.069)	0.483 (0.061)	0.511 (0.123)	0.533 (0.129)	0.573 (0.145) P<0.05	0.609 (0.155) P<0.01	0.516 (0.132)
LIM1215 72h	1.211 (0.168)	1.342 (0.245)	1.338 (0.166)	1.344 (0.209)	1.486 (0.22) P<0.05	1.555 (0.177) P<0.01	1.276 (0.091)
HT29 48h	0.567 (0.168)	0.593 (0.141)	0.593 (0.155)	0.609 (0.159) P<0.01	0.643 (0.163) P<0.01	0.607 (0.199) P<0.05	0.456 (0.027)
HT29 72h	1.214 (0.153)	1.271 (0.212)	1.310 (0.176)	1.297 (0.127)	1.405 (0.204) P<0.05	1.351 (0.184) P<0.05	1.266 (0.146)

The effect of ET-1 addition for 48 and 72 hours on colorectal cancer cell growth was measured using the methylene blue assay and the results (absorbance at 650nm, arbitrary units) expressed as the mean (standard deviation). Statistical analysis was carried out using a paired student's t-test and significance was taken as $p<0.05$.

4.3.3.2 Effect of antagonists on cell growth

Competitive ET_A antagonists BQ123 and BQ610 significantly inhibited ET-1 induced proliferation of both LIM1215 and HT29 at 48 and 72 hours respectively (Figures 4.4 and 4.5). ET_B antagonists had no effect on ET-1 stimulated proliferation of these cell lines.

