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The Insulin-like Growth Factor System and Adenocarcinoma of the Colon

Thesis submitted for the degree of Doctorate of Medicine
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For my parents

Namita and Nirmal

Abstract

The insulin-like growth factor (IGF) system is important in normal growth and development. However, it is also known to be involved with malignant transformation and cellular proliferation. IGF binding proteins modulate the biological activity of IGF-I, either potentiating or inhibiting its activity, as well as determining how much enters the circulation at any one time. IGF binding protein-4 (IGFBP-4), for example is believed to be inhibitory to the effects of IGF-I.

This thesis shows that the colon cancer cell lines Colo 205, HT29 and WiDR proliferate in response to IGF-I, and that IGFBP-4 at high concentrations inhibits their growth. However, it was found that with lower concentrationsof IGFBP-4, proliferation in HT29 and WiDR cells increased. Nevertheless in two cell lines, IGFBP-4 partially negated the proliferative effects of IGF-I. An antibody against IGFBP-4 was used to show that endogenous IGFBP-4 plays an important role in modifying cell growth.

In order to start *in vivo* experiments which required considerable quantities of IGFBP-4, this protein was produced in an expression system and purified using an immunoaffinity column method. The rhIGFBP-4 thus produced was shown to be functional and to inhibit colorectal cancer cell growth *in vitro*.

A nude mouse model of colon cancer was produced and the expression of components of the IGF system in this model determined using PCR.

Experiments were performed using conditioned medium from Colo 205 cells to investigate IGFBP-4 protease activity.

This thesis shows that manipulation of the IGF system is a potential target for further research into treatment for adenocarcinoma of the colon.

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Glossary of Abbreviations

General Abbreviations

Ab	antibody
BCA	bicinchonic acid
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
dATP	2-deoxyadenosine 5-triphosphate
dCTP	2-deoxycytosine 5-triphosphate
ddH_2O	double distilled water
DEPC	diethyl pyrocarbonate
dGTP	2-deoxyguanosine 5-triphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	dATP, dGTP, dCTP, dTTP
dTTP	2-deoxythymidine 5-triphosphate
EDTA	ethylenediamine tetraacetate
IPTG	Isopropyl β-D-thiogalactoside
kb	kilobase
kDa	kilodalton
LB	luria-bertani
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
RNA	ribonucleic acid
RNase	ribonuclease
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TE	Tris-EDTA
TEMED	tetramethylethylenediamine

Tris	tris(hydroxymethyl)methylamine

Units of Concentration

mM	millimolar (millimoles/litre)
nm	nanomole
pm	picomole

Units of Length, Area, Volume, Mass, Time

cm	centimetre
mm	millimetre
µm	micrometre
ml	millilitre
µl	microlitre
µg	microgram
ng	nanogram

Physical and Chemical Units

g	acceleration of gravity
mA	milliamp
°C	degree Celsius
OD	optical density
U	unit
V	volt

Chapter 1 Introduction

The insulin-like growth factor system consists of a family of ligands, receptors, binding proteins and proteases. There are two growth factors – IGF-I and IGF-II, and two receptors – the type I receptor and the type II receptor. However, both growth factors exert their mitogenic effects via the type I receptor. Six binding proteins have been identified to date, and these act to modulate the activity of the IGFs. Proteases exist to degrade the binding proteins, and this in turn has an effect on IGF bioavailability.

IGF-I is regulated by many different factors but is mainly under the control of growth hormone. Insulin, IGF-II, thyroid hormones and steroid hormones also have a role in its regulation and in diseased states may play a more prominent part. Growth hormone and insulin also affect binding protein expression, which alters the concentration of circulating free IGF-I.

As well as mediating its effects via the type I receptor, IGF-I interacts with other growth factors such as TGFβ, VEGF and TNFα, as well as the p53 gene, cyclins and cyclin-dependent kinases[1].

In humans, IGF-I has been shown to be important in cellular proliferation in a number of different organ systems. If it is not kept in check by mechanisms such as those mentioned above, the control of cellular proliferation may be lost, and dysplastic change and subsequent malignancy may ensue.

1.1 Insulin-like growth factor-I

1.1.1 Structure of IGF-I

The human gene for IGF-I is located on the long arm of chromosome 12 and spans over 100 kilobase pairs[2,3]. The gene can be spliced to produce different peptides from pro-IGF-I which may have different actions.

Expression of the human IGF-I gene leads to the production of three different sizes of mRNA at 1.1, 1.3 and 7.6 kilobases[4]. The human IGF-I gene contains 6 exons[5]. Exons 1 and 2 are alternative leader exons and have different transcription sites. When exon 1 is spliced to exon 3, it gives rise to Class I IGF-I mRNA transcripts. When exon 2 is spliced to exon 3, it gives rise to Class II transcripts. Exons 3 and 4 are constant within both classes and code for the mature IGF-I peptide and the first 16 amino acids of the E domain. Variability again occurs with exons 5 and 6 with alternative splicing giving rise to Ea, Eb and Ec domains in both Class I and II transcripts. As a result of alternative splicing, 6 different IGF-I mRNAs may be produced.

Mature IGF-I is a single chain basic protein comprising 70 amino acids[6] and has a molecular weight of 7649[7]. It has 70% homology with IGF-II and 40% homology with pro-insulin[3]. IGF-I is translated as a pro-peptide and is composed of five parts:
The A chain
The B chain
The C connecting peptide
The D peptide with a COOH-terminal
The E peptide at the C-terminal end – this is of variable size

The A and B domains of IGF-I have 50% homology with those of insulin[8].

1.1.2 Expression of IGF-I

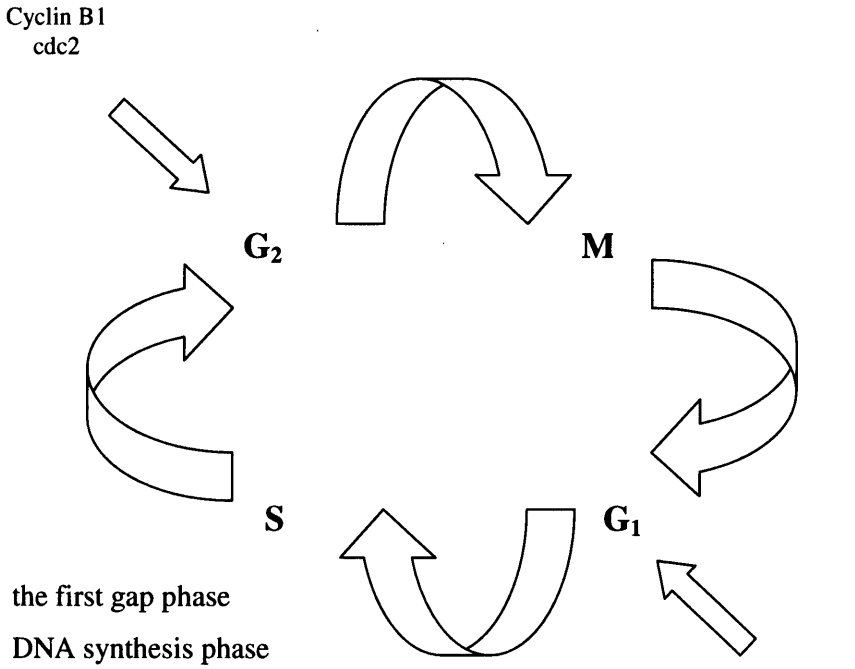
In the resting state the majority of IGF-I is produced in the liver. Muscle during intensive exercise can, however, account for an even greater percentage of circulating IGF-I than the liver. Active muscle produces an IGF-IEa as well as MGF, a form that is mechano-sensitive[9]. This has been shown to have a different expression and different biological actions[10].

IGF-I RNA is expressed in almost all tissues in the human, including the gastro-intestinal tract, muscle, kidneys, pituitary and chondrocytes[11-13]. In the rat, the levels of IGF-I mRNA in the liver are 30-fold greater than in the second highest tissue which is the uterus[11]. IGF-I mRNA levels are low in the brain and testes.

1.1.3 Function of IGF-I

IGF-I is an anabolic and mitogenic peptide. It has different specialised functions in different organ systems of the body, such as in the gastro-intestinal system, the reproductive system, the cardiovascular system and the peripheral and central nervous systems.

IGF-I exerts its anabolic effects by stimulating the synthesis of glycogen, lipids and proteins. It increases glucose metabolism in adipose tissue and also inhibits lipolysis[14].



G_1	the first gap phase
S	DNA synthesis phase
G_2	the second gap phase
M	mitosis phase

Figure 1 The cell cycle

The mitogenic effects of IGF-I arise as a result of increased DNA synthesis. IGF-I upregulates the expression of cyclin D1 (a G_1 cyclin)[15], cyclin B_1 (a G_2 cyclin), and two cyclin-dependent kinases (cdc2 and cdk2) . The activation and degradation of cyclins and the activation of cyclin dependent kinases control the progession of the

cell cycle from one stage to the next. Oesophageal, breast and gastric cancers have been shown to be associated with overexpression of cyclin D1[16].

IGF-I increases cell number by a combination of inhibiting apoptosis, as well as promoting cellular proliferation[17,18].

1.1.4 Regulation of circulating IGF-I

The regulation of IGF-I is mainly under the control of growth hormone which is produced by the pituitary. Growth hormone itself is regulated by growth hormone releasing hormone (GHRH) from the hypothalamus. A negative feedback mechanism occurs between these three factors (see figure 2).

Growth hormone stimulates the synthesis of IGF-I in the liver as well as in other tissues. Administration of growth hormone to hypophysectomized rats, led to an increase in serum IGF-I levels which peaked 12 hours after the administration of growth hormone[19]. In addition to growth hormone, insulin is involved in stimulating the production of IGF-I[20-23]. Other hormonal influences on IGF-I and the GH/IGF-I axis are thyroid status, oestrogens and androgens. Catabolic states such as illness and trauma also have a role in the regulation of IGF-I levels[24]. Nutritional deficiency impairs growth hormone induction of IGF-I[25].

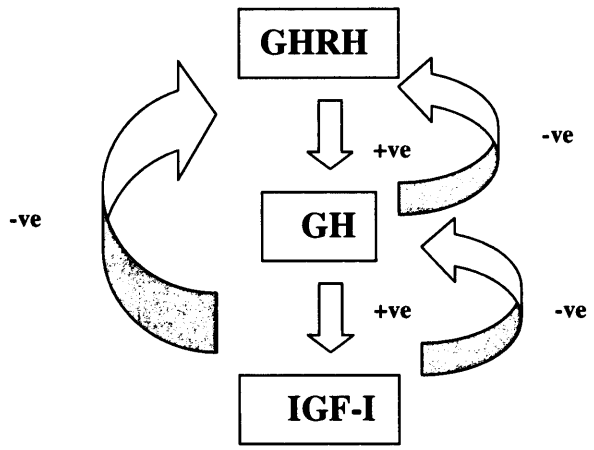


Figure 2 Negative feedback loop for the GH/IGF axis

Circulating concentrations of IGF-I remain fairly stable due to its constitutive pattern of secretion. Another factor involved in maintaining IGF-I levels are the IGF binding proteins. 99% of IGF-I is carried by binding proteins and at any one time only 1% may be biologically active. Alterations in the proportion of the binding proteins or their affinity for IGF-I affects the regulation of IGF-I[26-29].

1.1.5 Role in embryonic growth and development

IGF-I plays an important role in the growth and development of the foetus. The majority of work in this field has been done using mouse embryos with various mutations. Normal foetal growth consists of cell hyperplasia, cell differentiation and hypertrophy.

Mice embryos with null mutations for IGF-I have been created. These animals have an increased mortality. A significant proportion of the deaths are secondary to muscle hypoplasia and insufficient lung maturation[8]. Those that survive have a decreased rate of growth and as adults only reach 30% of the normal weight as compared with controls. They are infertile and have delayed bone development[30].

It has been shown that IGF-I is not significant in the very early stages of foetal development of the mouse, as the IGF-I receptor does not respond to IGF-I until embryonic day 13.5[30].

IGF-I receptor gene expression has been detected in placental tissue, however, the presence or absence of IGF-I has no effect on placental weight[30].

Unlike in the adult, IGF-I in the embryo is growth hormone independent[31,32]. This has been seen in animal studies where absence of growth hormone in the embryo has no effect on its growth.

The levels of human foetal IGF-I are constant throughout the majority of the gestation period until 34 weeks, after which they start to increase until birth[14]. The levels of IGF-I in the foetal serum and in amniotic fluid are equal. It is interesting to

note that this is higher than maternal serum IGF-I levels. It would appear unlikely that maternal IGF-I is able to cross the placenta and enter the foetal circulation.

There is a direct correlation between foetal IGF-I levels and birthweight, however, there is no link between maternal IGF-I levels and birthweight[14].

1.1.6 Role in post-natal growth

Mice with a null mutation for IGF-I have a decreased growth rate, not only during the gestation period, but also after birth, compared with wild-type animals[30]. They achieve only 60% of the normal birthweight, and at eight weeks old they have only managed to reach 30% of the normal adult weight. Despite their small size, they are proportionate in size and exhibit normal behaviour.

As well as slow post-natal growth, they have a decreased rate of long bone ossification. Both male and female mice have small reproductive organs and are infertile.

IGF-I has a significant role in post-natal growth, as demonstrated by the fact that acromegalics have high levels of circulating IGF-I and pituitary dwarfs have low levels.

1.1.7 IGF-I and ageing

Serum IGF-I levels show no seasonal or diurnal variation. However, levels do alter with age. In childhood, IGF-I increases slowly until puberty when there is a steep rise in IGF-I concentration. After puberty IGF-I slowly starts to decrease. In both men and women, serum IGF-I decreases with age in a linear fashion. For all other ages, IGF-I levels are lower in women than in men up to the age of 85 when they become equal[33].

1.1.8 IGF-I and acromegaly

Acromegaly is a condition characterised by raised levels of growth hormone. There is excessive growth of bone and soft tissues, as well as organomegaly due to the increased growth hormone production. Growth hormone is the major regulator of liver IGF-I synthesis and therefore these subjects also have raised levels of IGF-I. IGF-I is a potent mitogenic factor and acromegalics are subject to an increased rate of diseases related to this, such as colonic adenoma and adenocarcinoma[34].

Growth hormone can exert its proliferative effects both directly and indirectly. C-myc is a proto-oncogene which codes for a transcription factor regulating the early part of cell proliferation[35]. Overexpression of this oncogene induces dysplastic change in colonic mucosa, and growth hormone has been shown to increase c-myc mRNA levels in the liver 12-fold after 1 hour[19]. Growth hormone can also indirectly exert its effects via IGF-I.

Increased cellular proliferation is thought to be the first step in the adenoma-carcinoma sequence and can be seen as a marker for the risk of development of colon cancer.

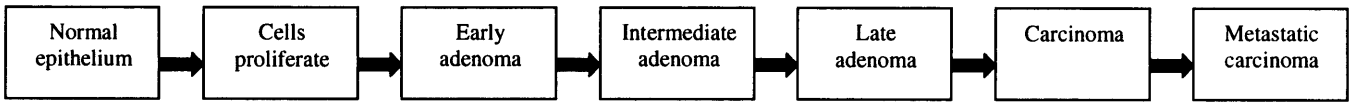


Figure 3 The adenoma-carcinoma sequence

A prospective study of 30 people with acromegaly showed that 54% of them had increased epithelial proliferation in the sigmoid colon compared with normal levels of epithelial proliferation[28]. This study showed a correlation between growth hormone levels and epithelial cell proliferation, as well as between IGF-I levels and cellular proliferation.

Not only is there increased epithelial cell proliferation in acromegalics, but they also progress to later stages of the adenoma-carcinoma sequence, and have an increased risk of colonic adenomas and adenocarcinomas. There is an increased relative risk of

the development of colorectal cancer in acromegalics of between 13 and 92 fold[36]. In a prospective colonoscopic study of 155 acromegalics, Jenkins et al found 5% of patients had a colorectal cancer, and 25% had one or more colonic adenoma. For a reason as yet unexplained, they found a higher ratio of left sided adenomas in acromegalics than in the normal population. The occurrence of adenoma formation was age related, as is true of the normal population, with 39% of patients over the age of 70 having one or more adenoma. Acromegalics with an adenoma had higher levels of IGF-I than those who had a normal colonoscopy.

However, not all studies agree with these findings. A retrospective study of acromegalics found that they had a 2.5 fold increased risk of malignancies in general, but stated that there was no increased risk of colonic adenoma or carcinoma formation. However, this study had small numbers, and only looked at results from proctoscopy rather than colonoscopy[37].

A prospective study compared colonoscopic findings from acromegalics with a post-mortem study and a population screening study[38]. This study showed the incidence of colorectal cancer in the acromegalic population to be 2.6% with the incidence of colon tumour formation (adenoma and adenocarcinoma combined) to be 12%. The highest incidence of colonic tumours was in people over the age of 60 years. Like the study above they found no difference in the incidence of either colorectal cancer or adenoma formation when compared with the two control groups. However, they did find significant pathological differences. In their study they found adenomas in acromegalics tended to be right-sided, larger and of more advanced histological grade.

1.1.9 IGF-I and the regulation of VEGF

Vascular endothelial growth factor (VEGF) is an angiogenic peptide that stimulates capillary formation and is mitogenic and chemotactic for vascular endothelial cells. One of the major factors in the regulation of VEGF is hypoxia, which results in its upregulation. Malignancies rely on neovascularisation and angiogenesis for sustaining their growth and many malignancies overexpress VEGF[39].

Another factor which is involved in controlling VEGF levels is IGF-I which acts to increase VEGF expression. In the colon cancer cell lines HT29 and SW620, the addition of 100ng/ml IGF-I led to an increase in VEGF mRNA. There was a 7.3-fold increase in mRNA expression in HT29 (peaking 24 hours after IGF-I administration) and a 4.7-fold increase in SW620 (peaking at 8 hours)[39].

The need for VEGF to sustain tumour growth has been demonstrated by the administration of antibodies against VEGF to a mouse model of colon cancer. This led to decreased tumour growth and the inhibition of metastasis formation. Liver metastases from a primary colorectal cancer have been shown to have an increased number of VEGF receptors than the surrounding liver parenchyma[40].

1.1.10 Evidence for a role of IGF-I in carcinoma of the colon

IGF-I levels are related to the risk of developing colorectal cancer. A prospective case control study nested within the Physicians Health Study of almost 15,000 men looked at the relationship between colorectal cancer and IGF-I[41]. Of this cohort, in a 14 year follow-up period, 193 developed colorectal cancer. After controlling for IGF binding protein-3 levels, age, smoking status, body mass index and alcohol, they found that men in the highest quintile for IGF-I levels, had a relative risk of 2.51 of developing colorectal cancer compared with men in the lowest quintile. Moreover, they discovered that those in the highest tertile for IGF-I who were also in the lowest tertile for IGF binding protein-3, had a 4-fold increased risk of colon cancer than those in the lowest tertile for IGF-I and the highest tertile for IGF binding protein-3.

A similar study looked at the risk of colon cancer in women and found that there was a small but not statistically significant positive association between IGF-I levels and risk of colon cancer in women[42].

IGF-I leads to colonic epithelial proliferation. Out of 8 colon cancer cell lines tested, 5 showed increased proliferation at five days with the administration of IGF-I[43]. This occurred in a dose-dependent manner, up until a maximum cell density was reached. In some of the remaining cell lines, there was a decrease in proliferation,which may have been due to cellular overgrowth and the accumulation of toxic metabolites.

As well as being involved in epithelial cell proliferation, IGF-I is important in the development and maintenance of the connective tissue component of the intestine. Exogenous IGF-I at 500μg/day was given to rats with a left-sided colonic anastomosis[44]. This led to an increase in anastomotic collagen content by 23% in the treated animals versus the controls. However, there was no difference in the anastomotic breaking strength between the two groups.

Somatostatin is a naturally occurring hormone with anti-proliferative properties. It has been shown to inhibit cellular proliferation in the rat and rabbit in the stomach, intestinal mucosa, and the pancreas, and it decreases proliferation in human colorectal xenografts[45-48]. One of the mechanisms by which somatostatin exerts its effect is by inhibiting the expression of IGF-I[26]. This has been shown to occur *in vitro* and *in vivo*. It also decreases levels of VEGF[49,50]. It has a very short half-life of 1-3 minutes in plasma. For this reason, different synthetic analogues have been produced for both experimental and therapeutic uses. Two of these are octreotide and SMS 201.995.

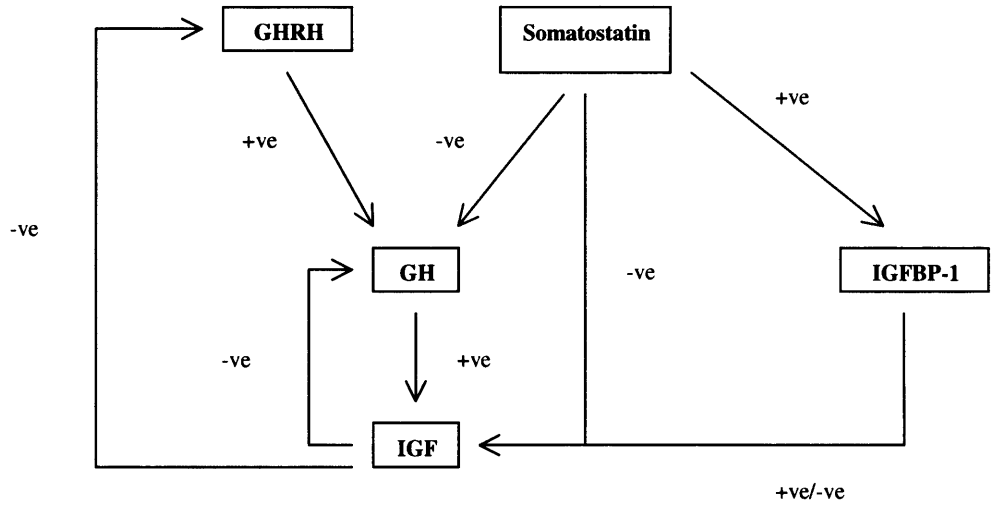


Figure 4 The effect of Somatostatin on the insulin-like growth factor system (adapted from Reichlin 1983[51])

SMS 201.995 is 3 times more potent *in vitro* than somatostatin, and is 70 times more potent *in vivo*. It has a much longer half-life than somatostatin of 113 minutes. Three

out of four colon cancer cell lines tested showed inhibition in a dose-dependent manner to the administration of the somatostatin analogue[52]. The same group also showed significant inhibition of growth in colon cancer xenografts treated with SMS 201.995.

Patient studies using octreotide have shown significant results. Seventy five patients with colorectal cancer were randomised to receive either octreotide at a dose of 200μg daily for 2 weeks prior to surgery, or to receive their usual medications only[26]. Octreotide was found to decrease serum IGF-I levels compared with the control group. Pre-treatment endoscopic biopsies were compared with the surgical specimen, and a statistically significant reduction in the mean percentage of the S-phase fraction (DNA replication phase of the cell cycle) out of the total number of tumour cells was found. This is likely to be a direct effect of octreotide on IGF-I as other studies have shown that octreotide has no effect on growth hormone levels[53,54]. Octreotide directly inhibits hepatic IGF-I gene expression and leads to a decrease in serum levels. It also partially inhibits the effect of growth hormone on IGF-I synthesis. Octreotide does not have any effect on the regulation of growth hormone receptors in the liver.

Octreotide also has a beneficial effect on patient survival. 107 patients with a range of advanced gastro-intestinal malignancies which were refractory to chemotherapy were randomised to receive either octreotide or best supportive care only[55]. Octreotide was administered at 200μg three times per day for 5 days per week, the mean duration of treatment being 12 weeks (range 6-32 weeks). The treatment group had a median survival of 20 weeks versus 11 weeks for the control group. Moreover, 45% of the treatment group showed stable disease as compared to 15% in the control group. 40% of the octreotide group had decreased pain as measured by a decrease in analgaesia requirements.

Another somatostatin analogue, somatostatin 14, inhibited growth in 2 out of 3 colorectal cancer cell lines *in vitro*. The same compound administered to a mouse model of colorectal cancer decreased the final tumour volume and weight[56].

Somatostatin and its analogues may exert their anti-proliferative effects either directly via the somatostatin receptor. Gastro-intestinal tissues such as the colon, stomach and pancreas have all been shown to have somatostatin receptors.

As well as having an effect on IGF-I, octreotide affects IGF binding protein-1 levels. Normal and growth hormone deficient subjects were given subcutaneous injections of either octreotide or saline[57]. In both the normal and growth hormone deficient groups, octreotide led to elevated binding protein-1 concentrations. This was maximal at 3 hours and remained raised for 6 hours. The increase in IGFBP-1 production is independent of growth hormone.

1.1.11 Evidence for a role of IGF-I in carcinoma of the breast

IGF-I is associated with breast cancer in pre-menopausal women. In a prospective case control study nested within the Nurses' Health Study, the relationship between IGF-I and breast cancer was examined[58]. The results as a whole showed no association between the two. However, when it was subdivided into pre- and post-menopausal women there was a significant association. In pre-menopausal women the relative risk of breast cancer for those in the highest versus the lowest tertile for IGF-I levels was 2.33. This rose to 4.58 for pre-menopausal women under the age of 50 years. After adjustment for IGF binding protein-3 levels this increased to relative risks of 2.88 and 7.28 respectively.

Other factors that are associated with breast cancer risk are birthweight and height[34,59-61]. These can be thought of as surrogate markers for IGF-I levels. Women who had a birthweight of 2500-2999g had an odds ratio for breast cancer of 0.55 compared with women with a birthweight of 4000g[59]. Serum IGF-I has been shown to predict height velocity in children and adolescents[61].

Further evidence linking the importance of IGF-I to breast cancer is that the IGF-I receptor appears to be required for breast cancer metastasis. A metastatic breast cancer cell line was transfected with a dominant negative form of the IGF-I receptor[62]. This inhibited cellular adhesion to laminin (in the basement membrane)

and type I collagen (in the interstitial matrix) and inhibited invasion through type IV collagen (in the basement membrane). Cellular adhesion to laminin and type I collagen was inhibited by 94% and 88% respectively. The development of metastases *in vivo* was also inhibited.

IGF-I, IGF-II and the IGF-I receptor have all been shown to be increased in breast cancer. IGF-I and IGF-II have been found to increase proliferation in a variety of breast cancer cell lines. IGF-I has also been shown to protect pre-neoplastic cells from apoptosis[62].

The IGF-I receptor is overexpressed in 52% of breast cancers and this has an effect on prognosis. Patients who have high levels of IGF-I receptor have a higher rate of ipsilateral recurrence within the first 4 years than those with lower levels[63]. There is no difference in rates of late recurrence. The high levels of IGF-I receptor confer a resistance to radiotherapy on the breast cancer cells.

IGF-I is involved in cell motility in a number of different malignant cell lines *in vitro*, including some breast cancer cell lines. This is mainly due to chemotaxis. Motility and cell migration can be inhibited by antibodies to the IGF-I receptor and also by tyrosine kinase inhibitors[64,65].