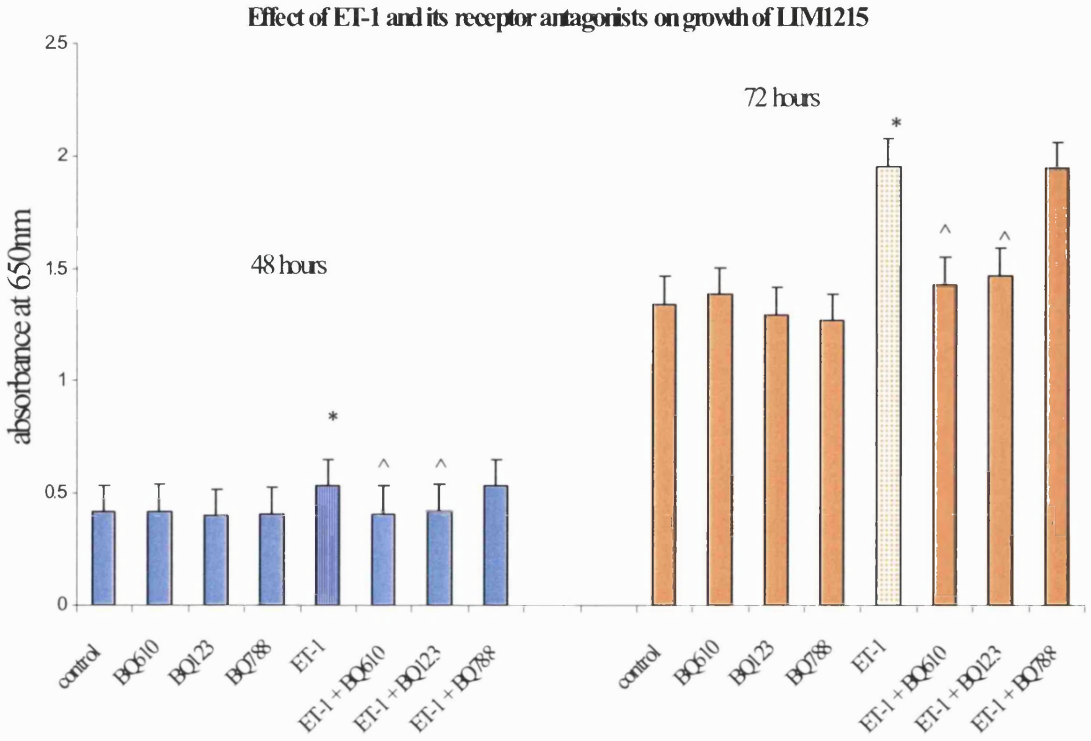


Figure 4.4 *Cells were allowed to settle overnight and then the media removed and serum-free media added containing ET-1 (10^{-8}M) with or without its receptor antagonists, either the ET_A receptor antagonists (BQ123 or BQ610) or ET_B receptor antagonists (BQ788) all at 100nM. After 48h or 72h the cells were fixed with formalin and the number of cells determined by the methylene blue assay. Cell numbers were significantly higher in the ET-1 group compared with controls (*$p<0.05$, Student's t-test) whereas cell numbers in the groups treated with ET-1+ET_A antagonists were similar to the control group and significantly lower than the ET-1 only group (^ $p<0.05$, Student's t-test).*

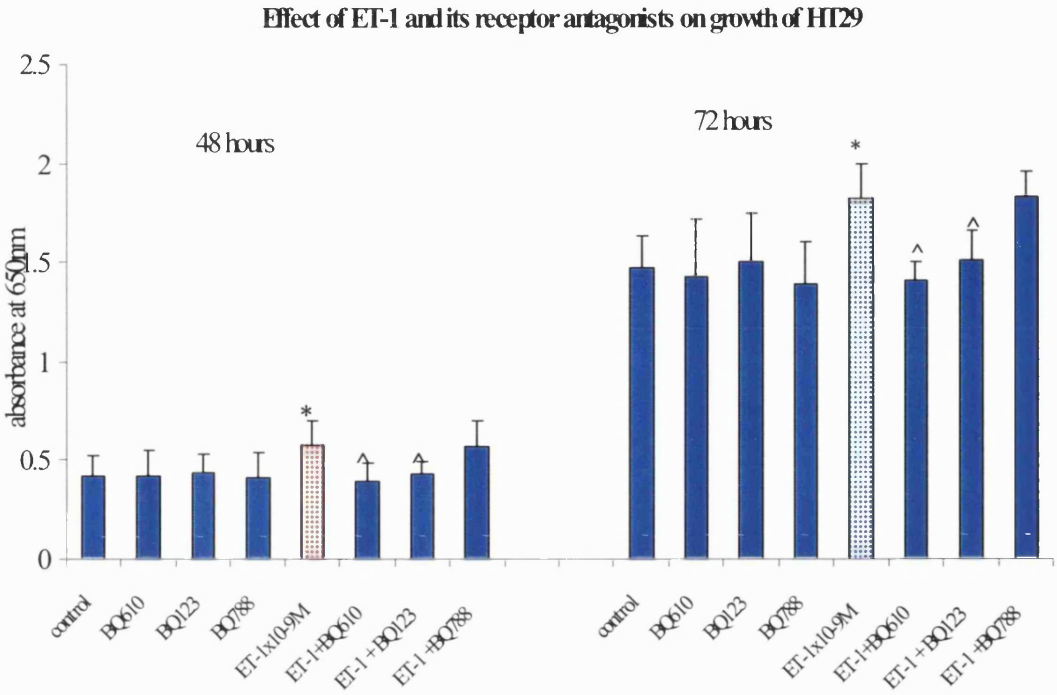


Figure 4.5 *Cells were allowed to settle overnight and then the media removed and serum-free media added containing ET-1 (10^{-9}M) and/or its receptor antagonists, either the ET_A antagonists, BQ123 or BQ610, or the ET_B antagonist, BQ788, all at 100nM. After 48h or 72h the cells were fixed in formalin and the number determined using the methylene blue assay. Cell numbers were significantly higher in the ET-1 group compared with controls (*p<0.05, Student's t-test) whereas cell numbers in the groups treated with ET-1+ET_A antagonists were similar to the control group and significantly lower than the ET-1 only group (^p<0.05, Student's t-test).*

4.4 Summary

Tissue culture work presented in this thesis investigated, firstly the production of ET-1 and expression of ET_A and ET_B receptors by two colorectal cancer cell lines, secondly the ability of ET-1 to stimulate the proliferation of these cell lines and finally the potential of ET receptor antagonism as an inhibitor of growth.

All colorectal cancer cell lines tested produced ET-1 and the cells ranged from "low" to "high" secretors. HT29 was selected for further study since this cell line secreted the highest level of ET-1; the amount measured was similar to that previously reported for HT29 of 23fmol/ml/10^6cells (Kushara et al 1990). Also LIM1215 was chosen; this was a moderate secretor and therefore addition of exogenous ET-1 might give further stimulation of growth. It could also be possible to inhibit any effects of endogenous ET-1.

ET-1 acts via two receptors, ET_A and ET_B, which were found to be present on LIM1215 and HT29 by autoradiography. This technique, by utilising radio labelled ligands that display high affinity for receptors, demonstrated the presence of the receptors and indicated that they occur in a functional state.

Exogenous ET-1 was found to significantly increase cell number for both cell lines with a higher rise for LIM1215 than HT29. The difference may be related to the greater endogenous production of ET-1 by HT29: If the mitogenic effect occurs via one or both of the receptors and these receptors are partially occupied by endogenous ET-1,

then addition of further ET-1 will have less of an effect than in a cell line where the receptors are available for binding.

The effect of ET-1 on LIM1215 and HT29 was mediated the ET_A, and not the ET_B receptor, as demonstrated by the ability of BQ123 and BQ610 to prevent an increase in cell number on addition of ET-1.

In summary, the colorectal cell lines studied expressed both ET_A and ET_B, produced ET-1 and grew better after being stimulated by exogenous ET-1. The growth stimulus was blocked by antagonising ET_A and not ET_B.

5 Discussion

Endothelin-1, a small vaso-active peptide originally isolated from endothelial cells, has been implicated in cancer growth and progression. Raised ET-1 levels have been detected in plasma and tissue samples from patients with solid malignant tumours including liver, lung, prostate and breast cancers (Giaid et al 1990, Yamashita et al 1992, Kojima et al 1995, Ishibashi et al 1993, Nelson et al 1996). Binding sites (receptors) for endothelin have been described in tissues from ovarian, prostate cancer phaechromocytomas (Watanabe et al 1997) and adrenocortical carcinomas (Rossi et al 1997). ET-1 is also produced *in vitro* by several cancer cell lines including colonic, pancreatic, ovarian, breast, stomach and prostate (Kusuhara et al 1990, Oikawa et al 1994, Baley et al 1990, Mathiew et al 1995), while receptors have been detected on ovarian and prostate cancer cell lines. The production of a biologically active peptide by the same tissue that expresses receptors for it raises the possibility of autocrine or paracrine growth loops. Indeed, Shichiri and colleagues proposed that ET-1 acts as an autocrine growth factor for cancer cells as early as 1991.

Previous work from the department of Surgery, UCL, focused on ET-1 in colorectal cancer: Raised levels of ET-1 were detected in the plasma of patients with either primary colorectal cancer or colorectal liver metastases. Furthermore, ET-1 was demonstrated immunohistochemically in tumour epithelial cells, stroma and the blood vessels of both primary colorectal cancer and liver metastases (Asham et al 1997, 1998, Shankar et al 1997). Further *in vivo* work using a rat model of liver metastasis Asham and colleagues (2001) has shown significant reduction in tumour mass following antagonism of the ET_A receptors. Other workers detected the presence of binding sites (putative receptors) for ET-1 in colorectal cancer specimens and normal tissue, including the nerve supply of colons, by autoradiography (Inagaki et al 1991, 1992).

Although colorectal cancer cells have been shown to secrete ET-1 *in vitro,* it is unknown if this peptide acts as a mitogen for these cells or indeed if these cells express the receptor for ET-1 and therefore have the potential to respond to ET-1.