Tamoxifen, which is a partial antagonist to the oestrogen receptor, is a widely used drug in the treatment of carcinoma of the breast. As well as acting via the oestrogen receptor, tamoxifen reduces serum IGF-I levels[66]. Tamoxifen decreases IGF-I gene expression in the liver 3-fold and causes a 25% decrease in serum IGF-I[67]. Using hypophysectomised, and hypophysectomised growth hormone replaced animals, the reduction in IGF-I was found to occur partly via downregulating growth hormone but also via a pituitary independent mechanism.

Another mechanism whereby tamoxifen has an anti-proliferative effect, is that it increases extracellular TGFβ produced by stromal fibroblasts[68]. TGFβ inhibits the growth of epithelial cells. This occurs irrespective of oestrogen receptor status, showing that tamoxifen exerts this effect via a mechanism other than acting through

the oestrogen receptor. TGFβ also leads to an increase in IGF binding protein-3 synthesis in certain tissues[69]. IGFBP-3 has been shown to mediate the apoptotic effect of TGFβ in a prostate cancer cell line[70].

IGF-I has also been shown to counteract the effect of commonly used chemotherapeutic agents used for breast cancer. *In vitro* studies have shown that the addition of IGF-I to breast cancer cell lines treated with 5-FU, methotrexate, tamoxifen or campothecin increases cell survival by approximately 25%[17]. This is due to the inhibition of apoptosis.

Breast cancer cells *in vivo* express low levels of IGF-II and the surrounding stroma expresses IGF-I[71]. The majority of breast cancer cells express the insulin receptor and IGF receptors. Different breast cancers express different IGF binding proteins and this varies according to their oestrogen receptor status. Oestrogen receptor positive cells produce mainly IGF binding protein 2. They do not produce IGFBP-3. Receptor negative breast cancers produce IGF binding proteins 1and 3[72,73].

1.1.12 Evidence for a role of IGF-I in carcinoma of the prostate

IGF-I has proliferative and anti-apoptotic effects on both normal and malignant prostate epithelial cells[74,75]. IGF-II and insulin also stimulate growth but much higher concentrations are required to produce an effect. Prostate epithelial cells have high levels of the IGF-I receptor. They produce IGFBP-2 and IGFBP-4. Prostate specific antigen (PSA) which is a biochemical marker used in screening for prostate cancer, as well as monitoring response to treatment is also a binding protein protease in prostatic tissue[76]. PSA is a serine protease found in semen and is able to cleave IGFBP-3. It has no effect on IGFBP-2 or IGFBP-4.

Epidemiological studies show high birthweight to be associated with an increased incidence of prostate cancer, and as mentioned previously, birthweight is related to foetal IGF-I levels[77].

A nested case control study within the Physicians' Health Study looked at the relationship between prostate cancer and the IGF system[78]. From the initial cohort of 14 916 subjects, 520 men developed prostate cancer. They found that men in the highest quartile for IGF-I levels had a relative risk of developing prostate cancer of 4.3 versus men in the lowest quartile. This result was independent of baseline PSA levels.

1.1.13 Evidence for a role of IGF-I in carcinoma of the lung

IGF-I and IGF-II have both been shown to have mitogenic effects on normal and malignant lung cells[7,79,80]. *In vitro* studies have shown that most lung cancer cell lines (small cell and non-small cell) express IGFs and binding proteins[81-84]. Non-small cell lines produce all 6 IGFBPs, the major one being IGFBP-3. IGF-I does not change IGFBP-3 mRNA expression but leads to an increase in extracellular IGFBP-3 and decreases the level of membrane associated IGFBP-3. In contrast IGF-I decreases IGFBP-4 levels[81]. Insulin-like growth factors release IGFBP-3 from the cell membrane.

High levels of IGF-I are related to an increased risk of lung cancer in a dose dependent manner[85]. The mean level of IGF-I in patients with lung cancer was 16% higher than controls. Increased levels of IGF binding protein-3, when adjusted for IGF-I, is associated with a decreased risk of lung cancer. IGF-II showed no association with lung cancer in this study.

1.2 Insulin-like growth factor-II

1.2.1 Structure of IGF-II

The gene for IGF-II is a completely separate gene from that of IGF-I, and in the human maps to the short arm of chromosome 11 which also contains the gene for insulin[86]. It spans over 30 kilobase pairs.

The IGF-II gene comprises at least 10 exons, including 4 leader exons, 1 alternative exon and 3 common protein-coding exons. The IGF-II gene has 4 promoters (P1-4) and these precede the common coding exons 7,8 and 9[3].

IGF-II is a straight chain polypeptide of 67 amino acid residues and is 7.5kDa in size[87]. The primary structure of IGF-II is similar to IGF-I and they share 62% amino acids in common. Both IGF-I and IGF-II have homology with human pro-insulin[88].

Like IGF-I, IGF-II has A and B domains with a connecting C peptide, as well as a D domain. The similarity in amino acid sequence with pro-insulin lies in the A and B domains. Insulin is produced by the β cells of the pancreas as pro-insulin which is then cleaved into insulin and the C peptide[89]. The C peptide is 8 residues long in IGF-II compared with 12 residues in IGF-I and 35 in pro-insulin. The D domain is 6 residues long in IGF-II compared with 8 in IGF-I.

The IGF-II pre-pro-hormone consists of a 24 amino acid signal peptide, a 67 amino acid mature peptide and a C-terminal peptide of 89 amino acids[90]. As a result of different RNA splicing, mRNA species exist of between 2.2 and 6.0kbp in size. The 5.3kbp RNA is synthesised by the liver, but other tissues express the 4.8 and 6.0 kbp RNA species[3].

IGF-II of differing molecular weights have been identified (10-15 kDa), and these may relate to the circulation of precursor forms of IGF-II which have been incompletely processed[3,91].

1.2.2 Expression of IGF-II

IGF-II is expressed in a wide variety of tissues in the foetus and in the adult. Several tissues in the foetus express IGF-II between 7 and 20 weeks of gestation. IGF-II mRNA levels in the foetus are higher than IGF-I mRNA in the liver, intestine, adrenal glands, skin, kidney and pancreas. In the liver levels of IGF-II mRNA levels are 650 times higher than IGF-I[3,92].

In adult tissues, IGF-II mRNA is often expressed at much lower levels than in the corresponding tissues of the foetus.

IGF-II mRNA distribution was examined in the adult rat and the highest levels were found in the brain. Other tissues expressing it were the uterus, heart, kidneys and skeletal muscle[11]. IGF-II mRNA was barely detectable in the liver, and was not found to be present in the lung, ovary, testes or mammary glands.

IGF-II may have a role in preventing apoptotic cell death[80]. Transgenic mice expressing the SV40 T antigen in the pancreatic islet of Langerhan cells, had an increased mortality and increased tumour formation in the absence of IGF-II[93,94].

1.2.3 Function of IGF-II

IGF-II plays an important role in foetal growth and development. However, it does not seem to exert an effect in normal post-natal growth.

The IGF-II gene expresses many different transcripts in several different tissues in the embryo. Human foetal IGF-II levels are stable from 23 weeks gestation until term, except for an increase at 30-34 weeks. At term, the level of IGF-II is equal in the foetal serum and the amniotic fluid. This level is lower than in the maternal serum[14].

Disruption of one of the IGF-II alleles in the mouse leads to heterozygous animals which achieve only 60% of the body weight of their normal counterparts[95]. The mice were otherwise normal, and unlike mice lacking IGF-I, they retained their fertility. The IGF-II mutation appeared to exert its effect before embryonic day 16. Mice which are deficient in IGF-II have also been shown to have decreased placental growth[96].

Mice which overexpress IGF-II and have a 2-3-fold higher concentration of serum IGF-II, show little change in weight or length compared with wild-type controls[97]. Despite not having an effect on growth in general, IGF-II does lead to an increased growth in some specific tissues such as in the skin and the uterus[98].

IGF-II appears to exert most of its biological actions via binding to the IGF-I receptor. It also binds to the IGF-II/mannose-6-phosphate receptor, but as yet no intracellular signalling pathway resulting from this interaction has been elucidated.

Like IGF-I, IGF-II circulates bound to binding proteins. As well as this it may also be bound to the soluble component of the IGF-II receptor.

The exact role of IGF-II in the adult remains unclear.

1.2.4 IGF-II and malignancy

Many tumours express high levels of IGF-II mRNA. These include Wilm's tumour (nephroblastoma)[99], rhabdomyosarcoma, hepatocellular carcinoma[100], phaeochromocytomas[101], liposarcoma[102], leiomyoma, leiomyosarcoma[103], breast cancer[104] and colon cancer[102].

Some mesenchymal tumours secrete excessive quantities of the pre-peptide form of IGF-II which is also known as 'big IGF-II'[103]. 'Big IGF-II' does not readily form a ternary complex with IGF binding protein-3 and ALS, and as a result leads to increased levels of free IGF-II. Increased levels of IGF-II in the circulation inhibit insulin and growth hormone secretion via a negative feedback mechanism. This in turn leads to a decrease in the amount of IGF-I, IGF binding protein-3 and acid labile subunit (ALS) produced.

Conditioned media from the HT29 colon cancer cell line was shown to have high concentrations of IGF-II, with 64% of it being in the high molecular weight form. The high concentration of IGF-II present does not seem to be related to gene over-expession, rather to the particular mRNA species transcribed. It may be that only certain mRNA transcripts are able to lead to protein production[91].

IGF-II has been shown to stimulate proliferation in 5 out of 8 human colon cancer cell lines tested. A concentration of 30ng/ml IGF-II led to a 3-fold increase in cell number. The increase in growth was equal to that seen by the addition of IGF-I[43]. This effect was dependent on the cell density, with the greatest effect occurring at low cell density and no effect at higher cell concentrations.

Out of 20 colon cancers examined, 40% had a 10-50-fold increase in IGF-II mRNA compared with the normal colon[102]. The colon cancers tested secreted IGF-II. The majority of this is in the pro-peptide form which is 15 kDa in size. 40% of IGF-II produced was in the mature peptide form of 7kDa.

In one study, IGF-II mRNA was found in all normal colonic epithelium and all malignant colonic samples tested[44]. However, the expression of IGF-II mRNA was 40 times greater in colon cancer than in the adjacent normal epithelium. The concentration of the IGF-II peptide in the malignant tissue was twice as great as in the normal tissue. This corroborates research from other workers in this field who have found that IGF-II is overexpressed in 30% of colorectal cancers tested. They divided their results into a moderate increase (2-15-fold) and a very marked increase (200-800-fold) [102,106].

However, in a different study looking at the difference in IGF-II between normal and malignant colon, it was found that IGF-II mRNA was not present in any of the normal samples. They detected IGF-II mRNA in 3 out of 10 malignant tissues. Interestingly, immunohistochemistry was negative for IGF-II protein in the samples positive for IGF-II mRNA[107].

IGF-II is regulated in part by growth hormone. The HT29 colon cancer cell line showed a reduction in proliferation and a 40% decrease in IGF-II production after being exposed to growth hormone releasing hormone (GHRH) antagonists. Mice with colon cancer xenografts were also treated with GHRH antagonists[108]. Although this did not alter the serum IGF-II levels, it did lead to a significant decrease in IGF-II mRNA expression and a decreased amount of IGF-II in the tumours. It also led to a decrease in tumour volume and weight. The decrease in tumour size was related to a decrease in cellular proliferation, as well as an increase in apoptosis.

Levels of IGF-II mRNA were found to be higher in tumours of the rectum and recto-sigmoid than in tumours from the rest of the colon. 60% of rectal and 50% of rectosigmoid tumours had elevated levels. Moreover, within this group, the majority of rectal/rectosigmoid tumours which had enhanced mRNA levels were of Dukes' C stage, and those which had normal levels were Dukes' B[102].

1.3 Insulin-like growth factor Type I Receptor

1.3.1 Structure of the IGF-I Receptor

The IGF-I receptor precursor has a predicted length of 1367 amino acids. A 30 residue signal peptide is removed from this to form the unmodified pro-receptor polypeptide. Cleavage of this leads to the formation of the α and β subunits.

The IGF-I receptor is a cell surface receptor that has a 70% homology with the insulin receptor[109]. It is a member of the tyrosine kinase family. It is a heterotetramic glycoprotein that contains the following components:
Two α subunits
Two β subunits

These are joined by disulfide bonds.

The α subunits of 132kDa are extracellular and bind the ligand (Insulin, IGF-I or IGF-II) [110]. The β subunits, which are 90-100kDa in size span the cell membrane and anchor the receptor to the cell. Most importantly the β subunits contain the tyrosine kinase activity as well as ATP binding sites. The tyrosine kinase domain has been shown to be important in the activity of other growth factors, such as epidermal growth factor receptor, PDGF receptor and the insulin receptor[3,111].

The IGF-I receptor is activated by the binding of IGF-I and IGF-II, and also by insulin at supra-physiological concentrations[112]. It binds IGF-I with high affinity and IGF-II with slightly lower affinity[110].

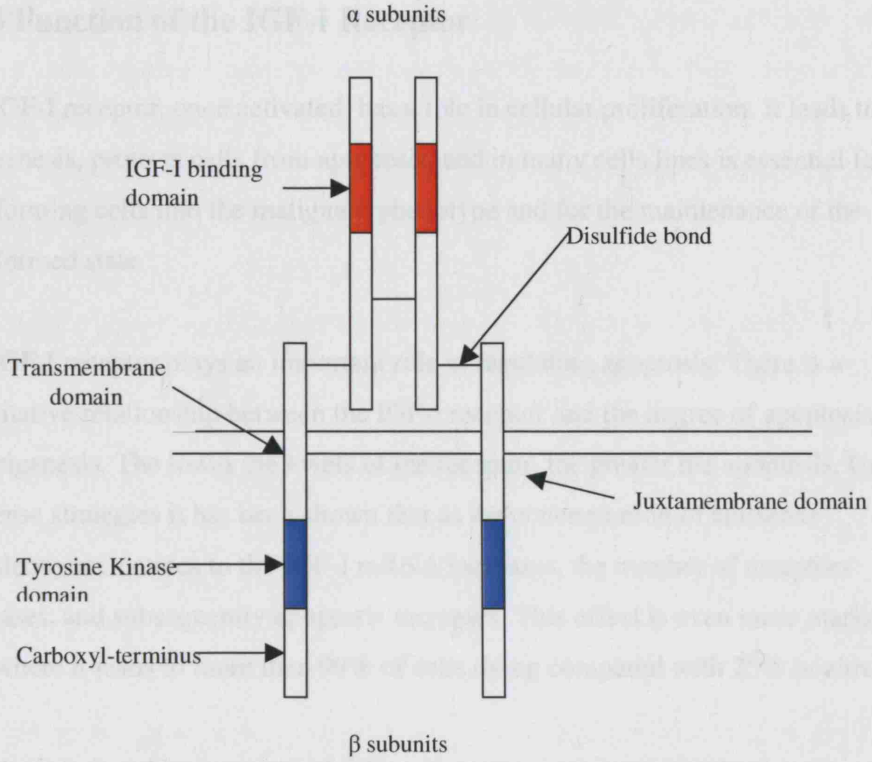


Figure 5 The structure of the IGF-I receptor (adapted from LeRoith 1992[111])

1.3.2 Signal Transduction

When the ligand binds to the α subunit of the IGF-I receptor, it leads to a conformational change which facilitates autophosporylation of the β subunits[111]. This autophosphorylation occurs as a transphosphorylation between the 2 β subunits of the same receptor[113]. This leads to the activation of the tyrosine kinase activity of the receptor. This in turn initiates a cascade of phosphorylations culminating in the activation of serine/threonine kinases.

Activation of the IGF-I receptor leads to the transmission of a signal to its two main substrates, namely insulin receptor substrate 1 (IRS-1) and *Shc*[112]. Both of these two proteins have a common signal transduction pathway acting via *ras* and *raf*[114,115]. The mitogen activated protein kinase cascade is also involved[116].

The effect of the activation of the receptor depends on the type of cell involved[71].

1.3.3 Function of the IGF-I Receptor

The IGF-I receptor, once activated, has a role in cellular proliferation. It leads to mitogenesis, protects cells from apoptosis, and in many cells lines is essential for transforming cells into the malignant phenotype and for the maintenance of the transformed state.

The IGF-I receptor plays an important role in regulating apoptosis. There is a quantitative relationship between the IGF-I receptor and the degree of apoptosis and tumorigenesis. The lower the levels of the receptor, the greater the apoptosis. Using antisense strategies it has been shown that as the concentration of antisense oligodeoxynucleotides to the IGF-I mRNA increases, the number of receptors decreases, and subsequently apoptosis increases. This effect is even more marked *in vivo* where it leads to more than 99% of cells dying compared with 25% *in vitro*[117].

Apoptosis induced by a variety of different agents, such as chemotherapeutic agents can be inhibited by the addition of IGF-I which then activates the type I receptor[17]. These agents include 5-fluorouracil and methotrexate which are both anti-metabolites, tamoxifen which is an anti-oestrogen and campothecin which is a topoisomerase 1 inhibitor.

Activation of the IGF-I receptor by IGF-I can inhibit apoptosis induced by TNFα[1,118] and p53[119]. p53 acts to decrease IGF-I receptor expression and tyrosine kinase phosphorylation of the receptor[120].

If the IGF-I receptor is impaired by antisense strategies or dominant negative mutants, then malignant cells *in vivo* undergo massive apoptosis and tumour growth and metastasis formation is halted[121].

1.3.4 The IGF-I receptor and malignancy

The IGF-I receptor has been found to be present in all of 12 colorectal cancer cell lines tested[122]. A monoclonal antibody against the IGF-I receptor inhibited

proliferation in 7 of these cell lines. The degree of inhibition was related to the diffentiation of the cell line. The moderately/well differentiated colon cancer cell lines had 85% inhibition with the antibody, whereas the poorly differentiated ones had 20% inhibition. This suggests that IGF-I may also play a role in the differentiation of malignancies. The inhibitory effect could be neutralised by the addition of excess exogenous IGF-I.

In an *in vitro* study it was shown that the majority of colorectal cell lines tested had specific binding of ^{125}I-IGF-I[123]. Half of these cell lines had a significant growth response to IGF-I. They found that the binding site was approximately 300kDa under non-reducing conditions on SDS-PAGE. Under reducing conditions this separated out into two bands of 240 and 130kDa. The 130kDa fraction corresponds to the α subunit of the IGF-I receptor, and the 240kDa fraction probably represents aggregates of this subunit that were incompletely reduced.

IGF-I receptor expression is upregulated in colon cancer[44]. Six patients with colon cancer were compared with 10 normal controls. IGF-I receptor mRNA was present in all the normal samples. However, there was a 2.5-fold increase in mRNA expression in those with colon cancer.

Not only does the IGF-I receptor have a direct effect on malignancy, it also has a bystander effect. Stable expression of a dominant negative mutant of the IGF-I receptor inhibited transformation of cells and inhibited tumour growth in nude mice[121]. It also had a bystander effect whereby when the cells expressing the mutant receptor were co-injected with wild-type tumour cells, it inhibited the growth of the wild-type cells. The mutant receptor that was created had a frameshift mutation that resulted in a stop codon at residue 486. The mutant receptor does not have a transmembrane region therefore it can be secreted into the surrounding medium. In all 5 cell lines tested with this mutant receptor there was a marked inhibition of growth of between 75-85%. When the same receptor was tested *in vivo* in a nude mouse model, the tumours expressing the mutant receptor showed a marked delay in the appearance of the tumour compared with controls and grew smaller tumours, with some mice never developing tumours.

The same mutant receptor as above, 486/STOP, was transfected into a metastatic breast cancer cell line. The soluble receptor was secreted extracellularly and was shown to have a bystander effect. It led to a marked inhibition of adhesion to laminin and collagen, and inhibited invasion through type IV collagen.

1.4 Insulin-like growth factor Type II Receptor

1.4.1 Structure of the IGF-II Receptor

The IGF-II receptor is a single chain polypeptide[124]. It is a different class of receptor to the insulin and the IGF-I receptors.

The gene for the receptor is on chromosome 6q24-q27[125]. The IGF-II receptor is highly conserved between species with an approximately 80% identity between human, bovine, rat and mouse receptors[126].

The IGF-II receptor consists of:
A large extra-cellular domain
A single transmembrane region
A small cytoplasmic domain

None of these domains share homology with either the insulin or IGF-I receptors. It has no tyrosine kinase activity and antibodies to the insulin and IGF-I receptors do not cross react with it[127].

93% of the receptor consists of the extra-cellular domain[128] which contains 15 contiguous segments of repeat sequences of approximately 150 amino acids each[3], totalling 2264 amino acids[8]. The transmembrane region consists of 23 amino acids and the cytoplasmic domain is 164 amino acids.

The extra-cellular domain binds the ligand and the intracellular part regulates movement between different cellular compartments.

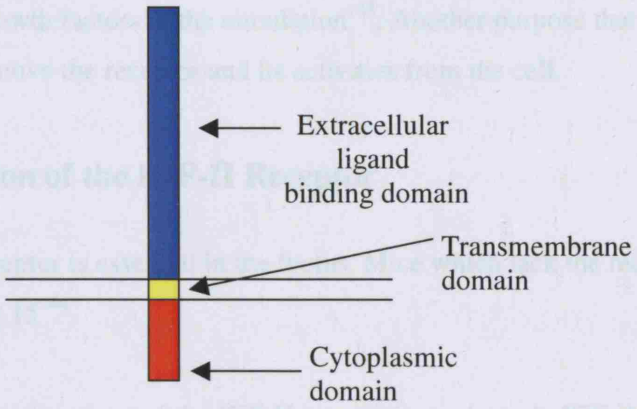


Figure 6 The structure of the IGF-II receptor (adapted from Stewart 1996[8])

The IGF-II receptor binds IGF-II with high affinity[127] and IGF-I with between 100-1000 times lower affinity[3]. Unlike the IGF-I receptor, it does not bind insulin even at the highest concentrations[111].

The IGF-II receptor is identical to the cation-independent mannose-6-phosphate receptor which has a role in the transport of lysosomal enzymes to the lysosome[129,130]. The binding sites for IGF-II and mannose-6-phosphate are different[131]. There is one binding site for IGF-II on the receptor and two sites for the binding of ligands containing mannose-6-phosphate[132]. The intracellular domain has sites for the sorting of lysosomal enzymes into different intracellular compartments[133,134], endocytosis of extracellular lysosomes and activation of GTP binding proteins[135,136]. The carboxyl terminal is the site for the sorting of the lysosomal enzymes. In the Golgi body it binds the newly synthesised lysosomal enzymes and carries them to a pre-lysosomal/endosomal compartment[132,137].

The IGF-II receptor is 220kDa on SDS-PAGE in non-reducing conditions and 240kDa under reducing conditions[3]. Non-glycosylated, the mature peptide is 270kDa[138]. The mature human protein is 2451 amino acids long[8].

The IGF-II receptor is membrane bound, however, the extra-cellular portion of it has been found in serum and conditioned media[139-141]. This arises by proteolysis of the receptor[142]. The soluble part of the receptor acts as a binding protein and transports

Insulin-like growth factors in the circulation[143]. Another purpose that this proteolysis serves is to remove the receptor and its activities from the cell.

1.4.2 Function of the IGF-II Receptor

The IGF-II receptor is essential in the foetus. Mice which lack the receptor die at embryonic day 15[144].

One of the main functions of the IGF-II receptor is to degrade IGF-II via receptor mediated internalisation of the ligand. Mouse embryos which lack the IGF-II receptor have increased levels of serum IGF-II and increased somatic growth in the foetal period.

The IGF-II receptor is coupled to a calcium channel by a G-protein. This leads to IGF-II stimulation of calcium influx in cell culture[131] for example in the competent Balb/c3T3 cell line[145].

The IGF-II receptor has anabolic properties and has been shown to stimulate glycogen synthesis in a human hepatoma cell line[146].

It is also involved in inositol triphosphate formation in the basolateral membrane of proximal tubular kidney cells[147] and cell proliferation in a human erythroleukaemia cell line which possesses IGF-II and insulin receptors but no IGF-I receptors[148].

Most of the effects of IGF-II are mediated via the IGF-I receptor, but in some cell types these effects may take place through the IGF-II receptor[3].

The IGF-II receptor has a role in the transport of lysosomal enzymes from their site of synthesis into an endosomal/pre-lysosomal compartment

IGF-II plays a role in the regulation of other mannose-6-phosphate proteins. It is involved in the uptake of thyroglobulin after it has been secreted by the thyroid follicular cells and in its subsequent degradation in lysosomes[149].

IGF-II receptor also binds the latent form of TGFβ1[8].

The IGF-II receptor appears to have a role in malignancy. All normal colon tissue examined contained the IGF-II receptor, but the was a 4-fold increased expression of IGF-II receptor in malignant tissue[44].

The role of the IGF-II receptor in growth factor signalling remains unclear[8].

1.5 Insulin-like growth factor binding proteins

Six binding proteins have been identified to date. They are able to bind both IGF-I and IGF-II. They act as a reservoir for Insulin-like growth factors in the circulation, prolong the half-life of the Insulin-like growth factors, and carry them to the target organ. The concentration of binding proteins in the circulation affects the transport and bioavailability of the Insulin-like growth factors.

Approximately 99% of IGFs are bound to binding proteins. More than 75% of IGFs circulate in a ternary complex composed of the Insulin-like growth factor, IGFBP-3 and acid labile subunit (ALS). The acid labile subunit binds to the C-terminal domain of IGFBP-3. IGFBP-5, once it has bound IGF-I also has the ability to form a 130kDa ternary complex with ALS. However, IGFBP-5 has only half the affinity for ALS that IGFBP-3 has[150,151].

IGFBP-3 is the largest of the binding proteins and is not able to traverse the capillary membrane.The other binding proteins are able to cross the capillary membrane due to their smaller size[150].

Different factors affect the affinity of the binding proteins for Insulin-like growth factors, including for binding proteins 1, 3 and 5 the degree of serine phosphorlylation of the central domain. This in turn regulates the activity of IGF-I[152]. The majority of IGFBP-3 is highly phosphorylated and therefore has a very high affinity for IGF-I, allowing less IGF-I to be free in the circulation to exert its effects. IGFBP-1 can act to either potentiate or inhibit the effects of IGF-I. When non-phosphorylated it potentiates the effects of IGF-I in some tissues, but when it

becomes phosphorylated the increased affinity it has for IGF-I converts it into an inhibitory protein.

1.5.1 IGF binding proteins and malignancy

The relative concentrations of the IGF binding proteins plays a part in some malignancies. High levels of IGFBP-1 are associated with a lower risk of the development of colorectal cancer as are high levels of binding protein-2.

Insulin inhibits the production of binding proteins 1 and 2[42]. Epidemiological studies show that one of the risk factors for colorectal cancer is obesity. People who are overweight develop insulin resistance and as a result the pancreas produces even greater amounts of insulin. This excess insulin inhibits the production of binding proteins 1 and 2, and this may be a mechanism whereby obesity increases the risk of colorectal cancer.

Cultured rat smooth muscle cells synthesise binding proteins 3, 4, and 5. Intestinal inflammation, such as a rat model of colitis, showed a 100% increase in the production of binding proteins 4 and 5[110]. The increase in binding protein-5 occurred specifically in the smooth muscle layer of the colon and the increase in binding protein-4 was in the lamina propria, the submucosa and in the smooth muscle. Binding protein-3 was localised to the lamina propria but its expression remained the same in inflammation.