Therefore, the first question this thesis addressed was whether tissues from patients with colorectal cancer express receptors for ET-1 and hence, are capable of receiving and responding to a signal from this peptide. After establishing that cells within tumours in the colorectum have endothelin receptors, I examined one of the possible growth stimulation loops by ET-1, namely autocrine stimulation of growth by employing the colorectal cancer cell lines, LIM1215 and HT29.

5.1 Profiles of ET_A and ET_B in colorectal cancer

The distribution of the two G-protein linked receptors ET_A and ET_B was investigated in normal colon and in specimens from patients with primary colorectal cancer. The techniques used were immunohistochemistry and autoradiography. Previous autoradiographic studies had detected the presence of binding sites (putative receptors) for ET-1 in colorectal cancer specimens and normal tissue, including the nerve supply of colons (Inagaki et al 1991, 1992). No immunohistochemical studies have been carried out previously.

In this study, immunohistochemical staining demonstrated a large increase in the expression of ET_A in tumours compared to distant normal tissue as staining for ET_A in normal tissue was restricted to the stroma and even here was seen in only 20% of patients. In contrast, in tumour samples, staining of tumour epithelial cells was observed in all samples and staining was also present in blood vessels and the stroma,

though not in every case. Interestingly, microscopically normal tissue adjacent to the tumour demonstrated a pattern intermediate to distant normal colon and tumour; it showed the same pattern of staining as for normal tissue in the stroma and blood vessels but was also similar to the tumour in that the epithelial cells expressed ET_A. This suggests that changes are occurring intracellularly in the epithelial cells adjacent to malignant cells but have not yet manifested in other normal cells types or affected the morphology. This could be due to factors released by the tumour affecting the adjacent epithelial cells.

A similar increase in the expression of ET_A receptors was demonstrated in ovarian cancer as compared to normal tissue using immunohistochemistry with a rabbit polyclonal ET_A antibody (Peninsula laboratories, Belmont, CA, USA) (Bagnato et al 1999). The distribution of ET_A receptors in ovarian cancer mirrored that of colonic cancer, with staining in the epithelial cells, blood vessels and stroma and also for the latter two areas in the normal tissue adjacent to the tumour. Also immunohistochemistry has demonstrated increase ET_A receptors in phaechromocytomas (Watanabe et al 1997) and in adrenocortical carcinomas (Rossi et al 1997).

Immunohistochemical staining for ET_B demonstrated an increased distribution in colorectal cancer; however, this was restricted to a greater percentage of epithelial cells containing ET_B compared to normal colon. As observed for ET_A, the levels of ET_B were similar in the adjacent normal tissue to the tumour. The ET_B distribution in the blood vessels and the stroma was the same in the three groups of tissue examined.

Immunohistochemistry for ET_B receptor has been carried out in malignant melanomas and an increase in their expression compared to normal skin demonstrated (Demunter et al 2001) and also in phaechromocytomas as compared to normal adrenomedullary tissue (Wattanabe et al 1997).

Similarly, to immunohistochemistry, a higher ET_A expression in cancers was observed compared to normal colon using autoradiography with semi-quantitation indicating twice as many receptors in tumours. Microautoradiography demonstrated a higher expression of ET_A receptors in tumour as compared to adjacent normal colon, with the epithelial cells of the tumour exhibiting the largest difference from normal colon. (Figure 3.2). However, *in situ* hybridisation for ET_A mRNA between normal colon and tumour shows no difference in mRNA levels and therefore, suggests that any differences in protein levels are due to post-translational modifications indicating the dynamic nature of expression of receptors and their activation depending on the cycle phase (Egidy et al 2000).

When ET_B receptors were studied by autoradiography they were reduced in colorectal cancer tissue as compared to normal colon and this was confirmed by microautoradiography. This is opposite to the results with immunohistochemistry.

The reduction in ET_B receptor expression demonstrated in colorectal cancer as compared to normal colon using the autoradiography technique was similar to that reported by Kobayashi et al (1994) and Nelson et al (1996) in human prostatic cancer as compared to normal prostate. However, *in situ* hybridisation of ET_B mRNA showed

high levels of the signal in colon cancer stroma compared to normal colon (Egidy et al 2000). There was high binding of the ET_B receptor in vascularised areas of the cancer.

The apparent discrepancy between the results obtained between autoradiography and immunohistochemistry staining for ET_B receptors, and indeed *in situ* hybridisation, may be due to the following: Autoradiography is examining receptors that can uptake ligands. Whereas with immunohistochemistry, it is possible that the receptors are present but not able to bind endothelin. And indeed, the main difference in the results is the staining of the epithelial cells in which immunolocalisation was seen intracellularly which would not be detected by autoradiography. This could suggest that ET_B is unable to bind endothelin in colorectal cancer.

Secondly, for ET_A all the receptors present may be active and able to bind endothelin. Further work is required to determine if the ET_B receptors are present but inactive or whether the difference between the immunohistochemistry and autoradiography is due to a technical problem such as lack of specificity of the antibody or cross-reactivity with another ET receptor as yet unidentified. The manufacturers quote a 98% sensitivity and specificity of the antibodies.

To date there is only one study in which structural abnormalities of the ET receptors have been described (Zhang et al 1998). Thirdly, therefore, it is possible that the discrepancies in the findings of the immunohistochemistry and the autoradiography for ET_B are due to changes in the receptor that prevent binding of the agonist used for the autoradiography but does not affect the binding of the antisera. The anti-ET_B was a polyclonal antibody and hence binds to several epitopes on the ET_B which are located

on the intracellular part of the receptor, whereas the agonist binds to the domains of the receptor which are located extracellularly. These different binding properties do give the potential for changes in the receptor to affect one technique without influencing the other.

These changes in ET_A and ET_B in tissue from patients with colorectal cancer compared to normal colon tissue does suggest that the increased levels of ET-1 in tissue of patients with colorectal cancer (Asham et al 1997) could be affecting the behaviour of the epithelial cells. Immunohistochemistry does not show whether the receptors are functional, however, this is implicated by the autoradiography for ET_A. Inagaki and colleagues demonstrated binding sites for ET-1 in human colonic tissue in 1992 that found an increase compared to normal colon especially in the blood vessels and stroma of the tumour. They did not distinguish between different sub-types of receptors or demonstrate by cell markers the cell type, which took up ET-1. The microautoradiographs used in this thesis localised the receptors and was combined with immunohistochemistry to identify the cell types, which expressed the receptor. This showed that ET receptors were present on epithelial cells and on nerves and blood vessels, which confirmed the supposition made from histological examination of the specimens by Inagaki and colleagues (1992).

The reason for the down-regulation of ET_B receptors in cancer as compared to normal colon remains unclear. Possibilities for the reduction in ET_B include it having an inhibitory effect on growth, that is functioning as a suppressor or having an apoptotic function, and therefore it is beneficial to the tumour to decrease ET_B. There is strong evidence for the apoptotic function of ET-1. Eberle et al (2000) have shown that ET-1

stimulated apoptosis and the mixed antagonist Bosentant blocked this stimulation in rat colon carcinoma cell lines. Okazawa et al (1998) demonstrated, in melanoma cell lines, that ET-1 induced apoptosis, probably via p53 up-regulation. The apoptotic effect was mediated by ET_B, since blocking the receptor with pertussis toxin abolished this effect. This is consistent with the fact that previously Eberle and his colleagues in 1999 found a down-regulation of the ET_B receptor in melanomas, suggesting that losing the ability to stimulate an apoptotic path confers a survival advantage on the tumour. On the other hand, Lahav et al (1999) demonstrated that cell proliferation of melanoma cell lines was mediated via the ET_B receptor. Administration of an ET_B antagonist can inhibit cell growth and in certain cell lines promote cell death. In a mouse subcutaneous melanoma model, blocking the ET_B resulted in growth arrest in 50% of the mice tumours. The evidence outlined above suggests that ET_B may have a dual role in growth, which is dependant on the existing conditions. In this case, its actions are not dissimilar to how it affects vessel tone, i.e. ET_B can mediate either dilatation or constriction