R3-IGF-I is an IGF-I agonist which is not able to be bound, and therefore not modulated by the binding proteins. In cultured human intestinal smooth muscle cells, the addition of R3-IGF-I led to an increase in cell proliferation. It also led to upregulation of binding proteins 3, 4 and 5[153]. In the same model, the addition of an antagonist to the IGF-I receptor led to a decrease in proliferation and a decrease in the same binding proteins. This shows that endogenous IGF-I has an important role in the regulation of binding proteins[154,155]. Cultured human osteoblast-like cells produce an IGFBP-4 protease that requires the presence of IGF-I to exert its proteolytic activity[154].

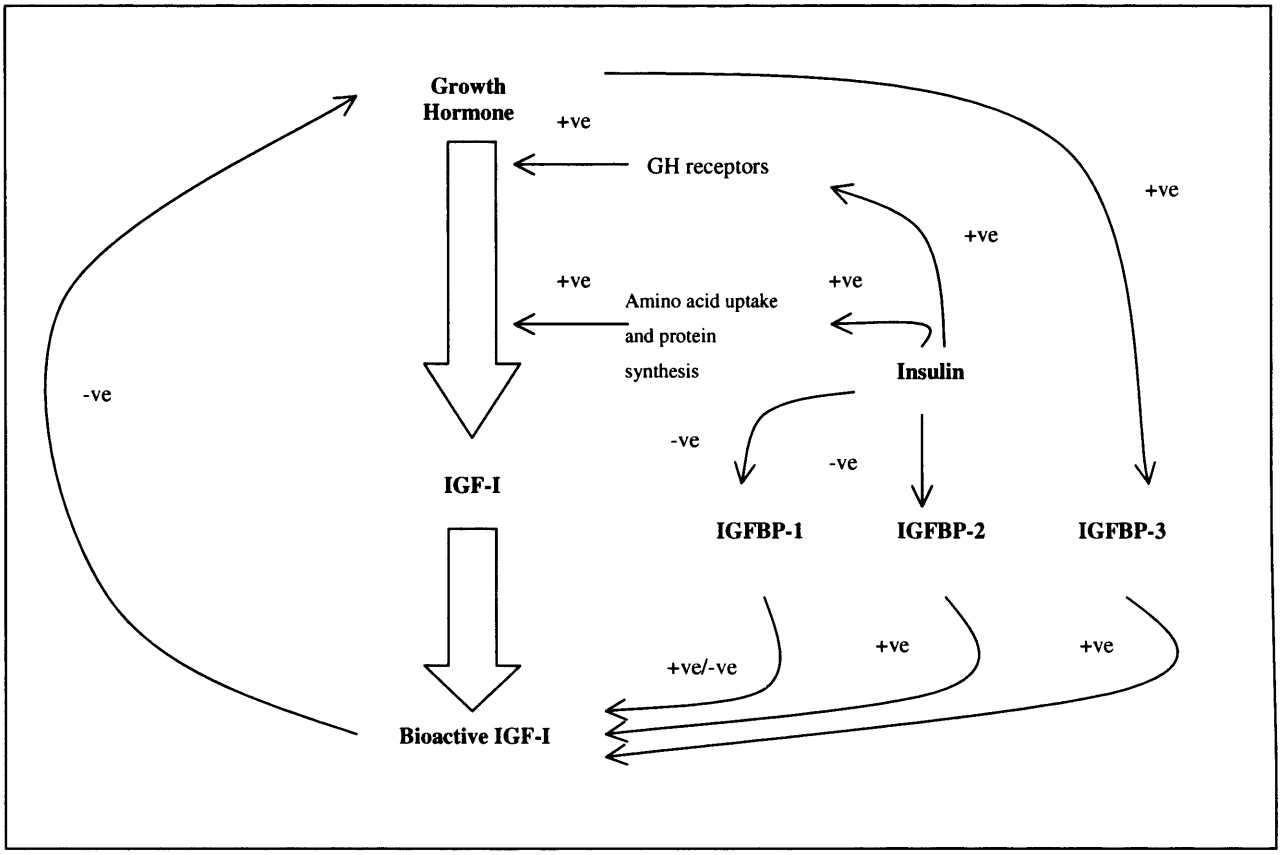


Figure 7 Regulation of bioactive IGF-I by Growth Hormone and Insulin (adapted from Kaaks 2000[42])

The effects of the binding proteins relate to the specific binding protein involved, and is dependent on the tissue in which it is acting[153].

Binding proteins can act in two ways[153,155]. They may be:
IGF dependent
IGF independent

Binding proteins regulate IGF-I dependent growth. At the same time IGF-I regulated proteolysis has been found for binding proteins 2, 3, 4 and 5. Therefore the two components regulate each other[79,154,156].

Vascular smooth muscle cells increase the production of IGFBP-4 with the addition of IGF-I and at the same time IGF-I decreases the activity of an IGFBP-4 protease[156].

In human osteoblast-like cells, endogenous IGF-I activates IGFBP-4 proteolysis and therefore limits its activity in the micorenvironment[154].

In human intestinal smooth muscle cells, IGF-I induced growth is inhibited by binding proteins 3 and 4 and increased by binding protein 5[157].

IGFBP-5 has been shown to stimulate mitogenesis in mouse osteoblast-like cells in the absence of both exogenous and endogenous IGFs[155].

1.5.2 Insulin-like growth factor binding protein-4

1.5.2.1 Structure of IGFBP-4

The IGFBP-4 gene spans 13.3kb and has 4 exons[158].

IGFBP-4 has two molecular forms – a 28kDa glycosylated form and a 24kDa non-glycosylated one. The glycosylation of IGFBP-4 does not seem to affect its affinity for IGF-I[159].

It was initially purified from the conditioned medium of human bone cells[160]. It exists as a soluble extracellular peptide and is not membrane associated.

In the rat, the main source of IGFBP-4 is the liver, but it is also synthesised by osteoblasts, the colon, smooth and striated muscle, the lung and the heart.

It is expressed as a precursor protein and has a single asparagine-linked glycosylation site.

Most IGF binding proteins have 18 highly conserved cysteines, but IGFBP-4 has two extra ones. The linkage of these cysteine residues plays an important part in the protein folding and therefore its activity[159].

Binding proteins 1-6 have cysteine rich N and C terminal domains. These are connected by a non-conserved central portion. In IGFBP-4 the N-terminal fragment has a high affinity for IGF-I and the C-terminal region has a low affinity[161]. Residues

72-91 in the N-terminal region are essential for the binding of IGF-I[162]. The IGF binding domain in IGFBP-4 contains a hydrophobic motif (Leu72-Met80) in the distal part of the N-terminal domain[163].

The cDNA for IGFBP-4 predicts a 237 amino acid mature peptide[164].

1.5.2.2 Function of IGFBP-4

IGFBP-4 is often thought of as being a protein that is purely inhibitory to IGF-I mediated growth. Although this is often true, it is not necessarily always the case.

The local administration of IGFBP-4 over mouse parietal bone blocked the growth inducing effects of local IGF-I[165]. When a fragment of IGFBP-4 which had a 50-100-fold decreased affinity for IGF-I was used there was no effect. In contrast when IGFBP-4 was injected systemically, it actually increased bone formation (as measured by alkaline phosphatase activity). A single injection of 200μg of IGFBP-4 led to a 50% increase in alkaline phospatase at 24 hours, and this remained elevated for 5 days. Subcutaneous injections of IGFBP-4 had no effect on co-injected IGF-I. This study shows that different effects can be gained from IGFBP-4 depending on whether it is administered locally or systemically.

IGF binding protein-4 competes with the IGF-I receptor for the binding of Insulin-like growth factors. Co-incubation of IGFBP-4 with ^{125}I-IGF-I and ^{125}I-IGF-II decreased the binding of both Insulin-like growth factors to the IGF-I receptor in a dose dependent manner[166].

Approximately 25% of Insulin-like growth factors circulate bound to binding proteins other than binding protein-3. These other binding proteins form an approximately 50kDa complex which is small enough to cross the vascular endothelial barrier and enter the tissues. It is possible that by injecting IGFBP-4 systemically, it decreases the proportion of IGF-I that is bound to binding protein-3 and increases the proportion bound to IGFBP-4. As a result more IGF-I is carried through the capillary membrane into the tissues. Once there, the IGFBP-4/IGF-I complex undergoes proteolysis and IGF-I can exert its effect.

Another hypothesis is that the injection of IGFBP-4 leads to a decrease in the proportion of free IGF-I. This may lead to a subsequent increase in growth hormone production via a negative feedback mechanism, and hence a rise in the concentration of IGF-I.

IGF-I is a mitogen in a variety of different tissues, including smooth muscle. It is said to have a role in bladder hypertrophy and arterial restenosis after angioplasty[25]. IGFBP-4 is the most abundant binding protein produced in rodent arterial wall. Overexpression of IGFBP-4 leads to muscle hypoplasia which is the converse effect of IGF-I.

In rat serum, binding protein-3 is the most abundant binding protein, followed by IGFBP-4[167]. The main site of production of IGFBP-4 is the liver. Partial hepatectomy in the rat leads to an increase in IGFBP-4 synthesis during the hepatic regeneration period. There is an increase in IGFBP-4 mRNA 6-12 hours after partial hepatectomy, and serum levels increase at 12-24 hours. There is also an increase in renal IGFBP-4 expression. The increase in IGFBP-4 may act to prolong the effect of IGF-I during the regeneration period. Another possibility is that IGFBP-4 is acting independently of IGF-I.

1.5.2.3 Regulation of IGFBP-4

The mode by which IGFBP-4 is regulated varies in different tissues. In general, IGFBP-4 is regulated by hormonal, nutritional and developmental factors that act at the gene expression or the protein synthesis level[167]. In bone, parathyroid hormone and cAMP lead to an increased expression of IGFBP-4 mRNA[164]. The steroid dexamethasone in the absence of IGF-I leads to a down-regulation of IGFBP-4 in fibroblasts[168].

The mean serum value of IGFBP-4 in men is 189μg/l and in women it is 193μg/l[160]. Growth hormone deficiency and acromegaly have no effect on IGFBP-4 levels. Serum IGFBP-4 levels are inversely related to the glomerular filtration rate.

The bodily fluids with the highest concentration of IGFBP-4 are amniotic fluid (391-717μg/l) and follicular fluid (249-500μg/l).

1.5.2.4 IGFBP-4 and malignancy

Most colon cancers express IGF binding proteins 2 and 4. Less than half express binding proteins 3, 5 or 6[169]. The expression of binding proteins may distinguish those cell lines which are responsive to the growth effects of IGFs.

1.5.3 Insulin-like growth factor binding protein-1

The gene for IGFBP-1 is located on chromosome 7p12-p14 and spans 5.2kilobases. The protein product is a 25kDa non-glycosylated protein. The mature protein is 234 amino acids long[3,170]. The COOH terminus has an Arginine-Glycine-Aspartic acid (RGD) sequence which is common in proteins which bind to cell surface receptors such as integrins[171]. IGFBP-1 binds IGF-I and IGF-II with equal affinity. Due to its small size it has the ability to traverse the capillary membrane.

IGFBP-1 synthesis is inhibited by insulin, glucose by a non-insulin dependent pathway, and growth hormone[57,172,173]. IGFBP-1 has the ability to inhibit IGF-I and growth hormone production. People with diabetes mellitus have a 2-4-fold increase in IGFBP-1 than the general population. Treatment with insulin brings IGFBP-1 back to the normal range[172,174]. Overnight fasting leads to an increase in IGFBP-1 by 3.5-12-fold. Growth hormone deficient people have raised levels of IGFBP-1[175]. Acromegalics have low levels of IGFBP-1. Octreotide leads to an increase in IGFBP-1 and the suppression of growth hormone and IGF-I[176].

IGFBP-1 is expressed in most tissues in the foetus and was first purified from amniotic fluid. IGFBP-1 is expressed mainly in the liver in the adult but also in the endometrium and the ovaries[173].

Octreotide, a synthetic somatostatin analogue, stimulates the synthesis of IGFBP-1 independently of insulin and growth hormone. A subcutaneous injection of 100μg of octreotide markedly increased IGFBP-1 levels in both normal and growth hormone deficient subjects. Levels remained elevated for 6 hours[57].

IGFBP-1 has been shown to act to both potentiate and inhibit the effects of IGF-I[177-179]. In fibroblasts it increases the proliferative effects of IGF-I. In pelvic cartilage and in chick embryo fibroblasts it has the opposite effect[180].

1.5.4 Insulin-like growth factor binding protein-2

The gene for IGFBP-2 is on chromosome 2q33-44[181]. It is 30 to 35kDa in size on SDS-PAGE in non-reducing conditions. Like IGFBP-1, it has an RGD sequence near the C-terminus. The mature peptide is 289 amino acids long[3]. IGFBP-2 binds IGF-II in preference to IGF-I. IGF-II plays a major role in the foetus, and correspondingly, IGFBP-2 levels are high in foetal tissues.

IGFBP-2 was originally purified from a rat liver cell line[182] and has been found to be present in many other tissues including human malignant cell lines[183,184].

There is a 4-8-fold increase in IGFBP-2 mRNA in colon cancer compared with normal colon. The increase is related to Dukes' staging, with the greatest levels being seen in Dukes' C[182]. The increase in IGFBP-2 has been localised to the malignant epithelial cells rather than the surrounding stroma, thereby suggesting a possible autocrine role for IGFBP-2.

Western blotting of both normal and malignant colonic tissue reveals bands at 22 and 30kDa, although at much higher levels in the malignant tissue. These represent proteolytic fragments of IGFBP-2.

Another study found that IGFBP-2 mRNA was present in all normal and malignant colonic tissue samples examined. However, immunohistochemistry revealed IGFBP-2 to be present in only 2 out of 10 malignant samples but in 7 out of 10 normal samples, and western blotting showed IGFBP-2 in 3 out of 10 malignant samples and 8 out of 10 normal samples. Extracts were then made from both malignant and normal tissue, and it was found that the malignant extract had the ability to degrade IGFBP-2 but the normal extract could not[107]. This shows that although both normal and malignant colon produce IGFBP-2, only the malignant cells are able to degrade it by proteolysis. This may confer a growth advantage to the malignant cells.

1.5.5 Insulin-like growth factor binding protein-3

The gene for IGFBP-3 is on chromosome 7 and is contiguous with the gene for IGFBP-1[3]. IGFBP-3 has a 30% homology with IGFBP-1[185].

IGFBP-3 is the most abundant binding protein in the circulation. Approximately 75% of Insulin-like growth factors circulate in a 150kDa trimeric complex composed of Insulin-like growth factors, IGFBP-3 and acid labile subunit (ALS)[186]. IGFBP-3 is 40-45kDa and ALS is 85kDa in size. Differential glycosylation of IGFBP-3 leads to 2 bands on SDS-PAGE of 41.5 and 38.5kDa[3]. The non-glycosylated form is 28.7kDa. ALS itself has no intrinsic binding capacity for IGFs and has no RGD sequence. It is thought that IGF and IGFBP-3 initially bind to each other, and then ALS binds the binary complex[187,188]. However, IGFBP-3 and ALS have been shown to bind to each other in the absence of IGFs[186] in adult rat serum *in vitro*.

The majority of IGFBP-3 is produced in the liver under the stimulus of growth hormone. This explains why acromegalics who have high levels of IGF-I also have high levels of IGFBP-3. Therefore, although they have higher levels of colorectal cancer compared with the general population, it is not as high as would be expected looking at the IGF-I levels alone.

IGF-I also affects the levels of circulating IGFBP-3. In human neonatal fibroblasts, the addition of IGF-I to the cells led to an increase in extracellular IGFBP-3 and a decrease in cell-associated IGFBP-3[69]. The IGF-I binds the cell associated IGFBP-3 and then releases it into the extracellular space.

The ternary complex forms a reservoir of IGFs in the circulation and limits its biological activity which is related to its free concentration. IGFBP-3 levels correlate with IGF-II levels and are inversely correlated with age[41]. IGFBP-3 levels peak at puberty and subsequently decrease with age.

IGFBP-3 has been found in the conditioned medium of many different cell types, for example, vascular endothelial cells[189], fibroblasts[190,191], chondrocytes, osteoblasts, and breast cancer cells[72,192,193].

IGFBP-3 has been shown to inhibit the proliferative effects of IGF-I in many different systems. IGFBP-3 sequesters IGF-I and competes for binding to the IGF-I receptor. IGFBP-3 is as potent as IGF-I at displacing the truncated IGF-I analogue ^{125}I-des(1-3)IGF-I from binding to the IGF-I receptor[194]. Proteolytic fragments of IGFBP-3 have no effect. It is possible that the binding of IGFBP-3 to the receptor leads to a conformational change which decreases the affinity of the receptor for IGF-I or even inhibits its binding.

It also exerts some of its effects via IGF-independent mechanisms[70,195]. Many cells including some breast and prostate cancer cell lines have specific cell surface binding sites for IGFBP-3. Binding of free IGFBP-3 to these sites inhibits cell growth *in vitro*[70 196,197].

IGFBP-3 is involved in the mechanism of p53 action[198]. The activation of p53 leads to the induction of IGFBP-3 transcripts as well as regulating IGF-I receptor levels. TGFβ increases synthesis of IGFBP-3[69] which has been shown to mediate the apoptotic effect of TGFβ1 in a prostate cancer cell line[70].

Anti-oestrogens, such as tamoxifen which is used in the treatment of breast cancer, upregulate IGFBP-3 as well as IGFBP-5[199,200], and patients with colorectal cancer have been shown to have lower levels of IGFBP-3 than controls[41].

1.5.6 Insulin-like growth factor binding protein-5

The gene for IGFBP-5 is located on chromosome 5. The mature peptide is 28kDa in size. IGFBP-5 mRNA is present in most tissues[201].

IGFBP-5 potentiates the effect of IGF-I[29,202,203].

IGFBP-5 exerts its effect by binding to the extracellular matrix and increasing the local concentration of IGF-I adjacent to the cells[203].

The highest levels of IGFBP-5 mRNA have been detected in the kidney. Levels are also high in the intestine and stomach. The lowest levels are in the liver[201].

1.5.7 Insulin-like growth factor binding protein-6

The gene for IGFBP-6 is on chromosome 12. It is 216 amino acids long and 22.8kDa in size[3]. It binds IGF-II with a 10-fold greater affinity than it binds IGF-I.

1.6 Insulin-like growth factor binding protein proteases

Several IGFBP specific proteases exist. Some are present in the circulation, but others are tissue specific. Prostate specific antigen (PSA) is a kallikrein enzyme with specificity for IGFBP-3[3,204,205].

Protease activity varies during different stages of life, for example, increased proteolysis of IGF binding proteins occurs during pregnancy and this increases bioactive IGF-I[206].

1.6.1 IGFBP-4 protease

IGF-I leads to a concentration dependent activation of IGFBP-4 protease in human intestinal smooth muscle cells. Protease activity is highest in proliferating cells and lowest in post-confluent cells. IGF-I antagonists inhibit IGF-I activated IGFBP-4 proteolysis. Proteolysis leads to the production of 18 and 14kDa fragments. IGFBP-4 proteolysis is inhibited by EDTA which is a cation dependent protease inhibitor. IGFBP-4 protease is likely to be a divalent cation-dependent member of the serine proteases[207].

The IGFBP-4 protease produced by fibroblasts has been found to be the same as pregnancy-associated plasma protein-A (PAPP-A)[206]. This protease has been shown to act in both an IGF-II dependent and independent manner. IGFBP-4 was unable to

inhibit the growth of an osteosarcoma line in the presence of pregnancy serum alone, or with pregnancy serum with IGF-II. However, a protease resistant form of IGFBP-4 was able to inhibit proliferation.

The abundance of proteases in pregnancy serum is related to the fact that as the foetus needs to grow, it needs high concentrations of Insulin-like growth factors. Therefore the more protease there is, the more Insulin-like growth factors can be released from their binding proteins.

PAPP-A circulates as two subunits of 200kDa. These subunits have a disulfide bond to two mutually disulfide bridged 50-90kDa pro-forms of eosinophil major basic protein (pro MBP) subunits. The total complex is 400kDa in size and is present in pregnancy serum from 4-6 weeks after conception. It increases in concentration until term and then decreases after birth.

Decidual cells also have increased IGFBP-4 proteolysis with the addition of IGF-I. There is no change in IGFBP-4 mRNA expression. It is possible that when IGF-I binds IGFBP-4 there is a conformational change that makes it more susceptible to proteolysis.

1.7 Summary

The Insulin-like growth factor system consists of IGF-I, IGF-II, the type I and type II receptors and a family of binding proteins and proteases. IGF-I has 70% homology with IGF-II and both mediate their biological actions via the IGF-I receptor. Both of these growth factors are vital for normal foetal growth and development.

In the adult, IGF-I has powerful anabolic and mitogenic actions. Its expression is mainly under the control of growth hormone, but many other factors also play a role in its regulation.

Increased IGF-I levels are associated with an increased risk of certain malignancies such as those of the colon, breast, prostate and lung. IGF-I leads to an increase in colonic epithelial proliferation, and higher serum levels of IGF-I are associated with increased risk of developing adenocarcinoma of the colon. Somatostatin analogues which inhibit IGF-I expression have been shown to increase patient survival in advanced gastro-intestinal malignancies. Increased IGF-I levels are associated with breast cancer risk in pre-menopausal women. IGF-II also has a role in malignancy. It leads to epithelial cell proliferation and is overexpressed in some colonic adenocarcinomas.

Insulin-like growth factor binding proteins transport IGFs and help to regulate their bioavailability. Some act to inhibit the actions of IGFs and others act to potentiate it. Binding proteins are in turn regulated by factors such as growth hormone and insulin, and specific proteases are present to degrade them.

The insulin-like growth factor system is intimately linked to normal growth and development in both foetal and post-natal life. If alterations occur to one part of the system, the fine balance of cell proliferation and cell death is lost and the potential exists for excessive proliferation and malignant transformation.

Colorectal cancer is the second commonest fatal malignancy in the United Kingdom with an incidence of approximately 32,000 new cases per year and approximately 17,000 deaths per year[208]. It may be either sporadic or familial, such as with familial

adenomatous polyposis. The development of a colorectal cancer may take several years as it passes through the adenoma-carcinoma sequence as described by Vogelstein.

Clinically the prognosis of a patient with colorectal cancer is determined by Dukes' staging whereby Dukes' A is when the tumour has not invaded the muscularis propria, Dukes' B is when it has breached the muscularis propria, and Dukes' C is where there is lymph node involvement. The overall 1 year survival is 65% and the overall 5-year survival 44%[209].

Cure is dependant on early detection and studies are ongoing examining screening methods for colorectal cancer.

The mainstay of treatment is surgery, although chemotherapy and radiotherapy may also have a role. Chemotherapy regimens based on 5-fluorouracil are commonplace for Dukes' C and high risk Dukes' B colonic adenocarcinomas. Pre- or post-operative radiotherapy may be used for some rectal or rectosigmoid adenocarcinomas.

However, despite the best available treatments, mortality remains high and new methods of treatment need to be developed. Molecular biology using the Insulin-like growth factor system offers a potential target for attacking colorectal cancer.

1.8 Hypothesis

The hypothesis of this thesis is that the IGF system affects the rate of growth of adenocarcinoma of the colon. Furthermore, that by manipulating components of the IGF system, it is possible to effect a change in the rate of growth of adenocarcinoma of the colon *in vitro*.

1.9 Aims of the research project

It is the aim of this thesis to further evaluate the IGF system, in particular IGF-I and IGFBP-4, in a way that may be utilised to develop new modalities of treatment for adenocarcinoma of the colon.

In particular:
To produce a nude mouse model of colon cancer and to define the expression of components of the Insulin-like growth factor system within this model.

To produce a human recombinant IGFBP-4 which may then be used in subsequent experiments.

To examine what effect IGF-I, IGFBP-4 and the IGF-IR has on colon cancer *in vitro*.
To investigate the effect of IGF-I when endogenous IGFBP-4 is blocked.

To investigate IGFBP-4 protease and its regulation.

It is hoped that by discovering more information about the insulin-like growth factor system that it will help work progress into modifying this system *in vivo* to treat patients with adenocarcinoma of the colon.

Chapter 2 Cell Culture Experiments

2.1 Introduction

Normal and malignant cells are under the control of a complex mixture of factors *in vivo* which determine their rate of growth. By incubating cells in serum-free medium, the effect of a single added factor may be determined. Alternatively, a combination of factors may be added to investigate their cumulative effect.

The experiments in this chapter were performed to ascertain the effect of different components of the IGF system on the proliferation of colon cancer cell lines *in vitro*. Included in this set of experiments are those designed to investigate the effect of endogenously produced IGFBP-4. The cell lines used for these experiments were Colo 205, HT 29 and WiDR. These cell lines were chosen as they are representative colon cancer cell lines on which much research has previously been carried out.

2.2 Aims

The experiments with IGF-I were designed to determine what concentration of IGF-I is required for proliferation, and to investigate whether proliferation occurs in a dose-dependent manner.

IGFBP-4 has been shown to be inhibitory in other cell systems, and to see if this was also the case for the cell lines in this study, experiments were performed using exogenously added IGFBP-4.

Subsequently the next experiment was designed to establish whether IGFBP-4 could inhibit the proliferative effects of exogenous IGF-I.

To examine whether endogenous IGFBP-4 has a role in the growth status of the three different colon cancer cell lines, experiments were planned using antibody against human IGFBP-4.

To study whether endogenous IGFBP-4 partly inhibits the proliferative effects of exogenous IGF-I, experiments were carried out using IGFBP-4 antibody in conjunction with IGF-I.

Chemotherapy is now standard treatment for Dukes' C and some Dukes' B colon cancers, and one of the commonly used chemotherapeutic agents is 5-Fluorouracil (5FU). Experiments were designed to ascertain whether IGFBP-4 has a role in augmenting the inhibitory effects of 5FU.

2.3 Methods

2.3.1 Preparation of cells

Colorectal cancer cell lines were purchased from the European Collection of Cell Cultures. They were defrosted and 20mls of the appropriate medium (see appendix 7.1), containing 10% serum and 1% antibiotics, was added in $25cm^3$ flasks. Cells were incubated at 37°C in 5% CO_2. After 48 hours, the medium was extracted, the cells washed twice with 10mls PBS and 20mls of fresh medium was replaced.

2.3.2 Passaging of cells

When the cells were 90% confluent they were passaged. The medium was extracted and the cells washed twice in 10mls PBS. 10mls 0.25% trypsin-EDTA was added to the flask of cells and incubated at 37°C for 5 minutes. The flask was then shaken to release the cells. The trypsinised cells were centrifuged at 900g for 5 minutes. The supernatant was discarded and the pellet of cells resuspended in 10mls of fresh medium. Two millilitres of the cell suspension was replaced into a flask and made up to 20mls with the appropriate medium.

2.3.3 Preparation of frozen cell stocks

100µl of cells at a density of $5x10^6$cells/ml were frozen at -70° in 900µl DMSO.

2.3.4 Cell proliferation assays

Cell proliferation assays were performed using the Alamar Blue reagent system purchased from Serotec. It has an oxidation-reduction indicator which changes colour in response to reduction of the growth medium by proliferating cells.