Our studies suggest that in colorectal cancer ET_A is the major receptor through which ET-1 may act as a growth factor. This is suggested too in ovarian cancer tissue and cell lines in which binding of ET-1 to ET_A stimulated growth (Moraitis et al, 1997, Bagnato et al 1995, 1997, 1999). Similar findings were demonstrated in lung cancer (Ahmad et al 2000). Therefore ET-1, additionally, may be acting as a growth factor for epithelial cells via ET_A as well as its traditional role as a vasodilator. The role of ET receptors and its interaction is complex. This work demonstrates a difference in ET receptors in colorectal cancer but does not address the pathways through which these might be operating in colorectal cancer.

5.2 ET-1 growth stimulation of colorectal cancer cells

Tissue culture work presented in this thesis investigated the following. Firstly, the production of ET-1 and expression of ET_A and ET_B receptors by two colorectal cancer cell lines, secondly the ability of ET-1 to stimulate the proliferation of these cell lines and finally the potential of ET receptor antagonists as an inhibitor of growth.

The two colorectal cancer cell lines employed in this study secreted ET-1, as has been demonstrated for other cancers. The level of ET-1 measured in the medium was similar to that previously reported for HT29 of 23 fmol/ml/10^6cells. All colorectal cancer cell lines investigated have been shown to produce ET-1 in this study and include SKCO1, SKCO17, LoVo, SW480 and SW620 in amounts that are very similar. The endocrine paracrine role of ET-1 in cancer growth was established by Kusahara et al (1990). The amount of ET-1 produced by several cancer cell lines is within the same range, e.g. Bagnato et al (1995) measured levels of 56-74 fmol/10^6 cells produced by three ovarian cancer cell lines.

ET-1 acts via two receptors, ET_A and ET_B, which were found to be present on LIM1215 and HT29 by autoradiography. This technique has directly, by utilising radiolabelled ligands, which display high affinity for receptors, demonstrated the presence of the receptors and indicated that they occur in a functional state (Dashwood et al 1998, Mumtaz et al 1999). Several studies have indirectly demonstrated the presence of ET_A and ET_B using receptor antagonist studies e.g. Leach et al (1999) in

glioma cells and Lahav et al (1999) in melanomas. These studies imply the presence of these receptors but do not establish a clear link between presence of receptors and effect. Ovarian cancer cell lines have been shown to produce ET-1 and express ET_A and ET_B receptors using a similar method to our own (Mancina et al 1997). Moriatis et al, Bagnato et al and Rossi et al (1997) have demonstrated the presence ET_A and ET_B directly by demonstrating gene expression using the reverse transcription polymerase chain reaction.

The mitogenic potential of exogenous ET-1 on these cell lines was assessed. This peptide was found to significantly increase cell number for both cell lines with a higher rise for LIM1215 than HT29. The difference may be related to the greater endogenous production of ET-1 by HT29: If the mitogenic effect occurs via one or both of the receptors and these receptors are partially occupied by endogenous ET-1, then addition of further ET-1 will have less of an effect than in a cell line where the receptors are available for binding. The concentrations of ET-1, 10^{-9}M and 10^{-8}M, which resulted in an increase in cell growth, are ten thousand times higher than the circulating plasma levels of ET-1 in patients with colorectal cancer. However, locally in the tissues much higher levels may occur (Inagaki et al 1991, Shankar et al 1998, Asham et al 2001). For other cell lines, e.g. Swiss 3T3 fibroblasts, vascular smooth muscle and ovarian cancer cell lines, a mitogenic effect of ET-1 was produced at 10^{-10}M, only ten fold lower than that required to produce an effect on LIM1215 and HT29.

The effect of ET-1 on LIM1215 and HT29 was mediated via the ET_A, and not the ET_B receptor, as demonstrated by the ability of the ET_A antagonists, BQ123 and BQ610, to prevent an increase in cell number on addition of ET-1. ET_A has also been

demonstrated to be the receptor through which the mitogenic effect of ET-1 is mediated for ovarian cancer cell lines and for a number of other cell lines. E.g. rat neuroblastoma cells (Heinroth-Hoffmann et al 1998, Zhou et al 2001), Choriocarcinoma cell lines (Mauschitz et al 2000), lung cancer cells (Ahmed et al 2000). In contrast, BQ788, the ET_B antagonist had no effect on cell number.

The only *in vitro* cancer model in which ET-1 mitogenic signalling has been studied used ovarian cancer cells. Binding of ET-1 to the ET_A G protein coupled receptor resulted in activation of phospholipase C activity and Ca^{2+}/PKC signalling, which are the classical effectors of G protein signalling. Furthermore, other intracellular targets activated included; tyrosine kinases (e.g., focal adhesion kinase $p125^{FAK}$), p42 mitogen activated protein kinase (MAPkinase), and immediate-early response genes (e.g. fos). This suggested that ET-1 does not just utilise phospholipase C/PKC pathways, but cross-talks with tyrosine kinase cascades. These intracellular steps have been implicated in mitogenic signalling via ET_A in a variety of cell types, including fibroblasts and vascular smooth muscle cells (Stahl et al 1992, Kirk et al 1999, McLellan et al 1991, Pretlow et al 1992)

5.3 Further Studies

This project does suggest the role of ET-1 in colorectal cancer and progression should be further investigated in order to understand its importance in this disease and to ultimately help identify specific steps, which could be selectively blocked in a therapeutic manner.

One aspect to study is the control of ET-1 levels, examining the production of ECE and EDE, which are enzymes that control at the cellular level, by measuring their levels in serum or tissue. This will indicate the availability of bioactive ET-1.

The study of the pathways through which ET-1 operates will also provides important information on how ET-1 exerts its effect on cells and, hence alternative methods of controlling its activity. The intracellular steps downstream of ET-1 leading to its mitogenic effect must be delineated. These include cross-talk with other pathways, e.g. MAPkinase. Knowledge of these steps may provide us with putative therapeutic options, especially since relevant specific blockers of other pathways have been manufactured and are now in clinical trials.

Although in this thesis, angiogenesis has not been investigated, ET-1 does have effects on neovascularisation, which is obviously crucial to the development and progression of a tumour, hence the effects of ET-1 on angiogenesis in colorectal cancer should form part of any subsequent studies.

5.3 Conclusion

This thesis has demonstrated that ET-1 can stimulate net cell growth of LIM1215 and HT29 colorectal cancer cell lines via the ET_A receptor. Whether this effect is mediated via a mitogenic stimulus, as in the case of ovarian cancer cells and other non-cancer cells, or an anti-apoptotic signal, or a combination of the two, has not yet been demonstrated in this model. However, the findings by Asham et al (2001) and Shankar et al (1998) that ET-1 is produced by colorectal cancers combined with the data from this study on the up-regulation of the ET_A receptor in colorectal cancer are consistent

with the proposition that ET-1 may act as a mitogen in colorectal cancer and that there may be some therapeutic potential in the use of ET_A antagonists. Therefore ET-1 and its receptors do appear to contribute to the development of colorectal cancer and further investigations are required to determine precisely the mechanisms through which these molecules operate.

5.4 Published work

5.4.1 Publications and presentations

Endothelin receptor expression in colorectal cancer.
Journal of Cardiovascular Pharmacology, 36 (Suppl. 1): S69-S71. 2000.

Endothelin receptor antagonism in colorectal cancer.
Stimulation of colorectal cancer cell line growth by ET-1 and its inhibition by ET_A antagonists. Gut. 2000 Nov; 47(5): 685-688.

Endothelin A receptor antagonism affects growth of colorectal cancer cells.
European Journal of Surgical Oncology December 1998, 24:622.

Endothelin Receptors in Colorectal Cancer.
University of London, Professor Charles Grant Clark Prize for Surgical Research (July 1998).