A cell suspension of $5x10^4$ cells/ml was made in the appropriate medium containing 10% foetal bovine serum and 1% antibiotic(see appendix 7.1). 200µl of the cell suspension (equivalent to $10 x10^3$ cells) was plated out into 96 well plates and incubated at 37°C in 5% CO_2 for 24 hours to allow the cells to adhere to the bottom of the wells. After this period, the medium was removed and the cells washed twice with 75µl PBS. Then 100µl of the appropriate serum-free media containing the test substance was added to each well. The cells were incubated for a further 20 hours. At this point, 10µl of Alamar Blue reagent was added to each well, and the plates incubated for a further 4 hours. The absorbance was read spectrophotometrically on a 96 well plate reader at 570nm and the background absorbance which was measured at 600nm was subtracted from it. The control for each experiment was cells without the test substance. Each experiment also had a lane of wells containing media alone with no cells, and the mean of these readings was subtracted from all the other readings in that experiment. All experiments were performed in triplicate.

2.3.5 Statistical analysis

All results are the pooled data from 3 experiments and each experiment has 6 repeats for each condition. Results were subjected to ANOVA and Least Significant Difference with 95% confidence intervals. Statistical analysis was performed using the UNISTAT program. All results are expressed as a percentage change in proliferation. This relates to the percentage change from the control which is cells grown in serum-free media alone. Results were deemed to be statistically significant when $p<0.05$. Error bars on the graphs represent the standard error of the mean.

2.4 Validation studies for cell culture experiments

2.4.1 Aims

Optimisation assays were performed to define the following parameters:

The optimum initial cell density for the Alamar blue assay

The optimum length of time after the addition of the test substance to the cells, to measure the absorbance

The optimum time after the addition of Alamar blue to measure absorbance

2.4.2 Methods

The cell proliferation assays were carried out according to the protocol in the Methods section above.

2.4.2.1 Optimisation of cell density for use in cell proliferation assays

The WiDR cell line was used at the following cell densities for this experiment:

$4x10^3$

$8x\ 10^3$

$16x10^3$

$32x10^3$

$64x10^3$

2.4.2.2 Optimisation of the duration of incubation of the cells

These experiments were performed to determine the optimum length of time after the addition of the test substance to the cells, to measure the absorbance. The cell line WiDR was used for this experiment. Cells were plated out at the following cell densities:

$4x10^3$

$8x\ 10^3$

$16x10^3$

$32x10^3$

$64x10^3$

The Alamar blue assay was used at 24 hours for the first experiment and at 48 hours for the second experiment.

2.4.2.3 Optimisation of the time from addition of Alamar blue to absorbance measurement

This was based on the manufacturer's protocol which suggested measuring absorbance between 1 and 4 hours after the addition of Alamar blue. To optimise this timepoint, experiments were performed and the absorbance measured at 1, 2, 3 and 4 hours after the addition of Alamar blue.

2.4.3 Results

2.4.3.1 Optimisation of cell density for use in cell proliferation assays

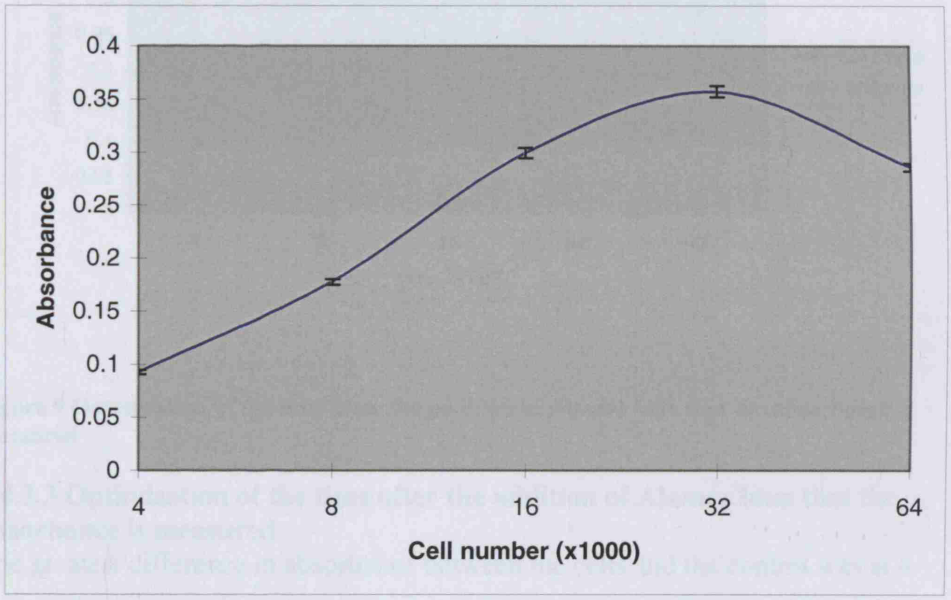


Figure 8 The effect of WiDR cell number on absorbance

As can be seen in figure 8, absorbance is directly proportional to cell number up to an initial cell number of $32x10^3$. Above this level absorbance decreases. It may be that the cell density is too high to permit further growth.After analysing the results from this experiment, it was decided that $10x10^3$ cells would be an appropriate number to plate the cells out at for subsequent experiments. At a lower cell number, if there was a decrease in cell growth, the absorbance would be too low, and at a higher value, an increase in cell growth may lead to an erroneous decrease in absorbance.

2.4.3.2 Optimisation of the duration of incubation of the cells with the test substance

At 24 hours there was a significant difference between the control and the different concentrations of IGF-I added (see figure 9). At 48 hours, at the higher cell densities, the cells were dying. Possible explanations for this are cellular overgrowth, or that the cells were unable to survive for 48 hours in serum-free medium. As a result of this experiment, the timepoint of 24 hours after the addition of the test substance was used for all subsequent experiments.

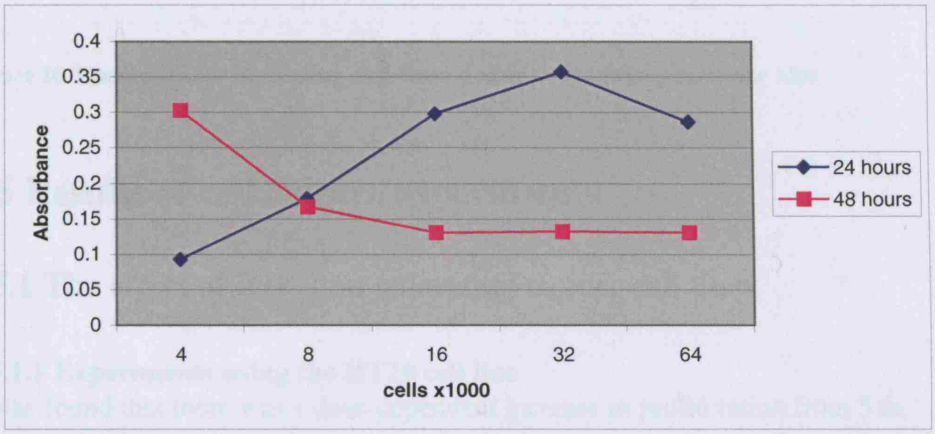


Figure 9 Optimisation of the time after the addition of Alamar blue that the absorbance is measured

2.4.3.3 Optimisation of the time after the addition of Alamar blue that the absorbance is measured

The greatest difference in absorbance between the cells and the control was at 4 hours, therefore this timepoint was used for the following experiments (figure 10).

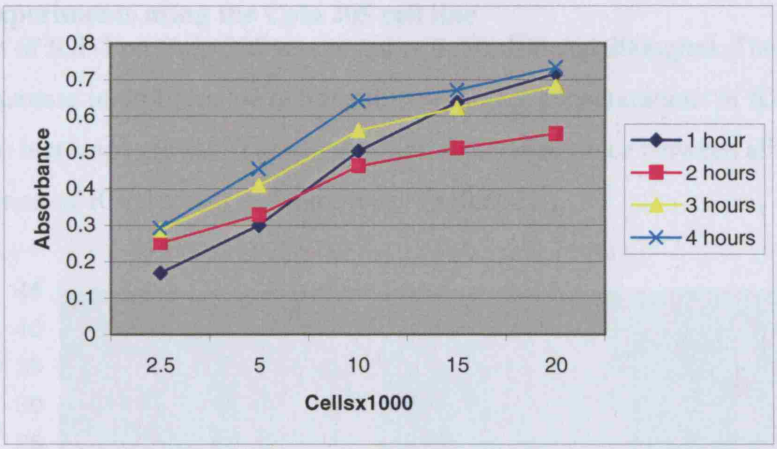


Figure 10 Timepoints for measuring absorbance after the addition of Alamar blue

2.5 Results of cell culture experiments

2.5.1 The effect of IGF-I on colorectal cancer cell lines

2.5.1.1 Experiments using the HT29 cell line

It was found that there was a dose-dependent increase in proliferation from 5 to 50ng/ml IGF-I. There was a slight decrease in proliferation at 100ng/ml compared with 25 and 50ng/ml. There was a significant difference between all concentrations and the control of 0ng/ml IGF-I (p=0.0163).

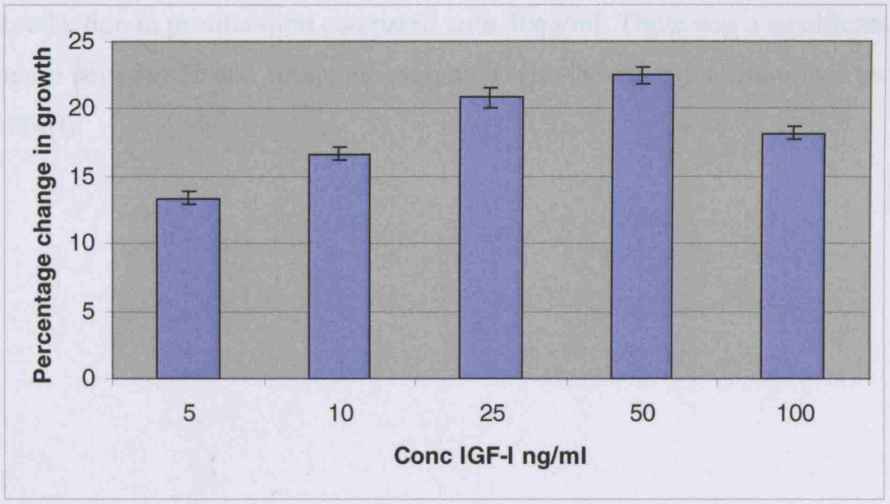


Figure 11 The effect of IGF-I on HT29 cell proliferation

2.5.1.2 Experiments using the Colo 205 cell line

The effect of IGF-I on Colo 205 was tested at 0, 50, 100 and 200ng/ml. There was a marked increase in proliferation at 50ng/ml, but higher concentrations of IGF-I did not lead to increased growth. There was a significant difference between all concentration of IGF-I tested and the control (p=0.0021).

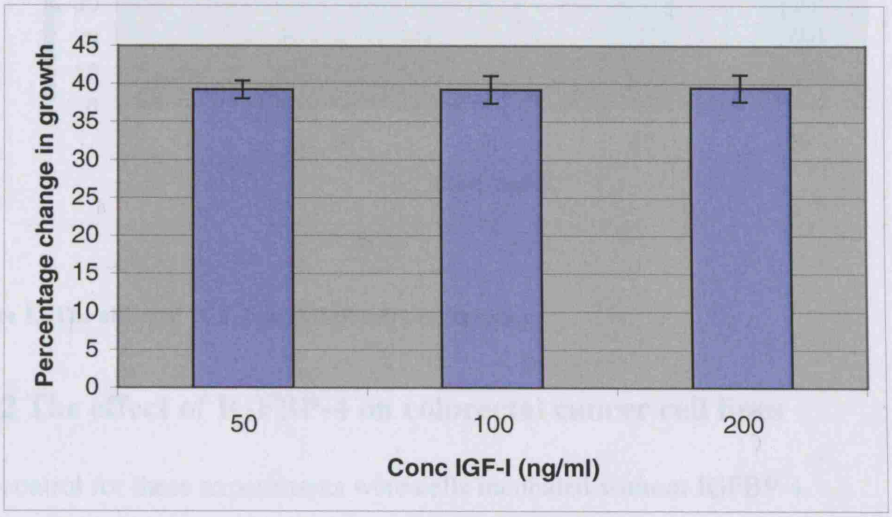


Figure 12 The effect of IGF-I on Colo 205 cell proliferation

2.5.1.3 Experiments using the WiDR cell line

IGF-I was tested at 5, 10, 25, 50 and 100ng/ml. There was a small increase in proliferation at 5-25ng/ml, but then a marked increase with 50g/ml. 100ng/ml led to a slight reduction in proliferation compared with 50ng/ml. There was a significant difference between 50 and 100ng/ml compared with the other concentrations tested (p=0.0003).

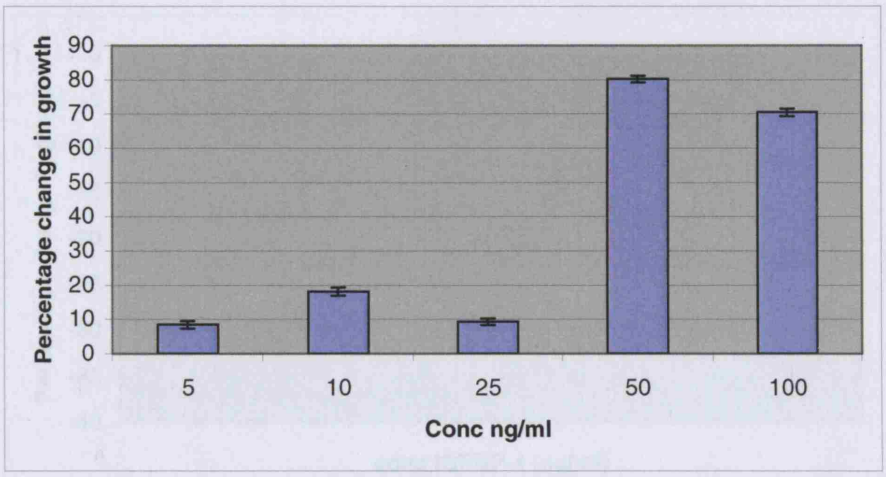


Figure 13 The effect of IGF-I on WiDR cell proliferation

2.5.2 The effect of IGFBP-4 on colorectal cancer cell lines

The control for these experiments were cells incubated without IGFBP-4.

2.5.2.1 Experiments using the Colo 205 cell line

IGFBP-4 was tested at 100, 300, 500 and 1000ng/ml. There was a dose-dependent decrease in proliferation. There was a significant difference between 500ng/ml and 100 and 0ng/ml IGFBP-4 ($p=0.0001$) and between 1000ng/ml and 0ng/ml ($p=0.0003$). The results for this experiment are pooled from one experiment using 0, 100, 300 and 500ng/ml IGFBP-4 (performed in triplicate) and another experiment using 0 and 1000ng/ml IGFBP-4 (also performed in triplicate) therefore it has only been possible to ascertain whether there is a statistically significant difference between 1000ng/ml and 0, and not between 1000ng/ml and the lower concentrations.

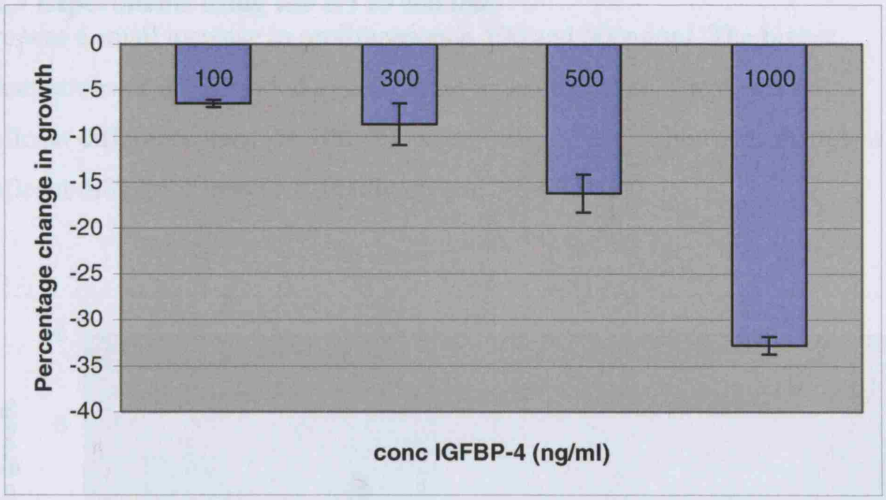


Figure 14 The effect of IGFBP-4 on Colo 205 cell proliferation

2.5.2.2 Experiments using the WiDR cell line

There was an increase in proliferation at the lower concentrations of IGFBP-4, but a marked decrease in proliferation at the highest concentration tested of 1000ng/ml. There was a significant difference between 100 and 300ng/ml and 0ng/ml, and between 500ng/ml and 100 and 300ng/ml (p=0.0001). There was a significant difference between 1000ng/ml and 0 (p=0.0000).

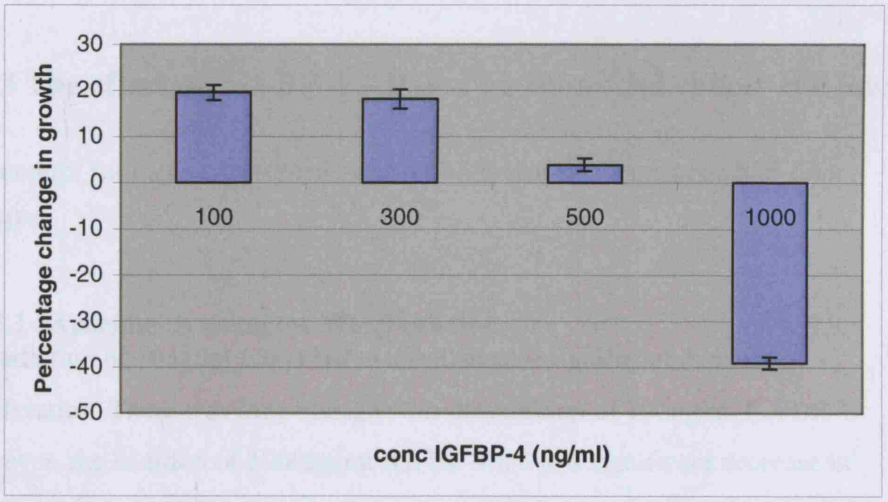


Figure 15 The effect of IGFBP-4 on WiDR cell proliferation

2.5.2.3 Experiments using the HT29 cell line

There was a small increase in proliferation at 100 and 300ng/ml. The higher concentrations of IGFBP-4 led to a decrease in proliferation. There was no significant difference between 100, 300 and 500ng/ml and 0, however there was a significant difference between 1000ng/ml and 0 (p=0.0429).

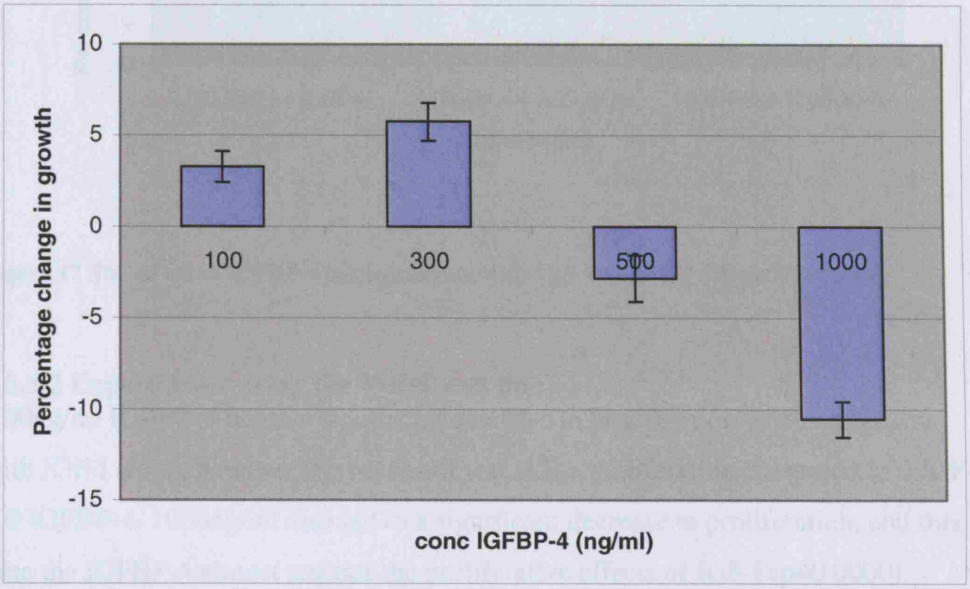


Figure 16 The effect of IGFBP-4 on HT29 cell proliferation

2.5.3 The effect of IGFBP-4 + IGF-I on colorectal cancer cell lines

The control for these experiments was cells incubated with neither IGF-I nor IGFBP-4.

2.5.3.1 Experiments using the HT29 cell line

The addition of 10ng/ml IGF-I led to a statistically significant increase in proliferation. There was little change with the addition of 100ng/ml IGFBP-4. However, the addition of 1000ng/ml IGFBP-4 led to a significant decrease in proliferation from IGF-I alone and also from IGF-I+10ng/ml IGFBP-4 (p=0.0000) but this still resulted in a net proliferation as compared with the control.

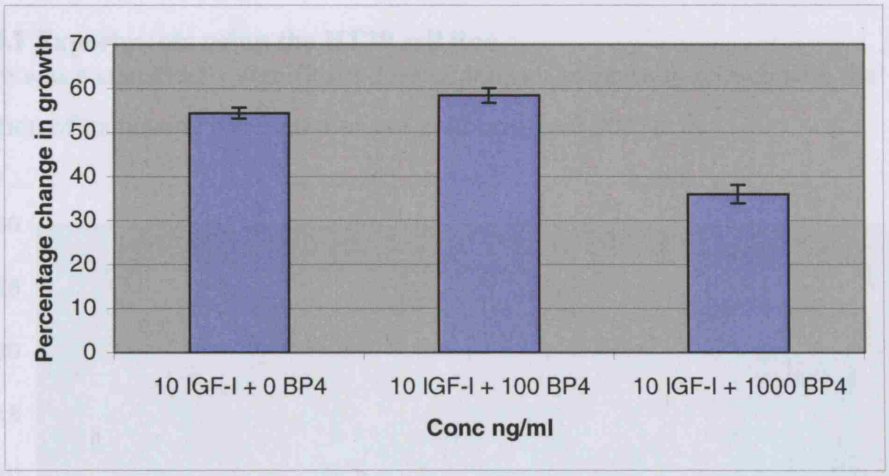


Figure 17 The effect of IGFBP-4 in conjunction with IGF-I on HT29 cell proliferation

2.5.3.2 Experiments using the WiDR cell line

100ng/ml IGFBP-4 led to a significant decrease in proliferation when compared with IGF-I alone, however the net result was still a proliferation compared to 0 IGF-I + 0 IGFBP-4. 1000ng/ml also led to a significant decrease in proliferation, and this time the IGFBP-4 almost negates the proliferative effects of IGF-I (p=0.0000).

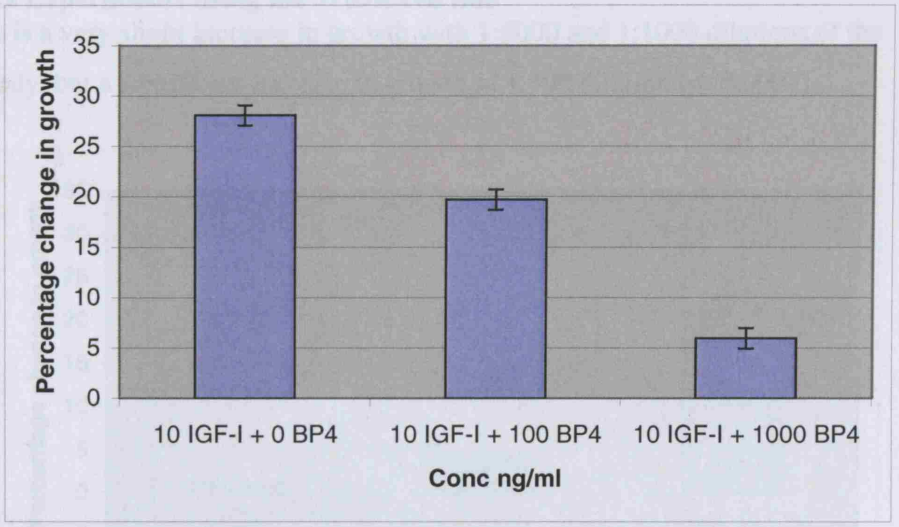


Figure 18 The effect of IGFBP-4 in conjunction with IGF-I on WiDR cell proliferation

2.5.4 The effect of IGFBP-4 antibody on colorectal cancer cell lines

The control for this experiment was cells incubated without IGFBP-4 antibody.

2.5.4.1 Experiments using the HT29 cell line

There was a statistically significant dose-dependant increase in growth with the addition of increasing concentrations of antibody (p=0.0057).

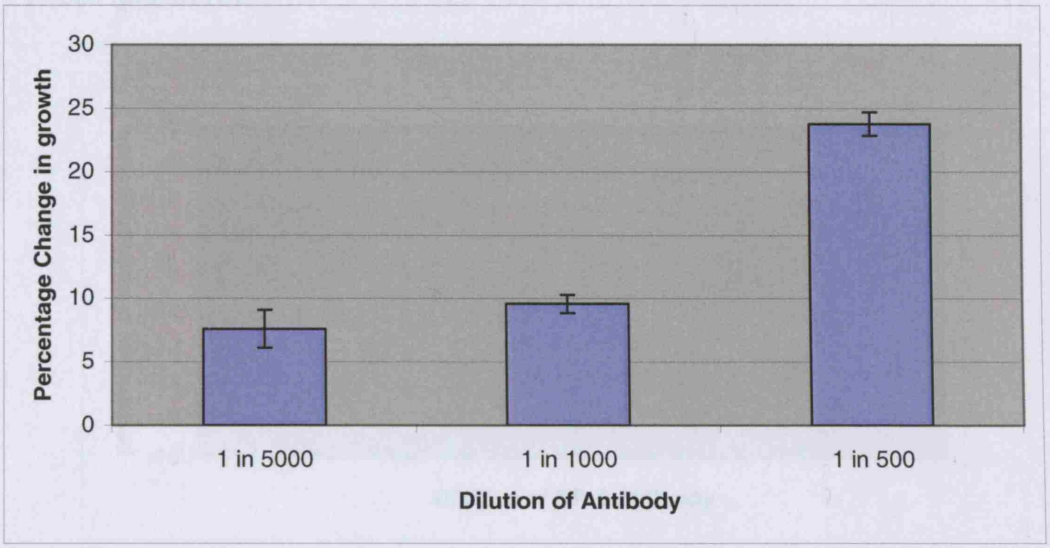


Figure 19 The effect of IGFBP-4 antibody on HT29 cell proliferation

2.5.4.2 Experiments using the WiDR cell line

There is a very slight increase in growth with 1:5000 and 1:1000 dilutions of the antibody, but a significant increase in growth at 1:500 dilution (p=0.0001).