6 Appendix

6.1 Patients's specimens for immunohistochemistry for ET_A and ET_B staining

Table 6.1 *Histological details of patients used for immunohistochemistry and the results of staining for ET_A.*

No.	Duke's Grade	Histological differentiation	ET_A epithelial staining for tumour	ET_A epithelial staining for adjacent normal colon
1	A	M	2	
2	C	P	2	1
3	A	M	3	0
4	B	M	2	2
5	B	M	3	
6	B	P	2	3
7	B	M	2	
8	C	M	3	1
9	A	M	1	2
10	A	M	2	
11	B	W	3	2
12	C	M	3	0
13	B	W	2	1
14	C	M	3	
15	A	M	1	3
16	C	M	2	3
17	A	M	2	2
18	A	M	2	2
19	A	M	2	
20	C	P	1	1
21	C	W	3	2
22	A	P	2	
23	A	M	3	2
24	B	M	1	1

25	C	W	2	2
26	A	M	2	2
27	B	M	1	
28	C	M	2	3
29	A	M	2	3
30	B	P	2	
31	C	M	3	2
32	A	W	1	
33	B	M	3	3
34	C	M	3	2
35	A	M	2	2
36	B	M	2	2
37	A	M	2	3
38	C	M	3	1
39	A	P	2	3
40	C	W	1	3
41	A	M	2	2
42	B	M	2	1
43	C	W	3	0
44	B	M	3	2
45	A	M	1	3

Table 6.2 *Histological details of patients used for immunohistochemistry and the results of staining for ET_B.*

No.	Dukes's grade	Histological differentiation	ET_B epithelial staining for tumour	ET_B epithelial staining for adjacent normal colon
1	A	M	1	
2	C	P	2	1
3	A	M	1	2
4	B	M	2	1
5	B	M	1	
6	B	P	2	2
7	B	M	3	
8	C	M	1	2
9	A	M	2	2
10	A	M	2	
11	B	W	3	2
12	C	M	1	3
13	B	W	3	2
14	C	M	2	
15	A	M	2	2
16	C	M	2	2
17	A	M	1	2
18	A	M	2	1
19	A	M	2	
20	C	P	3	3
21	C	W	1	2
22	A	P	3	
23	A	M	3	2
24	B	M	2	3
25	C	W	2	2
26	A	M	3	2
27	B	M	1	
28	C	M	3	2
29	A	M	2	2
30	B	P	1	

Table 6.3 *Patients 31 to 45 were patients who had distant normal colon samples. The highlighted 9 patients were used for autoradiography studies.*

No.	Duke's grade	Histological differentiation	ET_B epithelial staining for tumour	ET_B epithelial staining for adjecent normal colon	ET_B epithelial staining for distant normal colon
31	***C***	***M***	***2***		
32	A	W	3		
33	B	M	1	1	
34	***C***	***M***	***1***		
35	***A***	***M***	***1***		
36	***B***	***M***	***2***		***0***
37	A	M	2		0
38	***C***	***M***	***2***	***1***	***1***
39	A	P	3	1	1
40	C	W	2	3	0
41	***A***	***M***	***2***		***0***
42	***B***	***M***	***2***		***0***
43	C	W	2		1
44	***B***	***M***	***2***		***0***
45	***A***	***M***	***2***		***0***

Preparation of gelatin coated slides

6.2.1 Materials

Decon Solution: 20ml decon in 10 litres water

Subbing solution: 0.5% gelatin solution prepared by heating to 40°C until dissolved. Following cooling, 0.05% chrome alum (chromic potassium sulphate) was added. After stirring the solution was filtered.

6.2.2 Method

Microscope slides were pre-cleaned by immersing in decon solution overnight, rinsed for 3 hours in tap water followed by a distilled water rinse. The slides were then dipped in subbing solution for a few seconds, allowed to drain overnight at room temperature in a dust-free environment. The slides were used immediately or stored at –70°C.

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