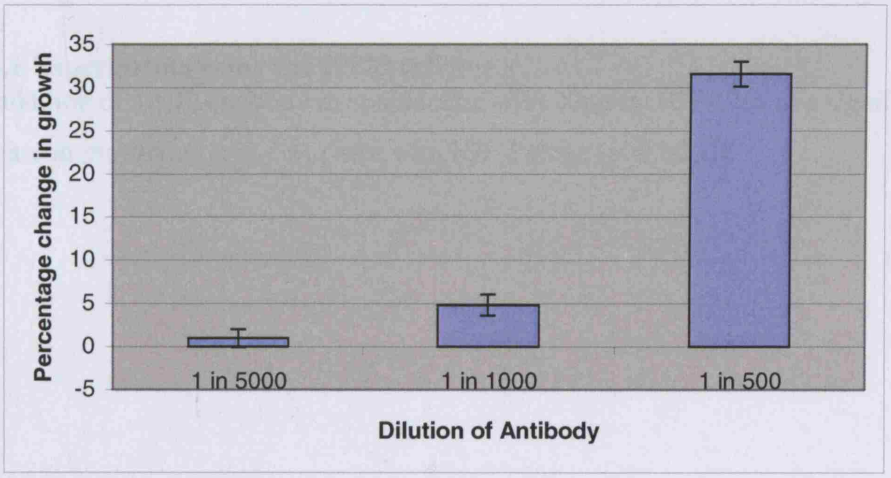


Figure 20 The effect of IGFBP-4 antibody on WiDR cell proliferation

2.5.4.3 Experiments using the Colo 205 cell line

The addition of antibody against IGFBP-4 led to a dose-dependent decrease in proliferation. There is a significant difference between 1:500 and 0, and 1:500 and 1:5000 (p=0.0118).

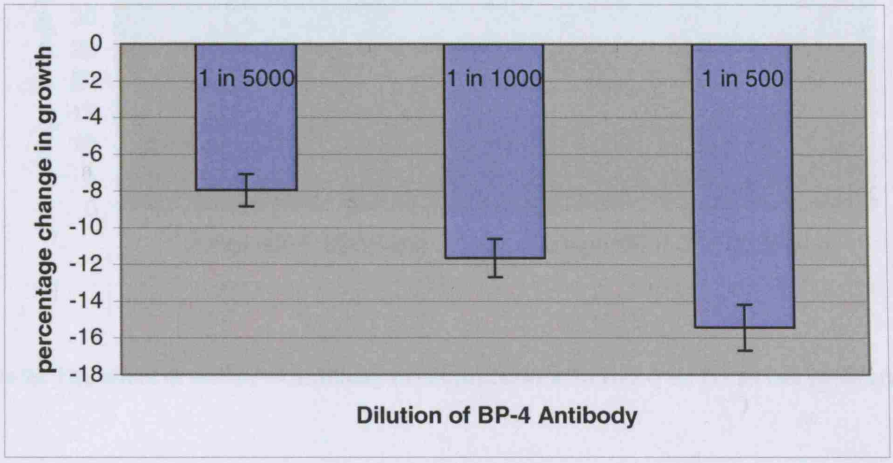


Figure 21 The effect of IGFBP-4 antibody on Colo 205 cell proliferation

2.5.5 The effect of IGFBP-4 antibody + IGF-I on colorectal cancer cell lines

The control was cells incubated with neither IGF-I nor IGFBP-4 antibody.

2.5.5.1 Experiments using the HT29 cell line

The addition of 1:500 antibody in conjunction with 20ng/ml IGF-I led to a significant decrease in proliferation as compared with IGF-I alone (p=0.0000).

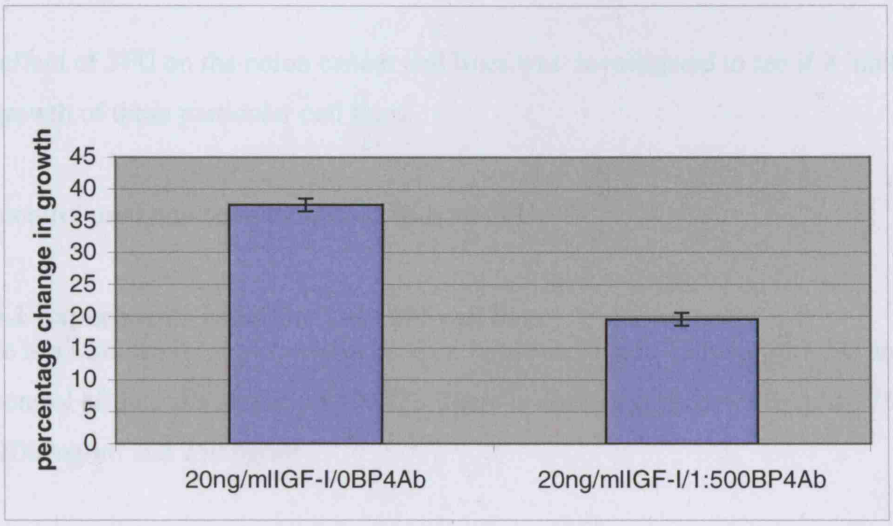


Figure 22 The effect of IGFBP-4 antibody in conjunction with IGF-I on HT29 cell proliferation

2.5.5.2 Experiments using the WiDR cell line

The addition of 1:500 antibody in conjunction with IGF-I led to a small but statistically significant change in growth compared with IGF-I alone (p=0.0001).

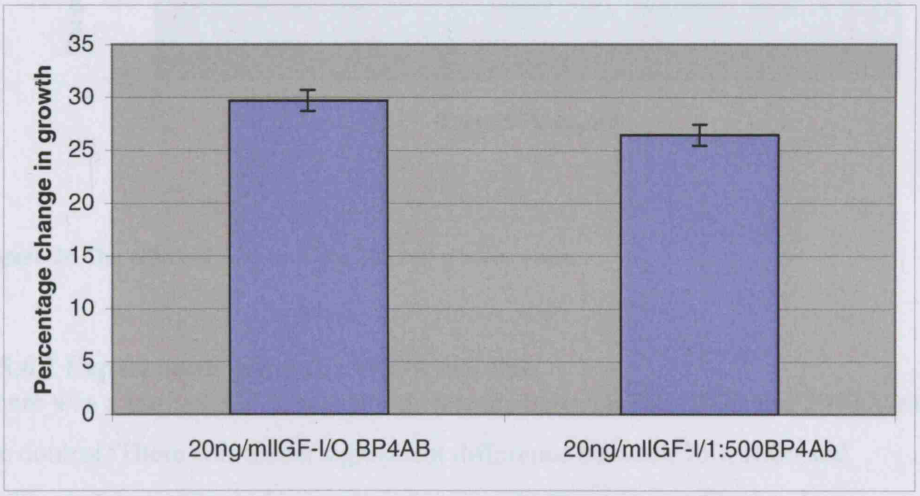


Figure 23 The effect of IGFBP-4 antibody in conjunction with IGF-I on WiDR cell proliferation

2.5.6 The effect of 5FU on colorectal cancer cell lines

The effect of 5FU on the colon cancer cell lines was investigated to see if it inhibited the growth of these particular cell lines.

The control used was cells incubated without 5FU.

2.5.6.1 Experiments using the Colo 205 cell line

There is a statistically significant difference between 750and 1000ng/ml 5-FU and the control of the cells alone (p=0.0282). There is also a significant difference 750 and 1000ng/ml and 250 ng/ml .

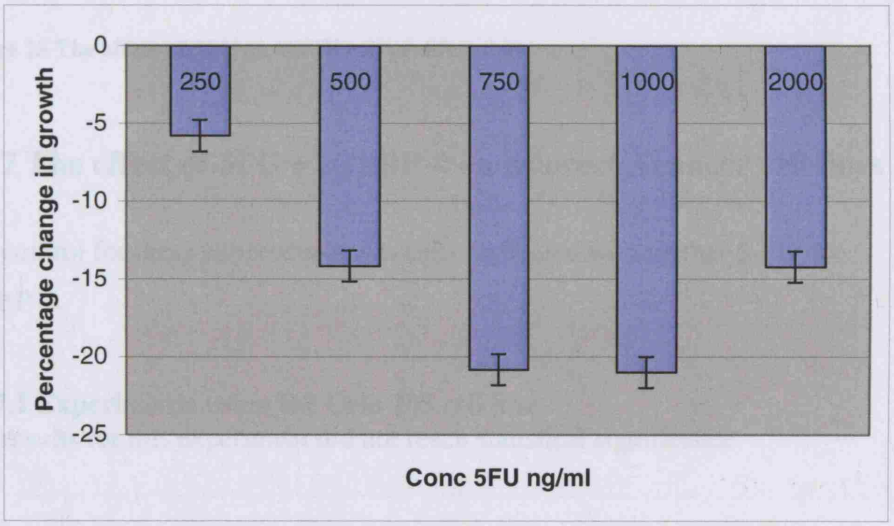


Figure 24 The effect of 5FU on Colo 205 cell proliferation

2.5.6.2 Experiments using the WiDR cell line

There was a statistically significant difference between 750, 1000 and 2000ng/ml and the control. There was also a significant difference between 750, 1000 and 2000ng/ml and 250ng/ml, as well as between 2000ng/ml and 500ng/ml.

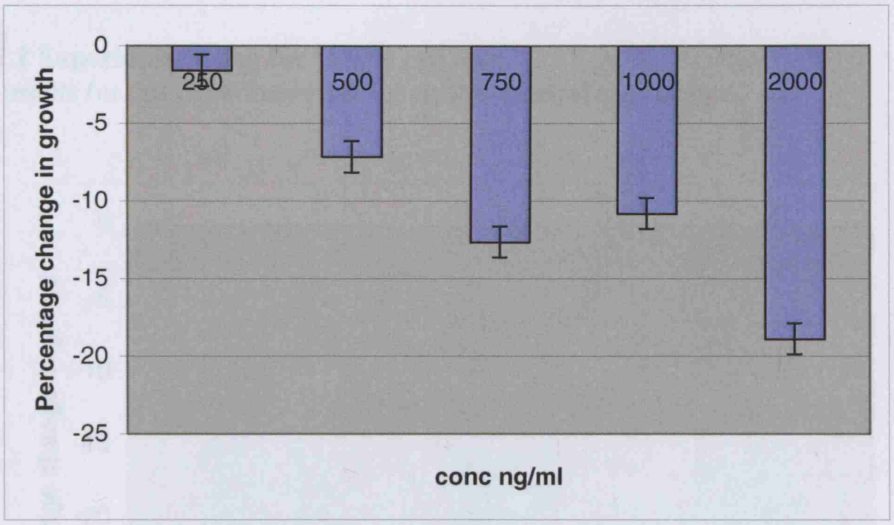


Figure 25 The effect of 5FU on WiDR cell proliferation

2.5.7 The effect of 5FU + IGFBP-4 on colorectal cancer cell lines

The control for these experiments was cells incubated with neither 5-FU nor IGFBP-4.

2.5.7.1 Experiments using the Colo 205 cell line

The results for this experiment did not reach statistical significance.

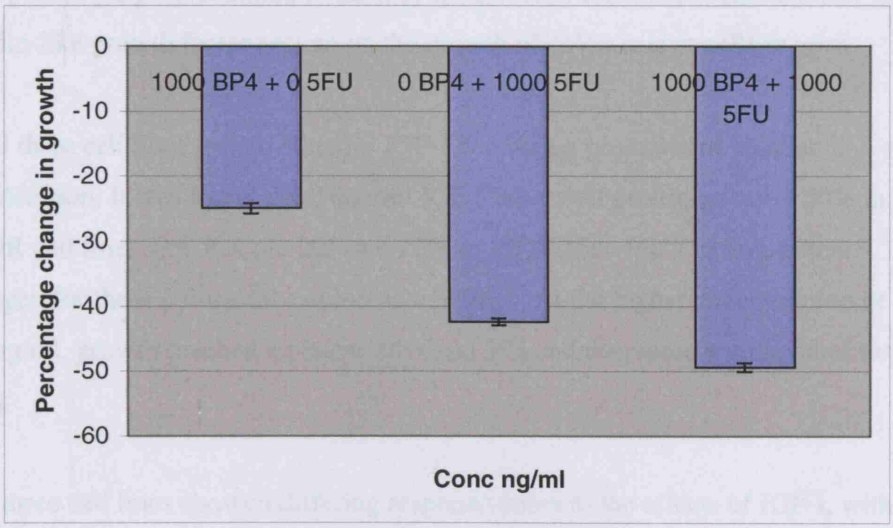


Figure 26 The effect of IGFBP-4 + 5FU on Colo 205 cell proliferation

2.5.7.2 Experiment using the WiDR cell line

The results for this experiment did not reach statistical significance.

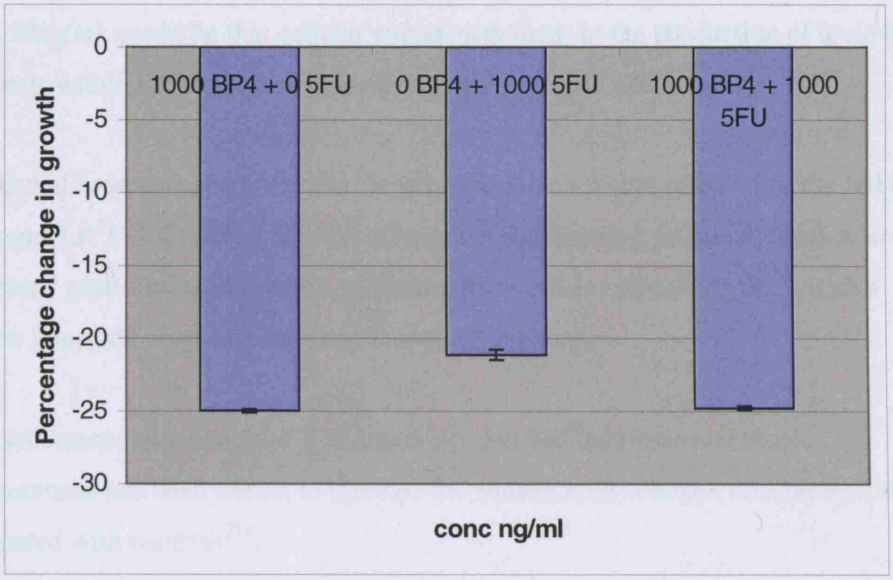


Figure 27 The effect of IGFBP-4 + 5FU on WiDR cell proliferation

2.6 Discussion

This part of the project aimed to identify the effect of different components of the Insulin-like growth factor system on the growth of colon cancer cells *in vitro*.

In all three cell lines tested, 50ng/ml IGF-I is a strong promoter of cellular proliferation. It was found that 50ng/ml IGF-I increased proliferation by 80% in the WiDR cell line, 39% in Colo 205 and 23% in HT29 showing it to be a potent mitogen for these colorectal carcinoma cell lines. At the higher concentration of 100ng/ml, growth reached a plateau for Colo 205 and decreased for the other two cell lines.

The three cell lines showed differing responsiveness to the effects of IGF-I, with WiDR being the most sensitive to its effects. It is possible that this cell line has a greater concentration of IGF-I receptors and therefore is more susceptible to a

mitogenic stimulus. It is reasonable to postulate that in humans, different colon cancers may react in a differential manner to the effects of the IGF system.

A possible explanation for the decrease in growth with 100ng/ml IGF-I compared with 50ng/ml, could be that cellular overgrowth leads to the production of toxic by-products within the media and this subsequently leads to cell death.

Steeb et al have demonstrated that the subcutaneous infusion of IGF-I or the IGF-I analogue LR^3IGF-I (which has less affinity for IGF binding proteins), leads to an increased gastrointestinal weight and increased small intestinal length[210]. It also causes intestinal crypt and villus epithelial hyperplasia.

The subcutaneous infusion of IGF-I into rats that had undergone a colonic anastomosis, has been shown to increase the anastomotic collagen content by 23%, compared with controls[211].

R3-IGF-I (an IGF-I agonist which has little affinity for binding proteins) causes a concentration dependent increase in growth of human intestinal smooth muscle cells. It also leads to an increase in production of IGFBP-4. Antagonists of the IGF-I receptor have been shown to decrease growth and decrease IGFBP-4 production. This group showed that the IGF-I regulation of IGFBP-4 takes place via the activation of MAP kinase and PI3-kinase pathways[153].

The results for IGFBP-4 were very interesting. IGFBP-4 at a concentration of 1000ng/ml leads to marked growth inhibition in all three cell lines tested. Colo 205 and WiDR are more sensitive to the effect of IGFBP-4 than HT29. There was a 33% decrease in growth for Colo 205, 39% for WiDR and 10% for HT29. With the Colo 205 cell line the effect of IGFBP-4 is dose dependent. With the other two cell lines, the lower concentrations caused an increase in growth, with only the higher concentrations of 500 and 1000ng/ml causing a decrease in proliferation for HT29, and only the highest concentration of 1000ng/ml causing a decrease in growth for WiDR.

At 1000ng/ml, IGFBP-4 has a marked inhibitory effect on cellular growth. With HT29 and WiDR, it may be that the lower concentrations of IGFBP-4 alter the regulation of other components of the IGF system which results in increased cellular proliferation, which is only overcome at the highest concentration of IGFBP-4. As for the experiments with IGF-I, the HT29 cell line is once again the least responsive to the effects of IGFBP-4. The differing responsiveness of colon cancer cell lines to the effects of IGF-I and IGFBP-4 may be related to variability in expression of different components of the IGF system. Although Colo 205 is sensitive to the stimulatory effects of IGF-I, it secretes a high concentration of IGFBP-4 into the conditioned medium[169]. It may be that the differential response of cells to IGF-I and IGFBP-4 may be as a consequence of variability in the co-secretion of IGF-I and IGFBP-4, and also to the differing concentration of IGF-I receptors. Another factor is that there may be a difference in both the concentration and activity of IGFBP-4 protease secreted by the cells.

Mohan et al showed that IGFBP-4 decreases the binding of ^{125}I-IGF-I to purified IGF-I receptor[166]. They also found that IGFBP-4 had no effect on decreasing cellular proliferation in bone cells induced by analogues of IGF-I and IGF-II which have greater than 100-fold decreased affinity for IGFBP-4. This suggests that part of the action of IGFBP-4 is to sequester IGFs away from the IGF-I receptor and hence decrease proliferation.

Having shown that IGFBP-4 is a potent inhibitor of cellular proliferation, experiments were designed to see if it could inhibit the powerful proliferative effects of IGF-I. Experiments using IGF-I and IGFBP-4 in tandem show that for the two cell lines tested – HT29 and WiDR –IGFBP-4 can in part inhibit the proliferative effects of exogenous IGF-I. IGFBP-4 at a concentration of 1000ng/ml can decrease the proliferative effects of IGF-I by 79% in WiDR, and by 34% in HT29 cells.

Overexpression of IGFBP-4 in the M12 malignant prostate epithelial cell line has been shown to significantly decrease IGF-II induced proliferation. It was also shown to lead to a significant decrease in colony formation compared to the control. This was shown to be due to the inhibition of IGFs rather than due to an IGF-independent

effect. When the IGFBP-4 transfected cell line was injected into nude mice, there was a significant delay in tumour formation[212].

A protease-resistant IGFBP-4 was found to be more potent than wild-type IGFBP-4 in inhibiting IGF-I induced mouse osteoblast cell proliferation *in vitro*. It was also more effective in inhibiting IGF-I induced bone formation as measured by alkaline phosphatase activity. The same group also showed that systemic administration of IGFBP-4 leads to an increase in serum IGF-I[213]. They suggest that the systemic administration of IGFBP-4 leads to an increase in bioavailable IGF-I in the circulation via an IGFBP-4 protease dependent mechanism.

They postulate the following:

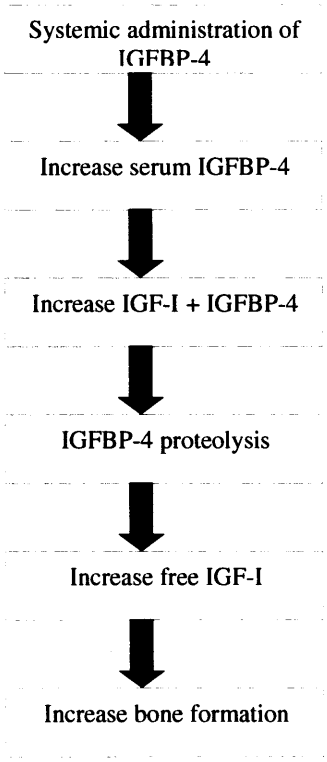


Figure 28 The effect of IGFBP-4 on free IGF-I

This hypothesis correlates with the findings in this thesis that lower concentrations of IGFBP-4 cause an increase in proliferation. It may be that this is due to an increased

bioavailability of IGF-I, and that at the higher concentrations of IGFBP-4, it is able to overcome the effect of the increased IGF-I bioavailability.

Singh et al attempted to see if they could titrate out the inhibitory effect of IGFBP-4 on HT29 cell proliferation by the addition of supra-physiological concentrations of IGF-I[29]. They found that this was not possible and concluded that the mechanism of action of IGFBP-4 was therefore unlikely to be solely due to the competitive binding of IGFs away from the IGF-I receptor.

It is known that many colon cancers overexpress IGF-I, and patients with colon cancer have higher serum levels of IGF-I than the normal population[41,42]. The results of this experiment indicate that if these patients were exposed to increased levels of IGFBP-4, it may have a beneficial effect in decreasing the rate of tumour growth.

The experiments using IGFBP-4 antibody were performed to isolate what effect endogenous IGFBP-4 has on growth. For the HT29 and WiDR cell lines, the addition of IGFBP-4 antibody led to a dose-dependent increase in growth. With HT29, 24% of cellular growth can be inhibited using a dilution of 1:500 of the antibody. This figure rises to 32% for WiDR cells. However, for Colo 205, IGFBP-4 antibody leads to a decrease in growth of up to 15.4% with the highest concentration of antibody tested.

These results illustrate that for the HT29 and WiDR cell lines, a significant proportion of cell growth is inhibited by endogenous IGFBP-4 and its importance in controlling excessive proliferation even within a malignant cell line. Other researchers in our laboratory using immunohistochemistry have shown that human colon cancer *in vivo* contains less IGFBP-4 than normal colonic tissue.

The results for Colo 205 are the exact opposite for the other two cell lines tested. It is possible that the IGFBP-4 antibody binds preferentially to the Colo 205 IGF-IR rather than to endogenous IGFBP-4. Alternatively, the endogenous Colo 205 IGFBP-4 is strongly bound to IGF-I and does not release it, allowing no ligand to bind to the IGF-IR.

This experiment shows what a significant factor endogenous IGFBP-4 is in controlling cellular growth for some colorectal cancer cell lines, and provides more evidence that this would be a worthwhile area to investigate further as a target for adjuvant therapy.

Other work correlates with these results. Singh et al found that IGFBP-4 antibody significantly increased both the basal and IGF-I stimulated growth of HT29 cells[29]. They developed a transfection model where HT29 cells were transfected with control vector, or with vectors containing sense or anti-sense IGFBP-4 cDNA. They found that the anti-sense vector led to a significant decrease in IGFBP-4 secreted into the medium and a subsequent increase in cellular proliferation. The insulin-like growth factors have a 40-fold greater affinity for the IGF binding proteins than for the IGF-I receptor . By decreasing the concentration of extracellular IGFBP-4, the bioavailability of IGF-I for the IGF-I receptor is greatly increased. There was 5-10 fold overexpression of IGFBP-4 by vectors containing sense IGFBP-4 cDNA, but this had no significant effect on cellular proliferation. This indicates that IGFBP-4 has no intrinsic inhibitory properties, but exerts its inhibitory effect via IGF-dependent mechanisms.

As the experiment above showed that the addition of IGFBP-4 antibody increased cellular proliferation in two cell lines, the next experiment was designed to see if it would augment the proliferative effect of IGF-I. This was done for the cell lines HT29 and WiDR. Contrary to expectation, the addition of the antibody led to a decrease in growth compared to the addition of IGF-I alone. Growth was decreased by 47.7% with HT29 and 11.1% with WiDR. A possible explanation for this may be that the antibody is interacting with IGF-I so as to prevent its binding to the IGF-I receptor and hence inhibiting proliferation.

Work by Singh et al to the contrary showed that IGFBP-4 antibody led to a significant increase in IGF-I induced cellular proliferation in the HT29 cell line[29]. They used 10nmM IGF-I with an antibody dilution of 1:5000 and 1:2000. Both of these dilutions of antibody caused an approximately 170% increase in proliferation.

5FU is a commonly used chemotherapeutic agent used for treating colon cancer. The experiments with 5FU show it has significant growth inhibiting effects on all three cell lines tested.

An experiment was designed to investigate whether this effect could be enhanced with the addition of IGFBP-4. For Colo 205 and WiDR cell lines there was no significant difference between 5FU alone and 5FU in conjunction with IGFBP-4. The results from this set of experiments show that IGFBP-4 does not augment the inhibition of growth by 5FU.

2.7 Conclusion

The cell culture experiments have shown that the IGF system plays an important role in the rate of proliferation of colon cancer cells *in vitro*.

IGF-I has been shown to be a potent mitogen in all three cell lines tested. These results correlate with results from other groups.

IGFBP-4 at 1000ng/ml causes a significant decrease in cellular proliferation. At lower concentrations of IGFBP-4 there is a transient increase in proliferation with the HT29 and WiDR cell lines. This could be due to an increase in bioavailable IGF-I as a result of increased IGFBP-4 proteolysis.

IGFBP-4 is able to inhibit the proliferative effects of exogenous IGF-I.

Endogenous IGFBP-4 has a powerful effect on inhibiting cellular growth in the HT29 and WiDR cell lines, as demonstrated by the increase in proliferation with the addition of IGFBP-4 antibody. However, the addition of IGFBP-4 antibody does not augment the proliferative effects of exogenous IGF-I.

This data supports the hypothesis that the IGF system plays an important role in the development and subsequent growth of colon cancers. Further work needs to be performed to see if by modulating the IGF system, it may in the future offer a treatment modality for carcinoma of the colon *in vivo*.

Chapter 3 *In Vivo* Experiments

3.1 Introduction

Having investigated the effects of the IGF system on colon cancer cells *in vitro*, the next stage was to prepare an animal model of colon cancer that could be used as the basis of future experiments to investigate the effects of the IGF system *in vivo*.

3.2 Aims

The first aim of this part of the project was to create two nude mouse models of colorectal cancer. The second aim was to then identify the components of the IGF system that were expressed in these models. This was done using polymerase chain reaction. The PCR products would be confirmed by gel purifying the DNA and ligating it into a vector which could be used to transform competent cells. DNA from the positive colonies grown was purified and a digest performed to confirm the correct inserts. The components of the IGF system investigated were the ligand IGF-I, the IGF-I receptor and IGF binding protein-4.

3.3 Methods

3.3.1 Preparation of nude mouse model of colon cancer

This work was performed under project licence PPL 70/5232 'The role of IGF-I in tumourigenesis and apoptosis'

Nude mouse models of colon cancer were developed in duplicate using the cell lines Colo 205 and HT29.

A cell suspension was made of $3x10^6$cells/ml of each of the two colon cancer cell lines. Five millilitres of each suspension ($15x10^6$cells) was centrifuged and the pellets resuspended in the appropriate medium with 10% foetal calf serum. 100µl of this suspension was injected into each flank of the nude mice.

After 3 weeks when the tumours had grown to a size of approximately 1.5cm diameter, the mice were sacrificed by a schedule 1 method and the tumours passaged. Further mice were anaesthetised with halothane and a small incision made over their flank. A $2mm^3$ piece of tumour was inserted subcutaneously via this incision and the incision closed with a single suture. The tumours were allowed to grow for a further 3 weeks before the mice were sacrificed and the tumours harvested.

Figure 29 Nude mouse bearing Colo 205 xenograft

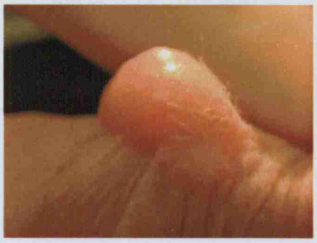


Figure 30 Nude mouse bearing HT29 xenograft

3.3.2 Polymerase chain reaction

3.3.2.1 Preparation of tumour tissue

After excision of the tumour from the animal model, the tissue was placed immediately into denaturing solution from Genosys at room temperature.

3.3.2.2 RNA extraction from tumour tissue

RNA was extracted using the Genosys RNA isolator kit. 100mg of tumour tissue was homogenised in 1ml Genosys denaturing solution. This was left to stand at room temperature for 5 minutes. 200µl of choloroform was added and mixed with the homogenate. This was incubated at room temperature for 15 minutes. The mixture

was centrifuged at 12 000g for 5 minutes. The aqueous phase was removed and 0.5ml of isopropanol added to it. This was incubated at room temperature for 10 minutes and then centrifuged at 15000g for 10 minutes. The RNA pellet was washed twice in 70% ethanol for 5 minutes at 15 000g. The pellet was allowed to air dry and the following were added: 200µl of DEPC water, 20µl 3M sodium acetate ph 5.2 and 550µl 100% ethanol. The solution was allowed to precipitate and the 50µl was removed and centrifuged at 15 000g for 20 minutes.

3.3.2.3 Measurement of RNA concentration

The concentration of RNA was measured using the optical density at 260nm and the ratio of absorbance at 260/280nm was calculated to ascertain RNA purity, as protein is mainly detected at 280nm.

3.3.2.4 First strand cDNA synthesis

The RNA was reversed transcribed to prepare cDNA and from there used for PCR. 10µg of RNA, 11µl DEPC water and 1µl of oligo dT were heated together at 70°C for 10 minutes and then put on ice. To this mixture, the following were added: 1µl RNAse inhibitor, 4µl 5x buffer, 2µl DTT, 1µl dNTP and 1µl superscript. This was then incubated at 42°C for 1 hour. The enzyme was inactivated by heating at 70°C for 10 minutes.

3.3.2.5 Polymerase chain reaction

Polymerase chain reaction (PCR) allows the amplification of specific sequences of DNA. Specific oligonucleotides which are complementary to sequences either side of the region of interest are required to prime the amplification of the intervening sequence of DNA. PCR consists of three stages:
Denaturation of the double-stranded DNA using a high temperature
Annealing of the oligonucleotide primers using a lower temperature
Synthesis of the new DNA using DNA polymerase at an intermediate temperature

DNA analysis was done using gel electrophoresis on an agarose gel to which ethidium bromide had been added. Samples run towards the positive electrode, with smaller DNA fragments migrating further down the gel. Ethidium bromide binds

between the nucleotides of DNA and fluoresces under ultraviolet light enabling visualisation of the DNA band.

It is a useful method to detect the expression of a particular gene within a particular tissue.

3.3.2.6 Sequence of oligonucleotide primers
Refer to Appendix 7.2

3.3.2.7 PCR protocol for IGFBP-4
Refer to appendix 7.3

3.3.2.8 PCR protocol for IGF-I using gold Taq
Refer to appendix 7.4

Experiment 1:

The enzyme was activated at 94°C for 7mins. This was followed by 35 cycles of 94°C (1 minute), 60°C (1 minute) and 72°C (1 minute). This was completed with a 10 minute extension at 72°C.

Experiment 2:

As the experiment using the protocol above did not give a band of the correct size, the protocol was optimised by altering the conditions. The magnesium concentration was decreased to 1.5mM and the cycles of 94°C, 60°C and 72°C were decreased to 30 seconds each.

3.3.2.9 PCR protocol for IGF-I using platinum Taq
Refer to appendix 7.5

3.3.2.10 PCR protocol for IGF-II
Refer to appendix 7.6

3.3.2.11 PCR protocol for IGF-IR
Refer to appendix 7.7

3.3.3 Preparation of agarose gel

1g of agarose was added to 100mls of TE buffer and heated for 90 seconds in a microwave. 1µl of ethidium bromide was then added and the gel set in a gel tank with a 12 lane comb to generate wells.

3.3.4 Conditions for running gel

The gel was run at 150v for approximately 30 minutes, or until the dye front was approximately 2cm above the bottom of the gel.

3.3.5 Purifying of DNA from agarose gels

The DNA extracted from the gel electrophoresis was purified using the QIAEX II gel extraction kit from QIAGEN. The PCR product was excised from the agarose gel with a clean scalpel and weighed. Three times this volumne of buffer QX1 was added to it. 10µl of QIAEXII (buffer and pH indicator) was added and incubated at room temperature for 10 minutes to solubilise the gel and the mixture vortexed every 2 minutes during this period. The mixture was then centrifuged for 30 seconds and the supernatant removed. The pellet was washed twice with 500µl of PE buffer and then air dried for 30 minutes. The pellet was resuspended in 30µl $dH_20$and incubated at room temperature for 5 minutes and then centrifuged for 30 seconds. The supernatant containing pure DNA was transferred into a clean tube.

3.3.6 Cloning of the PCR product

The DNA was then inserted into a cloning vector which was used to transform bacterial cells. The vector has a polylinker which contains multiple restriction enzyme sites allowing the vector to be cut at particular sites.

Cloning was performed using the Promega pGEM-T easy vector. The vector is pre-cut at the EcoRV site and a 3' terminal thymidine is added to both ends. This prevents the cut ends of the vector from rejoining. The successful cloning of the insert into the vector leads to the interruption of the coding sequence of β-

galactosidase (lacZ gene). This is visualised with the vector containing the insert forming white colonies and the empty vectors forming blue colonies.

3.3.6.1 Selection of positive clones

The vector has a gene conferring resistance to the antibiotic ampicillin. The bacterial cells are grown on agar plates containing ampicillin, and this allows for selection of positive clones. Bacteria which have not taken up the vector will die as they do not possess ampicillin resistance.

3.3.6.2 Screening for positive clones

Another method that the vector employs is to screen for β-galactosidase expression in the recombinant DNA plasmids. The plasmid contains the lacZ gene which codes for a subunit of β-galactosidase. β-galactosidase converts the colourless Xgal (which is present in the agar plates) into a blue precipitate. Plasmids which contain the recombinant DNA have an interrupted lacZ gene and therefore are unable to produce β-galactosidase. These clones grow as white colonies, as opposed to plasmids which have not taken up the DNA which grow as blue colonies. The white colonies are picked and tested further to confirm that the insert is present.

3.3.7 DNA Ligation into the vector

3µl of the PCR product was added to 5µl 2x rapid ligation buffer (60mM Tris-HCl ph7.8, 20mM Magnesium chloride, 20mM DTT, 1mM ATP 10% PEG), 1µl (50ng) pGEM-T easy vector and 1µl of T4 DNA ligase (3 Weiss units/µl) and incubated overnight at 4°C.

3.3.8 Transformation of competent cells

Promega JM109 High Efficiency E.coli Competent cells were used for transformations with the pGem-T easy vector. Selection for transformation was performed on 10 cm diameter LB agar plates containing ampicillin/IPTG/X-Gal (see appendix 7.8). 5µl of the ligation reaction was added to JM109 cells and the mixture incubated on ice for 20 minutes. It was then heat shocked for 45 seconds in a water bath at 42°C and then put on ice for 2 minutes. Then 950µl SOC medium at room

temperature was added. The mixture was incubated with shaking for 1.5 hours at 37°C. 100µl of the transformation reaction was plated out onto LB plates as above and incubated overnight at 37°C. Six white colonies were picked and each grown for 8 hours at 37°C in 5mls LB/Amp 50. The plasmids were isolated from these cultures using a commercial miniprep and the DNA resuspended in 50µl of nuclease free water and stored at -20°C. Digests were then performed to check the correct inserts in the plasmid.

3.3.9 Mini-prep (method 1)

The promega Wizard system was used. Two millilitres of each sample was centrifuged at 15 000g for 5 minutes to collect the cells. The supernatant was discarded and a further 2 mls of sample added and centrifuged for a further 5 minutes. The supernatant was once again discarded. 250µl of cell resuspension solution (50mM Tris-HCL pH 7.5, 10mM EDTA, 100µg/ml Rnase A) was added to the cell pellet and the mixture vortexed. 250µl of cell lysis solution (0.2M NaOH, 1%SDS) was then added and the tubes inverted four times. They were then incubated at room temperature for 5 minutes. 10µl of alkaline protease solution was added and the tubes inverted four times and incubated again at room temperature for 5 minutes. 350µl of neutralisation solution (1.32M potassium acetate) was added and the tubes inverted four times. The samples were then centrifuged at 14 000g for 10 minutes. The lysate was then transferred to a Promega Minicolumn and a vacuum was applied to draw the lysate through the column. The column was then washed by adding 750µl wash solution (95% ethanol) followed by 250µl wash solution, applying a vacuum each time. The Minicolumn was transferred to a new microcentrifuge tube and 50µl nuclease-free water was added. This was centrifuged for 1minute at 14,000g to elute the plasmid DNA.

3.3.10 Restriction digestion of PCR products using EcoR1

EcoR1 was purchased from New England BioLabs. EcoR1 cuts at the sequence GAATC. A digest was performed on the DNA prepared in the miniprep (method 1) using the enzyme EcoR1 (100u/µl). 5µl of DNA was added to 30 units of the enzyme

(0.27µl), 2µl of 10x buffer and made up to 20µl with 12.73µl of distilled water. The mixture was incubated in a water bath at 37°C for 2 hours. The digest was analysed on an agarose gel.

3.3.11 Restriction digestion of PCR products using Not1

Not1 purchased from New England Biolabs. Not1 cuts at the sequence GCGGCCGC. 1µl of enzyme was added to 2µl 10x buffer, 5µl DNA, 0.2µl 100x BSA and made up to 20µl with 11.8µl distilled water. The mixture was incubated in a water bath at 37°C for 2 hours. The digest was analysed on an agarose gel.

3.3.12 Mini-prep (method 2)

A commercially produced kit from Qiagen was used.
Two millilitres of culture was centrifuged at 15 000g for 10 minutes. The bacterial pellet was resuspended in 250µl of buffer P1 (to which RNAse A has been added). 250µl buffer P2 was added and the tubes inverted 4 times. 350µl buffer N3 was added and the tubes inverted 4 times. The mixture was then centrifuged for 10 minutes. The supernatant was then transferred to a spin column. This was centrifuged for 60 seconds and the flowthrough was discarded. The spin column was washed by adding 0.5mls buffer PB and centrifuged for a further 60 seconds and the flowthrough once again discarded. The spin column was then washed by adding 0.75 mls buffer PE and centrifuged for 60 seconds. The flowthrough was discarded and the column spun for a further 1 minute to remove any residual buffer. The column was then placed in a clean 1.5ml microcentrifuge tube. To elute the DNA, 50µl of buffer EB or dH2O was added to the centre of each column and allowed to stand for 1 minute. This was then centrifuged for 1 minute at 3 000g.

3.4 Results

3.4.1 The expression of IGF-I in the nude mouse model

IGF-I was expressed in the nude mouse models derived from both Colo 205 and HT29 colorectal cancer cell lines.

3.4.1.1 The expression of IGF-I in the Colo 205 model

The PCR was performed using gold taq with a magnesium chloride concentration of 2.5mM.

Lane 1 DNA ladder
Lane 2 positive control
Lane 3 Colo 205
Lane 4 negative control

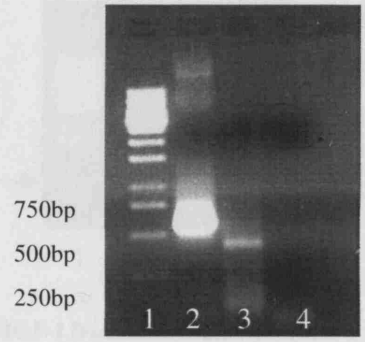
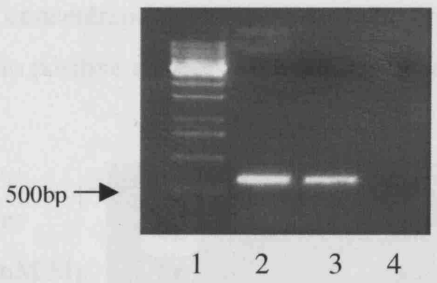


Figure 31 PCR amplification of IGF-I from Colo 205 tumour tissue

There is a band in experimental lane 3 of approximately 500bp (figure 31). This is slightly smaller than the expected 622bp which can be seen in the positive control in lane 2. It was postulated that the running conditions of the PCR led to this anomaly, therefore further experiments were performed to optimise the protocol.

The PCR was repeated using the modified protocol.



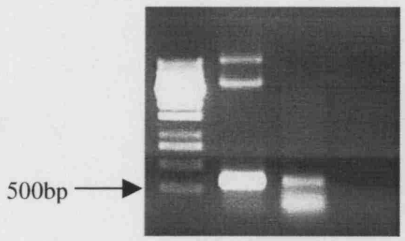
Lane 1 DNA ladder
Lane 2 positive control
Lane 3 Colo 205
Lane 4 negative control

Figure 32 PCR amplification of IGF-I from Colo 205 tumour tissue

As can be seen in figure 32, there are bands of the correct size of approximately 622bp in the positive control and the experimental lane (lanes 2 and 3). The negative control (lane 4) is blank.

3.4.1.2 The expression of IGF-I in the HT29 model

The PCR was performed using gold taq and a magnesium chloride concentration of 1.5mM.



Lane 1 DNA ladder
Lane 2 positive control
Lane 3 HT29
Lane 4 negative control

Figure 33 PCR amplification of IGF-I from HT29 tumour tissue

As can be seen, a band of the correct size is visible in lane 3 (figure 33), which corresponds to the band in the positive control.

3.4.2 The expression of IGF-IR in the nude mouse model

IGF-IR was expressed by both tumour xenografts.

3.4.2.1 The expression of IGF-IR in the HT29 model

The electrophoresis below shows the magnesium optimisation for HT29 IGF-IR. As can be seen there is a band of the correct size of 600bp in lanes 2, 3 and 4 which are the increasing concentrations of Magnesium. No band is visible in the negative control (lane 5). No positive control was available for this experiment.

Lane 1 DNA ladder
Lane 2 HT29 1.5mM Mg
Lane 3 HT29 3mM Mg
Lane 4 HT29 4.5mM
Lane 5 negative control

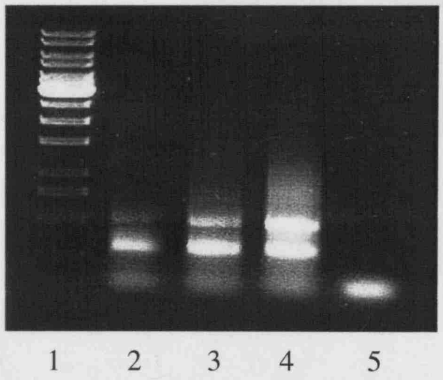


Figure 34 PCR amplification of IGF-IR from HT29 tumour tissue

3.4.2.2 The expression of IGF-IR in the Colo 205 model

The PCR was performed using gold taq and a magnesium concentration of 1.5mM.

As can be seen there is a band of the correct size of 600bp in lane 2.

Lane 1 DNA ladder

Lane 2 Colo 205

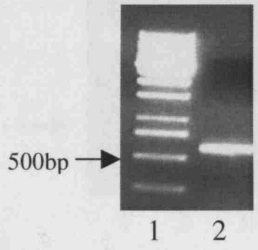


Figure 35 PCR amplification of IGF-IR from Colo 205 tumour tissue

3.4.3 The expression of IGFBP-4 in the nude mouse model

IGFBP-4 was also expressed by both nude mouse models.

3.4.3.1 The expression of IGFBP-4 in the Colo 205 model

The PCR was performed using gold taq and a magnesium concentration of 2mM.

As can be seen, a band of the correct size of 492bp is present in lane 2, as is present in the positive control (lane 3). There is no band in the negative control (lane 4).

Lane 1 DNA ladder

Lane 2 Colo 205

Lane 3 positive control

Lane 4 negative control

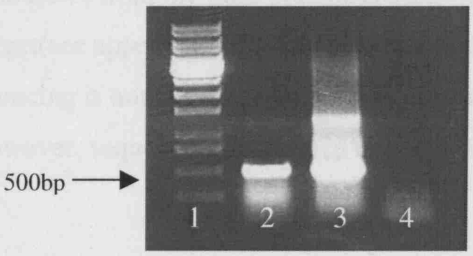


Figure 36 PCR amplification of IGFBP-4 from Colo 205 tumour tissue

3.4.3.2 The expression of IGFBP-4 in the HT29 model

The PCR was performed using gold taq and a magnesium concentration of 2mM. A band of the correct size of 492bp is seen in lane 2, corresponding to the positive control in lane 3. Once again the lane 4 (the negative control) is clear.

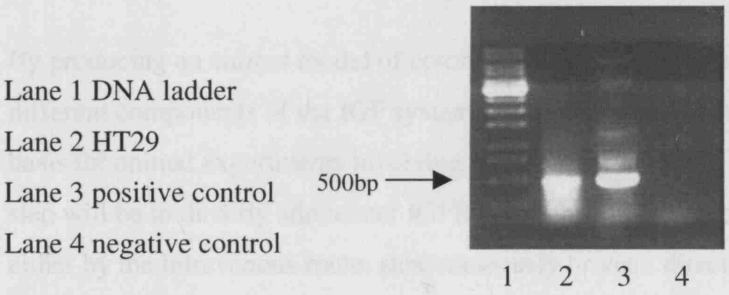


Figure 37 PCR amplification of IGFBP-4 from HT29 tumour tissue

3.4.4 Experiments to confirm the PCR product

To confirm that the correct DNA sequence had been amplified, the DNA band from the gel electrophoresis was cut out and ligated into the pGEM-easy vector (3015bp). The vector was then used to transform competent bacterial cells. The transformed cells were plated out onto agar plates (containing ampicillin and Xgal) and screened for ampicillin resistance and blue-white colony screening. Six white colonies were picked and grown in LB/ampicillin for 8 hours. DNA was purified from these cultures using a mini-prep.The DNA from the mini-preps were digested to check that the size of the insert was correct(see appendix 7.9 – 7.14) and the plasmids were sequenced. Data for the sequencing is not shown here as it was performed by another member of the laboratory, however, sequencing confirmed that the PCR products were all correct.

3.5 Discussion

Singh et al examined the cell lines Colo 205 and HT29 for the expression of IGF-I, IGF-IR and IGFBP-4[169]. They found that IGF-I expression was either absent or very low in these cell lines. However, they looked for the expression of the IGF-IA transcript, and it may be that although this was not being expressed, that the IGF-IB

transcript was expressed. They were also not able to detect IGF-I in the conditioned media of these cells. Using RT-PCR, IGF-IR mRNA was detected in both of these cell lines. IGFBP-4 mRNA was detected in both cell lines, as was the protein in the conditioned medium.

By producing an animal model of colon cancer and confirming the expression of different components of the IGF system within this model, it may now be used as a basis for animal experiments involving the modulation of the IGF system. The next step will be to directly administer IGFBP-4 to the animal model. This may be done either by the intravenous route, subcutaneously or via a direct intra-tumoural injection. As well as measuring any change in the rate of tumour growth, alterations in the expression of IGF-I, IGF-IR and IGFBP-4 should be examined. One method to do this could be by using quantitative PCR. In addition, Western blotting could be used to examine changes in concentration of the protein product.

The next step would then be to investigate other methods of increasing either local or systemic concentrations of IGFBP-4. This could be done via gene therapy methods or by the targeting of specific tumour markers. One area which would also need to be investigated, is the systemic effect of increasing serum IGFBP-4 levels, and the potential for decreasing proliferation in other non-target organs.

3.6 Conclusions

Two nude mouse models of colon cancer were established and examined to identify which components of the Insulin-like growth factor system they express. Polymerase chain reaction was used to detect mRNA for IGF-I, IGF-IR and IGFBP-4. It was found that all three components were present in both the Colo 205 and HT29 xenograft tumours. The PCR product was confirmed by cloning and sequencing.

Chapter 4 Production of rhIGFBP-4

4.1 Introduction

Recombinant DNA technology can be used to synthesise proteins. The gene coding for the protein of interest is cloned and then inserted into an expression vector. This is used to transform micro-organisms or transfect cultured mammalian cells. These cells will then produce the recombinant protein.

This technique is useful in research as well as in clinical medicine. Proteins produced in transfected cells include insulin, growth hormone, erythropoetin and tissue plasminogen activator which are all used in clinical practice.

4.2 Aims

The aim of this section of the project was to produce human recombinant IGFBP-4, which would be both pure and functional.

4.3 Methods

The IGFBP-4 DNA amplified from the tumour xenograft was cut out of the agarose gel and purified (see chapter 3). The DNA was ligated into the pBAD/His vector and cloned. This was then used to transform competent E. coli cells. The IGFBP-4 protein was extracted and purified from these bacterial cells. Its purity was checked using a silver stain and zinc stain. Its functional ability was determined using a band shift assay as well as a cell proliferation assay.

4.3.1 DNA ligation into the pBAD/His vector

The following were incubated at 4°C overnight:

5µl 2x buffer

1 µl pBAD/His vector

3 µl IGFBP-4 PCR product

1 µl t4 DNA ligase

4.3.2 Transformation of E. coli competent cells with the expression vector pBAD/His

The Invitrogen TOP10 E. coli competent cells were used for transformation by the pBAD/His A vector. 5µl of the ligation reaction was added to 50µl of TOP10 cells and mixed by tapping gently. The mixture was incubated on ice for 30 minutes and then heat shocked by immersion in a 42°C water bath for 30 seconds and then put on ice. 250µl of SOC medium at room temperature was added and the mixture was incubated on a shaker at 37°C for 1 hour at 225rpm. The cells were plated out on LB plates with 50µl ampicillin and 20µl Xgal added. These were incubated overnight at 37°C. The following day, white colonies which had taken up the DNA insert were selected and used to inoculate 5 mls LB. This was incubated overnight at 37°C with shaking.

4.3.3 Expression of rhIGFBP-4

5mls of LB was inoculated with 100µl of transformed bacterial cells and grown overnight at 37°C with shaking. Two millilitres of this culture was then used to inoculate 100mls LB and incubated at 37°C for 2 hours with shaking. One millilitre of 2% arabinose was added to this culture and then incubated for a further 5 hours at 37°C. The cells were harvested by centrifugation for 5 minutes. The pellet was then resuspended in 10mls of PBS.

4.3.4 Harvesting of rhIGFBP-4

The cells were sonicated on ice with 3 10 second bursts at a medium intensity setting. They were then flash frozen in a methanol/dry ice slurry and quickly thawed

at 37°C in a water bath. This sonication/freeze-thaw cycle was repeated a further 3 times. The bacterial cell lysate was centrifuged for 15 minutes at 3000g and the supernatant passed through a 0.8μm syringe filter. The lysate was either stored on ice for immediate use or was frozen at –20°C for later use.

4.3.5 Confirmation of rhIGFBP-4 expression

The cells from each of the five samples of E. coli culture were lysed with 50μl of protease inhibitor/reporter lysis buffer. An equal volume of Laemmli buffer was added and the samples heated at 90°C for 10 minutes, with vortexing every 2 minutes. 20μl of each sample were run on 2 separate 12% SDS gels at 200V and 50mA for 1 hour. One gel was stained with Coomassie Blue and the other was transferred to nitrocellulose membrane for use in a Western blot (ECL method). The membrane was exposed to film for 30 and 45 seconds.

4.3.6 Purification of rhIGFBP-4 using a polyhistidine column - Method 1

The recombinant protein was purified using the Invitrogen Xpress system of protein purification. This system is based on the fact that the recombinant protein has been produced with a polyhistidine tag which has a high affinity for the resin in the purification column. Initial buffers are used to wash out the bacterial proteins and then an eluting buffer is used to disassociate the protein of interest from the column.

4.3.6.1 Equilibriation of the purification column

The column was equilibriated by adding 7mls of native binding buffer and the resin resuspended by repeated inversion of the column. The column was then centrifuged at 800g and the buffer aspirated. This wash was repeated a further two times.

4.3.6.2 Application of the bacterial lysate

5mls of the bacterial lysate was passed through the purification column and rocked for 10 minutes to allow the poly-histidine tagged protein to bind to the resin. The column was then centrifuged at 800g for 5 minutes and the supernatant aspirated. This process was repeated with a further 5mls of bacterial lysate.

4.3.6.3 Column washing

The column was washed three times with 4mls of Native binding buffer pH7.8. It was then washed with 4mls of Native wash buffer pH6.0. This wash was repeated until the OD_{280} was less than 0.01

4.3.6.4 Elution of IGFBP-4

Refer to appendix 7.15

4.3.7 Purification of rhIGFBP-4 using a polyhistidine column - Method 2

To optimise the purification process the protocol was modified. The protocol was identical to Method 1 except that instead of Native binding buffer, PBS/1.5M NaCl was used.

4.3.8 Purification of rhIGFBP-4 using a polyhistidine column - Method 3 (Denaturing protocol)

4.3.8.1 Equilibriation of the purification column

The column was equilibriated by washing it with 7mls of Denaturing binding buffer (refer to appendix 7.16).

4.3.8.2 Application of the bacterial lysate

This was identical to that used in Method 1.

4.3.8.3 Column Washing

The column was washed twice with 4mls of denaturing binding buffer pH7.8 by resuspending the resin followed by rocking for 2 minutes and then centrifugation for 5 minutes at 8000g. The supernatant was removed and the column then washed twice with 4 mls denaturing wash buffer pH 6.0. Then the column was washed twice with 4mls denaturing wash buffer pH 5.3 (refer to Appendix 7.17).

4.3.8.4 Elution of rhIGFBP-4

The recombinant protein was eluted from the column by passing 5mls of denaturing elution buffer through the column (refer to appendix 7.18). Aliquots were collected

at 1ml intervals and the OD_{280} measured. The fractions with the highest OD_{280} were pooled, concentrated, a Bradford protein assay performed, and then the samples were subjected to Western blotting.

4.3.9 Purification of rhBP4 using an immunoaffinity column - Method 1

The HiTrap NHS-activated affinity chromatography system from amersham pharmacia biotech was used.

A single colony of pBAD/BP4 was grown in 5mls of LB/Amp and 3mls of this culture used to inoculate 100mls of LB/Amp 50 and grown overnight. 20% arabinose was added to a final concentration of 0.02%. The cells were grown for a further 6 hours.

The bacterial culture was centrifuged at 5000 rpm for 5 minutes and the pellet resuspended in 20mls PBS/Protease inhibitor/reporter lysis buffer pH7.4 and the mixture vortexed thoroughly to lyse the cells. The lysate was then sonicated for 3 10 second bursts on ice and then centrifuged at 3000rpm for 15 minutes to remove cellular debris. The lysate was passed through a 0.5µg syringe filter.

4.3.9.1 Preparation of the immunoaffinity column

The column was prepared by washing it with 3 column volumes of PBS pH 7.4 and then with 6 column volumes of 1mM ice cold HCl at no more than 5ml/minute. 2.5 mls of the antibody coupling mixture was added to the column and left to stand at room temperature for 30 minutes.

Excess active groups in the column which have not bound ligand were then deactivated and non-specifically bound ligands were washed out. To do this, the column was washed with 30mls of buffer A, followed by 30mls of buffer B, and then 30mls of buffer A. The column was then left to stand for 30 minutes. Then the column was washed with 30mls of buffer B, 30mls of buffer A, and a further 30mls of buffer B (see appendix 7.19).

The column was prepared for the purification process by washing it with 15 mls of start buffer followed by 15mls of elution buffer and 5mls of start buffer. Using a syringe, the bacterial lysate was passed through the column twice at 1ml/minute.

The column was washed with start buffer until the OD_{280} was less than 0.01. The rhIGFBP-4 was eluted with a pH gradient of elution buffer(refer to appendix 7.19). The 1st 10mls of elution buffer was at pH 5. The next 5mls were at pH 4 and the final 5mls at pH 3. Aliquots were taken throughout this process and the OD_{280} measured.

4.3.9.2 Regeneration of the immunoaffinity column

The column was washed with 15mls of binding buffer.

4.3.10 Purification of rhIGFBP-4 using an immunoaffinity column - Method 2

The protocol above was modified slightly so that the protein was eluted using 100mM glycine at pH5 only.

4.3.11 Measurement of protein concentration (Bradford method)

Protein content of samples was measured against dilutions of bovine serum albumin (BSA). A stock solution of BSA was made at 2mg/ml. Dilutions of the stock solution were made as below and 5µl of reporter lysis buffer/protease inhibitor was added. Each standard was made up to 50µl with PBS.

BSA 2mg/ml(µl)	1x RLB/PI(µl)	PBS(µl)	Conc mg/ml
0	5	45	0
0	5	45	0
2	5	43	0.08
4	5	41	0.16
6	5	39	0.24
8	5	37	0.32
10	5	35	0.4

The protein sample to be measured was diluted either x10 or x50 to a volume of 50µl in PBS. One millilitre of working reagent was added to each standard and to each sample which were then incubated at 65°C for 30 minutes(refer to appendix 7.20). The samples were allowed to cool and then measured at 562nm against the standards.

4.3.11.1 Concentrating of protein samples

Protein samples eluted from the polyhistidine column had a concentration of 17µg/ml. As it is usual to load approximately 15µl of protein per well (+15µl Laemmli buffer) this would give a protein load of only 255ng per well. Therefore the protein sample was concentrated in Vivaspin concentrating columns to a volume of 150µl. The protein sample was now at a concentration of 227µg/ml.

The proteins in the concentrated sample were separated using SDS-PAGE with 10µl of the concentrated sample loaded per well (equivalent to 2.27µg of protein).

4.3.12 Protein separation by SDS-PAGE

30µg of protein was made up to 10-15µl with PBS and an equal volume of Laemmli buffer was added. Samples were incubated at 90°C for 10 minutes and were vortexed every 2 minutes during this period. Samples were loaded into either precast 1mm thickness 12% tris-glycine gels (from Invitrogen) or SDS-acrylamide gels immersed in running buffer in gel tanks. A standard protein marker was run alongside the sampes. Gels were run at 200V and 50mA for 1-1.5 hours until the protein marker had run to 1cm above the bottom of the gel.

4.3.12.1 Preparation of 12% SDS-acrylamide gels

The solution for the resolving gel was poured into a gel cassette until the meniscus reached a level 4cm below the top of the cassette. Distilled water was gently poured on top of this to allow the top of the gel to set in a straight horizontal line. The resolving gel was allowed to set in a vertical position at room temperature. The water was then poured off, and the solution for the stacking gel was poured on top of it (refer to appendix 7.21). A comb to generate wells was inserted into the stacking gel and was left to set. There was a distance of approximately 0.5cm between the bottom

of the comb and the top of the resolving gel. Once it had set, the comb was carefully removed and the gel was now ready for use.

4.3.13 Protein transfer to nitrocellulose

After the proteins had been separated by SDS-PAGE, the gel was immersed in transfer buffer (refer to appendix 7.22). A semi-dry blot was used to transfer the proteins to Hybond-c nitrocellulose membrane (from amershampharmaciabiotech). The gel was laid over the nitrocellulose membrane and enclosed between sheets of 3MM paper (from Whatman). The protein transfer was performed at 25V and 95mA per gel for 1 hour.

4.3.14 Coomassie brilliant blue staining

The gel was placed in coomassie brilliant blue stain and shaken for 40 minutes at room temperature (refer to appendix 7.23) . The coomassie brilliant blue was removed and the gel washed in destain buffer, changing the destain buffer every 15 minutes until the bands appear clearly.

4.3.15 Antibody staining using the ECL plus method

The nitrocellulose membrane was blocked for 1 hour with 5% Marvel/TBS at room temperature on a rotary shaker at 1revolution/second. The blocking solution was removed and replaced with 5µl of primary antibody in 10mls of blocking solution for 1 hour. The primary antibody solution was then removed and the membrane washed 3 times with TBS for 5 minutes each time. The membrane was incubated for 1 hour in 10µl of the secondary antibody in 10mls of blocking solution. This was then removed and the membrane washed 3 times for 5 minutes each time in TBS and then washed a final time for 15 minutes in TBS. ECL Plus was added and then the membrane was exposed to film. The film was then developed (refer to appendix 7.25).

4.3.16 Antibody staining using the Western Breeze method

Western Breeze chemiluminescent Western Blotting immunodetection system was purchased from Invitrogen(refer to appendix 7.26). The membrane was immersed in blocking solution and agitated for 30 minutes on a rotary shaker at 1rev/s. The blocking solution was then discarded and the membrane washed twice for 5 minutes each time with 20mls of water. The membrane was then incubated for 1 hour with the primary antibody in 10mls of blocking solution. The membrane was then washed 4 times for 5 minutes with 20mls of antibody wash solution. The membrane was incubated with 10mls of secondary antibody solution for 30 minutes and then washed 4 times for 5 minutes each time with 20mls of antibody wash solution. The membrane was then washed three times for 2 minutes each time with 20mls of water. 2.5mls of Chemiluminescent substrate was added to the membrane and the reaction allowed to develop for 5 minutes. Excess solution was removed from the membrane with filter paper. X-ray film was exposed to the membrane for variable lengths of time and the film developed.

4.3.17 Band shift assay

A band shift assay was performed on the purified rhIGFBP-4 to confirm that it was functional.

5µg of IGF-I (2mg/ml) ie 2.5µl was incubated on ice for 10 minutes with varying concentrations of rhIGFBP-4 in 1mg/ml bovine serum albumin. Laemmli buffer was added and the samples loaded on SDS-acrylamide gels and run at 200V 50mA for 1 hour. The protein was then transferred to nitrocellulose and antibody staining performed probing for IGFBP-4.

Sample	IGF-Iμl	BP4μl	Laemmli μl	BSA μl
1	2.5	5	27.5	0
2	2.5	10	22.5	0
3	2.5	20	12.5	0
4	0	0	25	10
5	2.5	0	22.5	10
6	2.5	0	32.5	0

BSA at a concentration of 0.5mg/ml.

4.4 Validation studies

4.4.1 Aims

The first of these experiments was to find the optimum concentration of primary and secondary antibody for use in Western blotting.

The aim of the next set of experiments was to determine what concentration of arabinose induced the highest expression of rhIGFBP-4 and what the optimum duration of incubation is after the addition of arabinose.

The next aim was to confirm the correct DNA insert into the expression vector pBAD/His.

The final experiment of the validation studies was to confirm that the protocol for lysis of the bacterial cells released intact rhIGFBP-4.

4.4.2 Methods

4.4.2.1 Optimisation of IGFBP-4 antibody concentration for use in Western Blots

25ng IGFBP-4 in 10μl TBS was spotted onto nitrocellulose membrane. This was repeated so that there were 9 spots on the membrane. The primary antibody used was anti-hIGFBP-4 polyclonal antibody raised in goat (purchased from DSL) and was tested at 1:1000, 1:2000 and 1:4000 dilutions. The secondary antibody used was horseradish peroxidase conjugated anti-goat IgG (purchased from Sigma) and was

tested at 1:2000, 1:4000 and 1:8000 dilutions. The membrane was developed with ECL and the film exposed to the nitrocellulose membrane for 30 and 60 seconds.

4.4.2.2 Optimisation of arabinose concentration

5 flasks containing 10mls LB/Amp 50 were inoculated with 100µl of overnight culture of plasmid pBAD/BP4 and incubated for 2 hours at 37°C.
One millilitre of culture was then removed from each flask and centrifuged at maximum speed for 30 seconds and the supernatant removed. One hundred microlitres of arabinose was added to the pellet at concentrations of 0.002, 0.02, 0.2, 2 and 20%, giving final concentrations of 0.00002, 0.0002, 0.002, 0.020 and 0.2% respectively. The cultures were grown for a further 4 hours at 37°C with shaking. A 1ml aliquot was removed from each sample and centrifuged for 30 seconds. The supernatant was removed and the pellet frozen.

The pellets were then resuspended in 50µl protease inhibitor/reporter lysis buffer and a Western blot (ECL method) performed to identify what concentration of arabinose led to the highest expression of rhIGFBP-4.

4.4.2.3 Optimisation of duration of culture

Having ascertained the optimum arabinose concentration, the expression experiment was repeated using a final concentration of 0.02% arabinose and 1ml aliquots of culture taken at t=0 hours after the addition of arabinose, t=4 hours, t=5 hours and t=6 hours, the aliquots were centrifuged and the pellets frozen. Control samples were also prepared where no arabinose was added. Western blotting was performed on these aliquots to find the optimum time period of culture with arabinose.

4.4.2.4 Confirmation of the DNA insert in the expression vector pBAD/HisA

The vector pBAD/HisA was cut with kpn1 (cuts at position 451) and xho1 (cuts at position 431). This was run on an agarose gel to separate the fragments. The cut vector was excised and gel purified. The IGFBP-4 DNA was ligated into this vector and then used to transform competent Top10 cells . The transformation was plated out and positive colonies picked and grown further. The DNA was purified and an xho1 digest performed.

4.4.2.5 Confirmation of harvesting of IGFBP-4

The IGFBP-4 was harvested using the method described previously. An aliquot of the resuspended bacterial pellet and an aliquot of the bacterial lysate were used to perform a Western blot.

4.4.3 Results

4.4.3.1 Optimisation of IGFBP-4 antibody concentration for use in Western Blots

The optimal antibody dilutions were 1:4000 for the primary antibody and 1:4000 for the secondary.

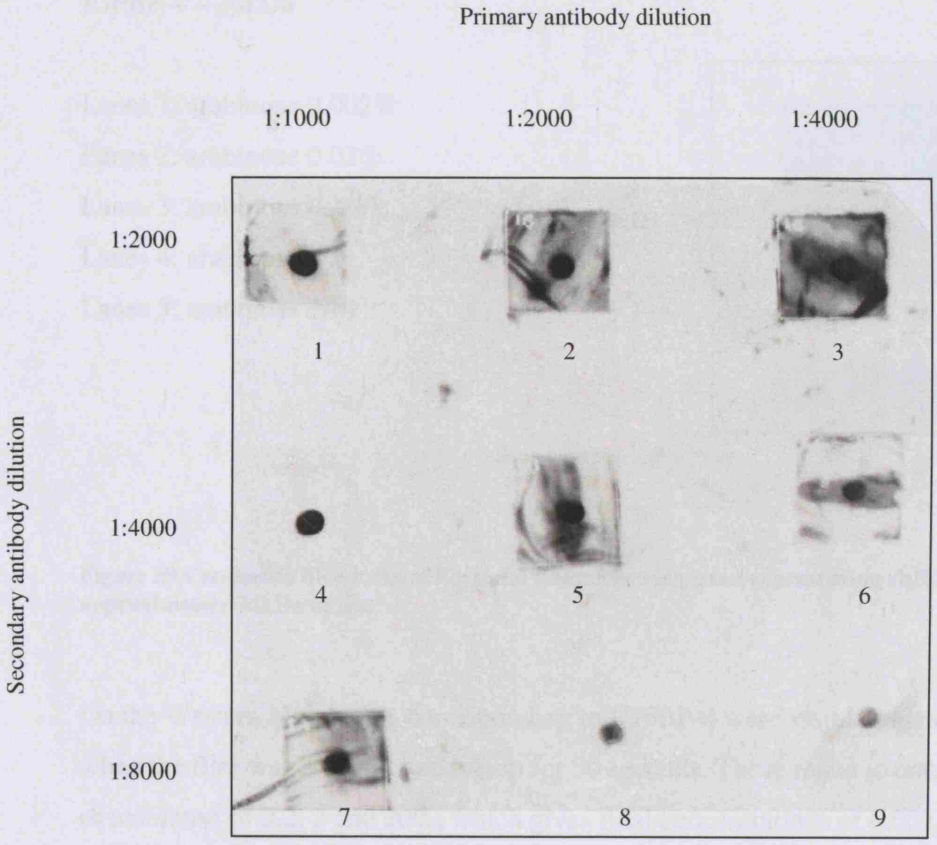


Figure 38 Spot blot showing optimisation of antibody concentration

4.4.3.2 Optimisation of arabinose concentration

The proteins in the bacterial lysate were separated using SDS-PAGE. One gel was stained with Coomassie brilliant blue and the other was used to perform a Western blot.

The Coomassie stained gel showed equal protein loading in all lanes. A strong band was visible at approximately 34kDa. This represents the recombinant IGFBP-4. The rhIGFBP-4 consists of 3 parts:

poly-histidine tag – 3kDa

signal peptide – approx 7kDa

IGFBP-4 – 24kDa

Lanes 1: arabinose 0.002%

Lanes 2: arabinose 0.02%

Lanes 3: arabinose 0.2%

Lanes 4: arabinose 2%

Lanes 5: arabinose 20%

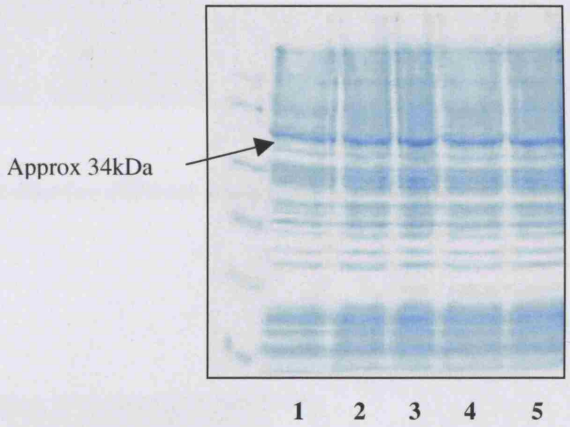


Figure 39 Coomassie blue stain of bacterial lysate showing band representing rhIGFBP-4 at approximately 34kDa in size

On the Western blot, bands corresponding to IGFBP-4 were visible in lanes 3,4 and 5 when the film was allowed to develop for 30 seconds. These relate to concentrations of arabinose of 0.2, 2 and 20%, which gives final concentrations of 0.002, 0.02 and 0.2% respectively. However the strongest band was found using 2% arabinose(lane 4). This relates to a final concentration of arabinose of 0.02%.

Lane	Conc of arabinose added	Final conc of arabinose
1	0.002%	0.00002%
2	0.02%	0.0002%
3	0.2%	0.002%
4	2%	0.02%
5	20%	0.2%

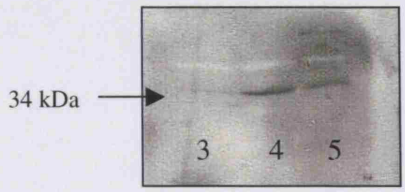


Figure 40 Western blot of bacterial lysate showing different arabinose concentrations after film exposed for 30 seconds

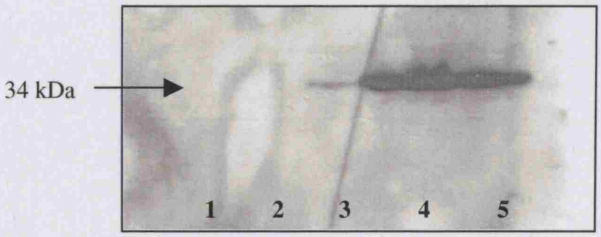


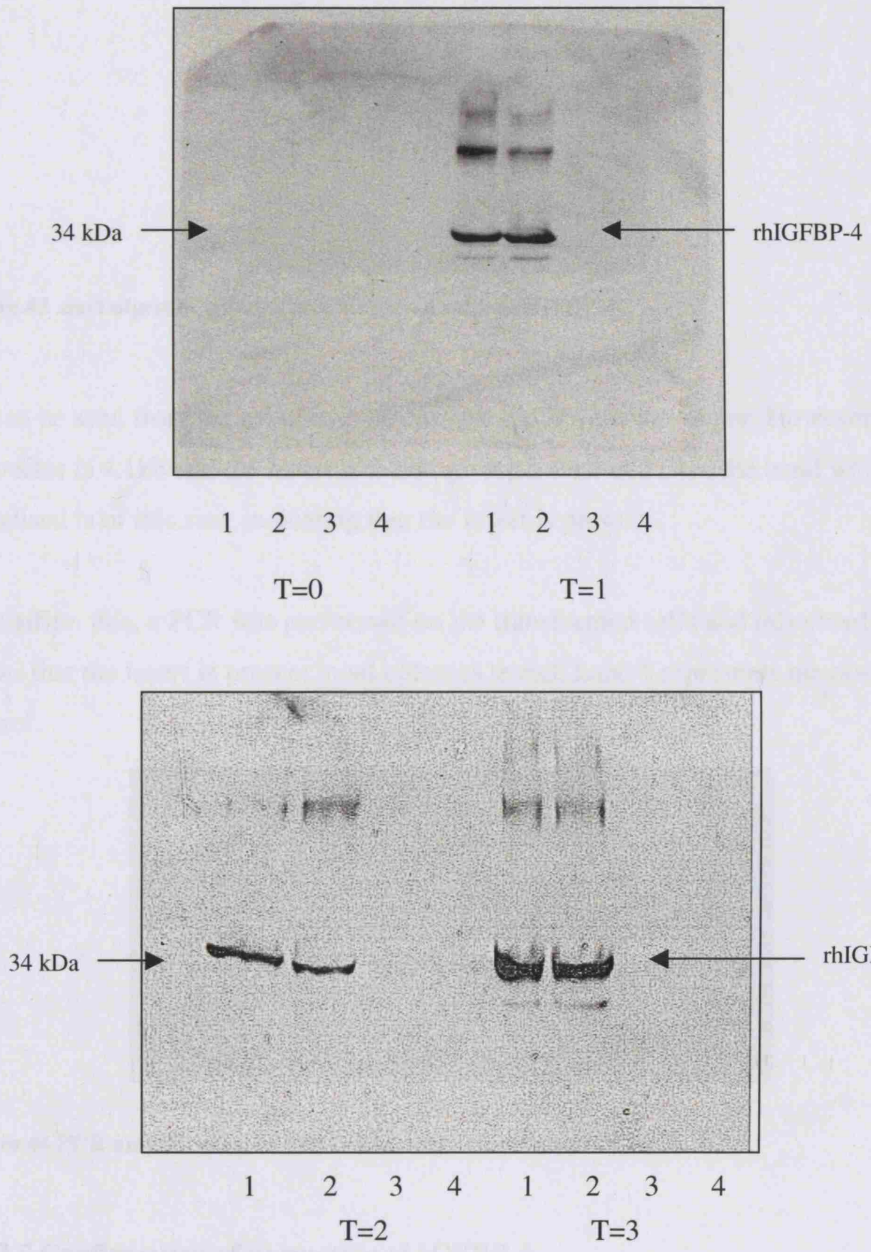
Figure 41 Western blot of bacterial lysate showing different arabinose concentrations after film exposed for 45 seconds

4.4.3.3 Optimisation of time for culture with arabinose

The expression experiment was repeated using the empty vector pBAD/HisA as a control. This experiment was performed to identify the optimum time for incubation after the addition of arabinose. Using the results from the optimisation of arabinose concentration above, the cultures were inoculated with arabinose to a final concentration of 0.02%.

T=	Timepoint
0	No arabinose added
1	4 hours after addition of arabinose
2	5 hours after addition of arabinose
3	6 hours after addition of arabinose

The bacterial samples were prepared as in the arabinose concentration optimisation experiment above and a Western blot performed with each lane loaded in duplicate.



Lanes 1 and 2 Vector with IGFBP-4 DNA insert

Lanes 3 and 4 Control vector without IGFBP-4 DNA insert

Figure 42 Western blots of Bacterial lysate showing different time points

From the Western blot above, it was decided that the optimum time for culture with arabinose was 6 hours (T=3).

4.4.3.4 Confirmation of the DNA insert in the expression vector pBAD/HisA

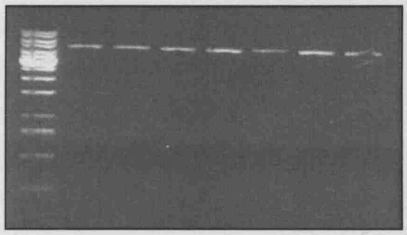


Figure 43 xho1 digest of pBAD/HisA vector containing IGFBP-4

As can be seen from the gel above the enzyme did not cut the vector. However, as the vector is 4.1kb and the insert is 1.2kb, giving a total of 5.3kb, the band which is visualised is of this size, indicating that the insert is present.

To confirm this, a PCR was performed on the transformed cells and this clearly shows that the insert is present in all colonies tested. Lane 1 represents the positive control.

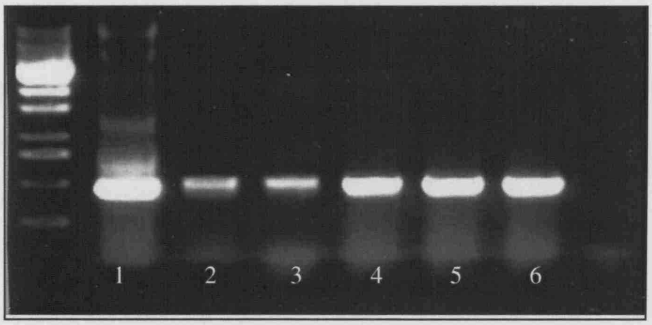


Figure 44 PCR amplification of pBAD/His vector containing IGFBP-4

4.4.3.5 Confirmation of harvesting of IGFBP-4

As can be seen in the Western blot in figure 4.5 there is a weak signal for IGFBP-4 in the bacterial pellet (lane 1) and a strong signal in the bacterial lysate (lane 2). This demonstrates that the protocol used effectively lyses the cells and allows for efficient harvesting of the protein.

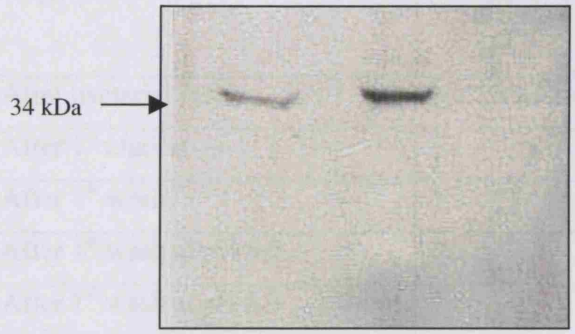


Figure 45 Western blot of rhIGFBP-4

4.5 Results

4.5.1 Purification of rhIGFBP-4 using the polyhistidine column

The sample was subjected to SDS-PAGE and one gel stained with Coomassie brilliant blue and the other used for a Western blot. No bands were seen on the Coomassie stained gel and no band was seen on the Western blot.

It is possible that the concentration of rhIGFBP-4 was too low to be detected by the Coomassie stain and Western blot methods. Therefore a protein assay was performed on the eluted sample which was subsequently concentrated. A further Western blot was performed but no band was visible.

The purification protocol was modified. Instead of using Native binding buffer, PBS/1.5M NaCl was used (Method 2). The optical density was measured at 280nm at every stage of the purification process, to identify at which point the recombinant protein was being lost.

Aliquot no.	Stage of Process	Absorbance	Protein concentration µg/µl
1	After bacterial lysis	Not measured	5.58
2	After 1st elution	3.1	0.6
3	After 1st wash	0.9	0.89
4	After 1st wash at pH 6.0	0.6	0.225
5	After 1st wash at pH 5.3	0.2	0.0961
6	1st ml collected after elution	0.316	0.663
7	2cnd ml collected after elution	0.049	-
8	3rd ml collected after elution	0.047	-
9	4th ml collected after elution	-0.008	-

Aliquot 6 was concentrated in a Vivaspin column.

Protein assays were performed on samples 1 to 6.

20µg of protein from each sample was loaded onto a gel for separation by SDS-PAGE. One gel was stained with Coomassie brilliant blue, and the other used for Western blotting. The Coomassie stained gel showed multiple bands in all lanes, including the 'purified'sample. The Western blot showed a large band in the 'purified' sample.

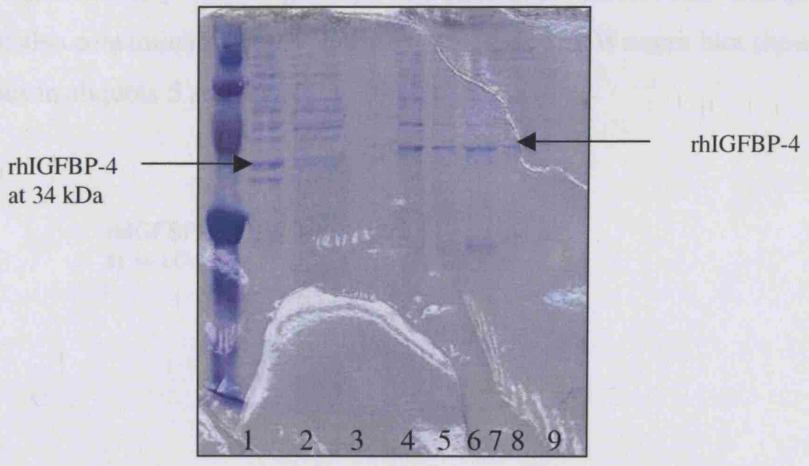


Figure 46 Coomasie blue stain of purified bacterial lysate

The rhIGFBP-4 was now present in the eluent, but it was not a pure sample. Therefore the purification process was repeated using a denaturing wash (method 3) to try to improve the purity of the eluent. Once again the optical density was measured throughout the procedure. Protein assays were performed on samples from each stage of the procedure. The first and second 3mls of eluent were concentrated in Vivaspin columns.

Aliquot	Stage of the process	Protein conc µg/µl
1	Wash at pH 6.0	0.027
2	Wash at pH 5.8	Too low to assay
3	Wash at pH 5.6	Too low to assay
4	Wash at pH 5.5	Too low to assay
5	1st 3mls of eluent	0.1454
6	2cnd 3mls of eluent	0.0059

Aliquot 1 was concentrated further. 20µg of protein from aliquots 1,5 and 6 were loaded onto a gel and subjected to SDS-PAGE.

One gel was stained with Coomassie blue, and another was used for Western blotting. The Coomassie stained gel showed a band at the correct size in aliquots 1,5 and 6, but also contaminants in the 'purified samples'. The Western blot showed large bands in aliquots 5 and 6.

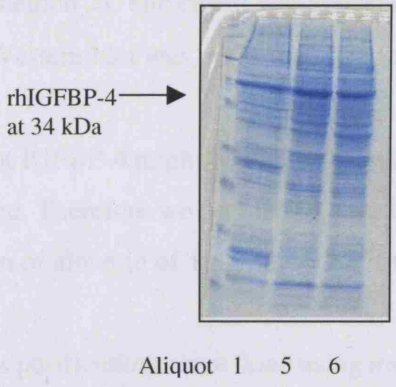


Figure 47 Coomassie blue stain of samples from the purification process

4.5.2 Purification of rhIGFBP-4 using an immuno-affinity column

It was decided to try a different method of purification, as firstly the amount of IGFBP-4 purified using the polyhistidine column method was very low, and secondly, the IGFBP-4 was not being fully purified.

The lysate was passed through the immuno-affinity column and eluted as per the immunoaffinity protocol with a pH gradient of 100mM glycine at pH 5,4 and 3. The eluent was spot blotted and signals were seen in spots 1, 2 and 4, with 4 being the strongest.

1 initial eluent
2 1st wash at pH 7.4
3 1st 5mls of sample
4 2cnd 5mls of sample

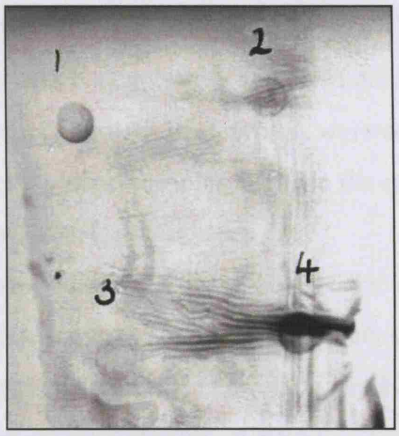


Figure 48 Spot blot of samples from the purification process

The purification process was repeated, this time eluting the protein with 100mM glycine at pH 5 (method 2). The eluent was concentrated to 4.464µg in 25µl (0.179µg/µl). A Western blot was performed but no band was seen.

We postulated that IGFBP-4 might be unstable, and degrading before the Western blot was performed. Therefore we decided to elute it into bovine serum albumin at a final concentration of albumin of 1mg/ml, to see if this improved its stability.

Two simultaneous purifications were done using method 2. One column was eluted into Tris pH7.5, and the other was eluted into Tris pH7.5 with 1mg/ml BSA.

10mls of eluent was concentrated 10-fold using vacuum adapted Vivaspin concetrating columns.

A Western blot was performed. The membrane had a lot of background but a small band could be seen in the bacterial lysate lane, but no band was visible in the eluent.

The immunoaffinity column purification (method 2) was repeated, this time performing all parts of the purification process at 4°C.

The columns were washed with PBS pH 7.4 and eluted with 50mM glycine/NaCl 0.15M (1ml) and neutralised with Tris-HCl pH 9.5 (5µl). The 28mls of eluent were divided into two. One half was concentrated, and the other half had 1ml of 10µg/ml BSA added before being concentrated. Both groups were concentrated 10-fold. SDS-PAGE was then performed on these samples and also the sample from the previous experiment. A zinc stain was performed on the gel.
The zinc stain showed a very faint band of the correct size in the sample without BSA. The sample with BSA showed a band of the correct size, and another band relating to the BSA. The sample from (3) showed multiple bands, however this sample had a 100-fold higher concentration of BSA.

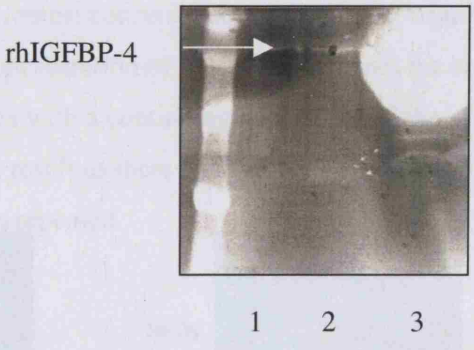


Figure 49 Zinc stain of eluent from purification process

The bacterial lysate was purified using the immunoaffinity column (method 2), and the bacterial lysate and eluent were concentrated 10-fold. A spot blot was performed with these two samples, and as can be seen, a signal is present in the eluent.

Figure 50 Spot blot of eluent and bacterial lysate

4.5.3 Is rhIGFBP-4 functional and does it bind to IGF-I?

A band shift assay was performed to see if the rhBP4 produced is functional. This was ascertained by investigating whether or not it was able to bind to IGF-I. Samples 4-6 are controls.

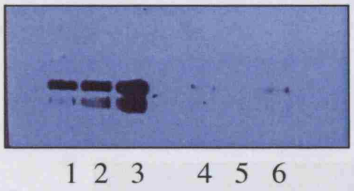


Figure 51a Western blot of band shift assay

Sample	IGF-Iμl	BP4μl	Albumin μl
1	2.5	5	0
2	2.5	10	0
3	2.5	20	0
4	0	0	10
5	2.5	0	10
6	2.5	0	0

As can be seen from the Western blot above, there are strong signals from samples 1, 2 and 3. At the lowest concentration of IGFBP-4, nearly all of it appears at the higher band. As the concentration of IGFBP-4 increases the signal of the lower molecular weight increases with a continuing band at the higher molecular weight. It is difficult to interpret this result as there is no control of IGFBP-4 alone, therefore the experiment was repeated.

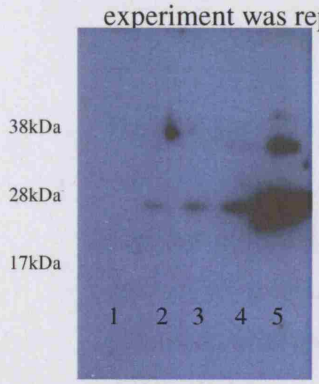


Fig 51b Western blot of band shift assay with film exposed for 60 seconds

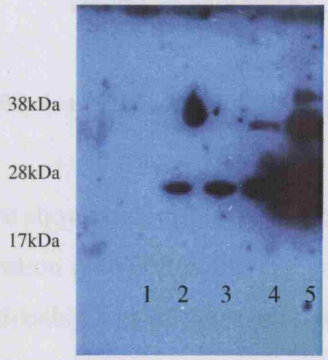


Figure 51c Western blot with film exposed for 90 seconds

Lane	IGF-I μg	IGFBP-4 μg
1	1	0
2	0	25
3	1	25
4	1	50
5	1	100

As can be seen from figures 51b and 51c above, there is no band in the negative control (lane1) but a band visible at just below 28kDa in lane 2 for the positive control (IGFBP-4 alone). As the concentration of IGF-I remains the same and the IGFBP-4 increases, a band can be seen to appear in lane 4 and a strong band is present in lane 5 both at just below 38 kDa. This suggests that the band at the higher molecular weight is due to IGF-I bound to IGFBP-4.

4.5.4 Is rhIGFBP-4 functional and does it inhibit colon cancer growth *in vitro*?

To confirm that the rhIGFBP-4 produced was functional another experiment was performed to see if it retained the ability to inhibit colon cancer cell growth *in vitro*. This was performed using a cell proliferation assay. Each concentration was repeated 4 times and the experiment itself was done in triplicate as with the previous cell culture experiments.

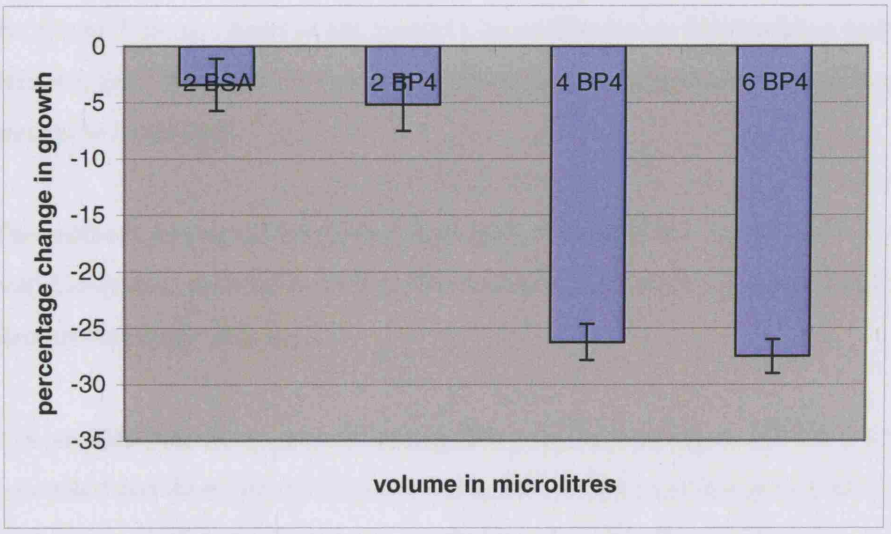


Figure 52 The effect of rhIGFBP-4 on WiDR cell proliferation

The data in the figure above shows that rhIGFBP-4 produced was very effective in preventing cellular proliferation of WiDR colorectal cancer cells. However, the precise dose/response relationship was difficult to establish, due to the fact that the rhIGFBP-4 had to be eluted into BSA to ensure its stability, and therefore it was not possible to quantify the concentration of the IGFBP-4. One of the aims of the

production of the recombinant protein was to use it in future experiments. However, the lack of quantification prohibited its use in future experiments, and instead commercially produced IGFBP-4 was used.

4.6 Discussion

rhIGFBP-4 was produced using the pBAD/HisA vector by transformation of competent Top 10 cells. Western blotting confirmed that rhIGFBP-4 was being expressed.

Protein purification was initially performed using a polyhistidine column. This relies on the polyhistidine tag of the rhIGFB-4 binding to the column, remaining there while other proteins are washed away, and then being eluted. Using the native E coli lysate protocol (method 1) there was no IGFBP-4 detectable in the eluent.

Subsequently the protocol was modified and as a result IGFBP-4 was identifiable in the eluent, both as a band of the correct size on Coomassie blue staining and also on Western Blot. However, on Coomassie staining many impurities were shown to be present in the eluent.

The protocol was modified further, and again, a band of the correct size was seen on both Coomassie staining as well as Western blot, but contaminating proteins were also present in the eluent.

It is possible that due to protein folding, the polyhistidine tag on IGFBP-4 was concealed and therefore not able to bind to the column. Another possibility is that the protein was binding to the column but that the elution buffer was not able to break this bond. Although various different buffers were used to try to optimise this method, there was no improvement in the result. Therefore a different method of purification was used.

The immunoaffinity column method of purification relies on the IGFBP-4 binding to IGFBP-4 antibody in the matrix. The initial experiment showed the presence of IGFBP-4 when a dot blot was performed, however, when a Western blot was done,

this signal disappeared. We hypothesised that the IGFBP-4 is unstable and degraded in the eluent before the Western blot was performed. Therefore the IGFBP-4 was eluted into bovine serum albumin to see if this stabilised it and this appeared to be the case. To confirm if our hypothesis regarding the instability of IGFBP-4, the western blots could be probed looking for fragments of IGFBP-4.

The zinc stain reveals a band of the correct size relating to IGFBP-4, however it does not enable us to confirm that the sample is pure. To do this it would be necessary to perform a Coomassie stain on this sample and compare it with the Coomassie stained gel from the previous purification process.

Having purified the rhIGFBP-4, the next step was to show that it was functional. This was confirmed by two methods. The first was to investigate whether it was able to bind to IGF-I. Although the band shift assay suggests binding of IGF-I to the rhIGFBP-4 this cannot be conclusive as there remains some uncertainty about the identity of the 34kDa band. This would have to be analysed using mass spectrometry. The second method was to investigate whether it could inhibit the proliferation of colon cancer cells *in vitro*. Although due to the sample size the results are not statistically significant, the addition of 6µl of rhIGFBP-4 led to a 28% decrease in cell growth. Due to the fact that the rhIGFBP-4 had to be eluted into bovine serum albumin, it was not possible to quantify its concentration.

4.7 Conclusion

A functional recombinant human IGFBP-4 was produced using an expression vector which was then used to transform competent bacterial cells. Initial purification methods using a polyhistidine column, did not produce a pure sample, therefore purification was undertaken using an immunoaffinity method. The results suggest that the rhIGFBP-4 produced retains the ability to bind to IGF-I as well as to be inhibitory to colon cancer cell growth *in vitro*.

Chapter 5 The regulation of IGFBP-4 protease activity

5.1 Introduction

The activity of IGFBP-4 is dependent on a balance of its expression and its degradation by IGFBP-4 protease. If factors can be identified which regulate protease activity they could be manipulated to prevent the degradation of IGFBP-4, allowing it to remain in the circulation for longer and potentially to inhibit the mitogenic effects of IGF-I on colon cancer cells.

5.2 Aims

The aim of this part of the research was to explore the area of IGFBP-4 protease regulation. The first area to be investigated was to see if protease produced by colorectal cancer cells would be able to degrade exogeneous IGFBP-4. The next aspect that was to be investigated was to determine whether or not IGFBP-4 protease production was IGF-I dependent, and finally, whether or not it was IGFBP-4 dependent.

5.3 Methods

5.3.1 To ascertain if protease from colorectal cancer cells can degrade exogenous IGFBP-4

2mls of WiDR cells at a concentration of $1.25x10^4$cells/ml were plated out in 6-well plates in medium containing 10% serum and incubated for 24 hours. The medium was then removed and the cells washed twice with 4ml of PBS. Two millilitres of serum-free medium containing the following was added to each well:

Well	Containing
1	0ng/ml IGFBP-4
2	100ng/ml IGFBP-4
3	300ng/ml IGFBP-4
4	500ng/ml IGFBP-4

The cells were incubated for a further 24 hours, after which the conditioned medium was removed and concentrated 10-fold in 0.5ml Vivaspin columns and frozen at -70°C. The protein concentration in each sample was measured using the BCA method (see methods section in chapter 4). A volume containing 10µg of protein was taken from each sample and concentrated down to 15µl using vacuum centrifugation. A western blot was performed on these samples and probed for IGFBP-4.

5.3.2 Preparation of conditioned medium to ascertain if Colo 205 cells have protease activity

20mls of a cell suspension at a concentration of $7x10^5$ cells/ml were placed in a $25cm^3$ flask and incubated for 48 hours. The medium was then removed and the cells washed three times with 10mls PBS. 20mls of serum-free medium was added and the cells incubated for a further 24 hours. The conditioned medium was removed and centrifuged for 5 minutes to remove any dead cells. It was then concentrated 135-fold in Vivaspin columns at 5000rev/min for 30minutes. A BCA protein assay was performed and the concentrated conditioned medium was found to have a protein content of 8.735mg/ml.

5.3.3 Preparation of conditioned medium to see if IGF-I induces protease activity in Colo 205 cells

Colo 205 cells were plated out in medium containing 10% serum for 48 hours. They were then washed three times in 10mls PBS. 10mls of serum-free medium was added and incubated for 48 hours. The cells were fairly sparse and in the proliferative phase. The medium was removed and centrifuged at 4°C for 5 minutes at 500rev/min to remove cellular debris. The medium was then concentrated 10-fold at 4°C in 20ml

Vivaspin columns at 5000rev/min. A BCA protein assay was performed and the concentrated conditioned medium had a protein content of 4.305mg/ml.

5.3.4 To ascertain if IGF-I induces protease activity in Colo 205 cells

Conditioned medium containing 120µg of protein (28µl) was added to 2.5ng IGFBP-4 (final concentration of 50ng/ml), IGF-I at final concentrations of 50,100 or 500ng/ml (2.5, 5, 25 ng IGF-I) and made up to 70µl with PBS.

	CMµl	BP4µl(100ng/ml)	IGF-Iµl (2ng/ml)	PBSµl
1 control 1	0	25	2.5	22.5
2 50ng/ml IGF-I	28	25	1.25	16.75
3 100ng/ml IGF-I	28	25	2.5	15.5
4 500ng/ml IGF-I	28	25	12.5	5.5
5 control 2	28	25	0	18
6 control 3	28	0	0	43

The above were incubated at 37°C for 24 hours. 15µl of the reaction mix was removed and 15µl Laemmli buffer was added. These samples were prepared and the proteins separated on 12% tris-glycine gels, which were then used for Western blotting using the Western Breeze method and probed with IGFBP-4 antibody.

The remaining reaction was incubated for a further 24 hours and the process repeated.

5.3.5 Preparation of conditioned medium from the HT29 cell line

10^7 cells were plated out into each of two $25cm^3$ flasks and incubated for 48 hours. The medium was removed and the cells washed 5 times with 5mls of PBS. Flask 1

had 20mls of serum-free medium added, and flask 2 had 20mls of serum-free medium containing 100ng/ml IGF-I added. They were incubated for a further 24 hours. The conditioned medium was removed and 2mls of 10x protease inhibitor (purchased from Sigma) was added to each. The medium was centrifuged to remove any dead cells and then concentrated in 20ml Vivaspin columns. A BCA protein assay was performed. Flask 1 (without IGF-I) had a protein concentration of 4.635mg/ml and flask 2 (with IGF-I) had a protein concentration of 5.905mg/ml.

5.4 Validation studies

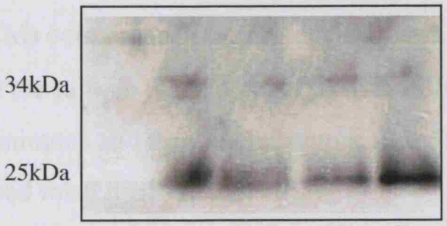
The validation studies for Western blotting are as for those in Chapter 4.

5.5 Results

5.5.1 To ascertain if protease from colorectal cancer cells can degrade exogenous IGFBP-4

This experiment was performed to see if conditioned medium from WiDR cells contained IGFBP-4 protease, and if so, would it be able to degrade exogenous IGFBP-4.

The western blot below shows strong bands in all four lanes. Even in the control (lane 1) which has no exogeneous IGFBP-4 there was a strong band. Therefore this is due to endogenously produced IGFBP-4. Lanes 1 to 4 show a strong band at approximately 25kDa which represents the intact IGFBP-4. There is also a faint band at approximately 34kDa. This may represent IGFBP-4 which is bound to endogenous IGF-I.



Lane 1 control (conditioned media + 0ng/ml IGFBP-4)

Lane 2 conditioned media + 100ng/ml IGFBP-4

Lane 3 conditioned media + 300ng/ml IGFBP-4

Lane 4 conditioned media + 500ng/ml IGFBP-4

Figure 53 Western blot of conditioned media incubated with IGFBP-4

5.5.2 To ascertain if Colo 205 cells have protease activity

This experiment was performed to investigate whether Colo 205 cells have IGFBP-4 protease activity, and if so, what concentration of exogenous IGFBP-4 the protease can degrade.

This experiment was initially performed with the incubation period taking place on ice. A similar experiment was subsequently performed at room temperature to see if this has an effect on the stability of the IGFBP-4.

A 12% SDS-PAGE was run using the following samples:

Lane	Containing
1	100ng/ml IGFBP-4
2	CM
3	CM + 0.5ng/ml IGFBP-4
4	CM + 1ng/ml IGFBP-4
5	CM + 5ng/ml IGFBP-4
6	CM + 10ng/ml IGFBP-4
7	CM + 50ng/ml IGFBP-4
8	CM + 100ng/ml IGFBP-4

Conditioned medium (CM) containing 30µg protein was used. IGFBP-4 was added to the sample on ice and the sample made up to 20µl with PBS. Each sample was incubated on ice for 15 minutes and then loaded onto a 12% SDS gel. A Western blot was performed and probed for IGFBP-4.

Bands of similar intensity were seen in all lanes at approximately 34kDa including lane 2, which represents the endogenous BP4 possibly bound to IGF-I. No bands were seen at a smaller size which would represent degraded fragments of IGFBP-4.

Figure 54 Western blot of conditioned media incubated with IGFBP-4 on ice

For the experiment at room temperature, the samples were made up as below.

Sample	Containing
1	CM
2	Blank
3	1ng/ml IGFBP-4
4	CM + 1ng/ml IGFBP-4
5	10ng/ml IGFBP-4
6	CM + 10ng/ml IGFBP-4
7	100ng/ml IGFBP-4
8	CM + 100ng/ml IGFBP-4
9	500ng/ml IGFBP-4
10	CM + 500ng/ml IGFBP-4

A faint signal can be seen in the original film which reproduced below, in lanes 1,4,6 and 8. There is no signal in lane 10 which was at the edge of the gel.

Figure 55 Western blot of conditioned media incubated with IGFBP-4 at room temperature

It is possible that there is something in the conditioned media that is stabilising the IGFBP-4, and that the IGFBP-4 alone is unstable and degrades. This would tie in with the results from the purification process of rhIGFBP-4 where the protein had to be eluted into BSA to stabilise it.

5.5.3 To see if BSA stabilises IGFBP-4

In previous western blots, the positive control of BP4 alone did not give a signal. Therefore it was postulated that this could be because IGFBP-4 is unstable and degrades. This experiment was designed to determine whether the addition of bovine serum albumin (BSA) would stabilise IGFBP-4.

This experiment was performed using conditioned medium from HT29 cells.
CM1=conditioned media without IGF-I
CM2=conditioned media with IGF-I
concentration of BSA=2mg/ml

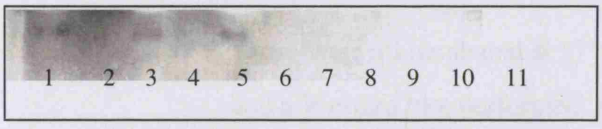


Figure 56 Western blot of IGFBP-4 incubated with bovine serum albumin

Sample	Containing
1	BSA
2	2µg/ml BP4
3	2µg/ml BP4 + BSA
4	0.2µg/ml BP4
5	0.2µg/ml BP4 + BSA
6	CM1
7	CM1 + 2µg/ml BP4
8	CM1 + 0.2µg/ml BP4
9	CM2
10	CM2 + 2µg/ml BP4
11	CM2 + 0.2µg/ml BP4

Samples were incubated at room temperature or on ice for 15 minutes.
Bands were seen in samples 1, 3 and 5 indicating that there was non-specific binding to BSA. Once again, no signal was seen for IGFBP-4 alone.

5.5.4 To see if IGF-I induces protease activity in Colo 205 cells

The aim of this experiment was to establish whether IGF-I added to Colo 205 cells would increase the activity of IGFBP-4 protease in the cells. The activity of the protease was to be determined by its ability to degrade exogeneously added IGFBP-4. Two time points were used – 24 hours and 48 hours after the addition of IGF-I and IGFBP-4.

Conditioned media containing 120µg of protein (28µl) was incubated with 2.5ng IGFBP-4 (final concentration of 50ng/ml) and 2.5, 5 and 25ng IGF-I (final concentrations of 50, 100 and 500ng/ml). The samples were made up to 70µl. There were three controls of no conditioned media , no IGF-I and no IGF-I nor BP4.

Sample	Containing
1	BP4 + 5ng/ml IGF-I
2	CM + BP4 + 2.5ng/ml IGF-I
3	CM + BP4 + 5ng/ml IGF-I
4	CM + BP4 + 25ng/ml IGF-I
5	CM + BP4
6	CM

These were all incubated at 37°C. After 24 hours 15µl of each sample was removed and a Western blot performed. After 48 hours a further 15µl was removed and another Western blot performed.

At 24 hours when the film was allowed to develop for 10 minutes there were bands in samples 2-6 at approximately 34kDa, however the band for sample 5 was of a much lower intensity. The absence of a band in sample 1 may be explained by the degradation of the IGFBP-4. The IGFBP-4 in the other samples are stabilised by the

conditioned medium. Sample 6 shows that there is a significant production of endogenous IGFBP-4 by the Colo 205 cells. The decreased signal in sample 5 cannot be explained.

When the film is left to develop for 15 minutes, another signal is seen in lanes 2 to 6 at approximately 14kDa. This represents a proteolytic fragment of IGFBP-4. The intensity of this band is the same is samples 2 to 6. This indicates that the conditioned medium contains proteolytic activity, and that this is not influenced by the presence or absence of exogenous IGF-I.

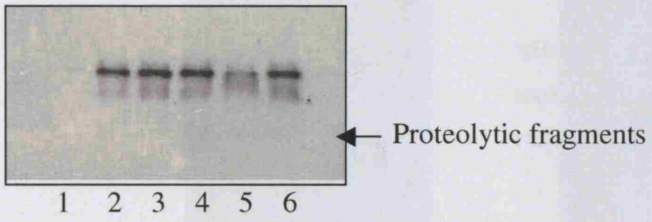


Figure 57 Western blot at 10 minutes exposure - samples from 24 hours

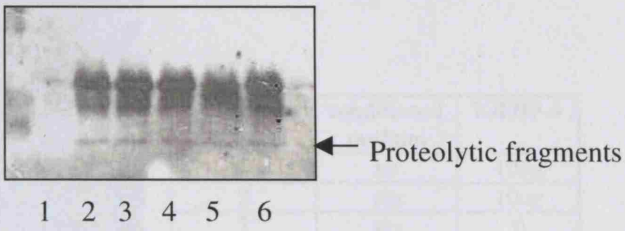


Figure 58 Western blot at 15 minutes exposure - samples from 24 hours

A Western blot was repeated for the samples collected at 24 hours. The picture below shows the signals when the film has been left to develop for 10 minutes. Only the 34kDa band is visible. In this Western blot the sample intensities are equal for lanes 2 to 6. Once again there is no signal for sample 1.

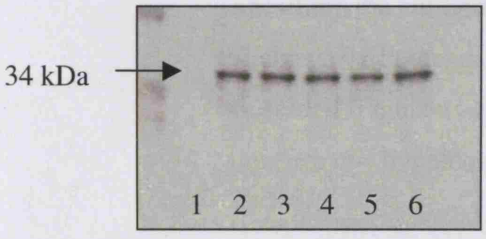


Figure 59 Western blot at 10 minutes exposure - samples from 24 hours.

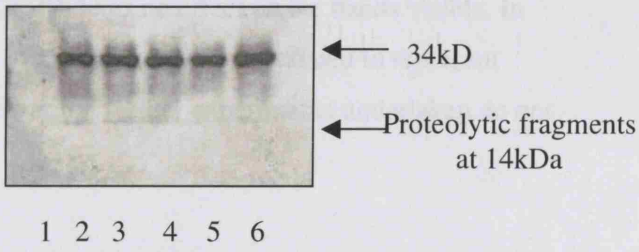


Figure 60 Western blot at 15 minutes exposure - samples from 24 hours.

When the film is developed for 15 minutes, the proteolytic fragments once again becomes visible. At 48 hours there are still signals for lanes 2-6 but these are at a lower intensity than at 24 hours. The bands at approximately 14kDa can still be seen. There is no difference between the different concentrations of IGF-I.

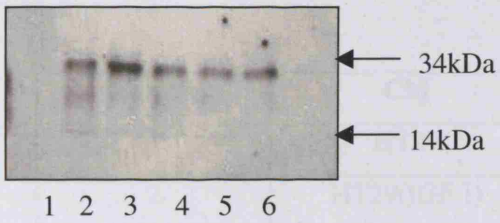


Figure 61a Western blot - samples from 48 hours

In figures 57-61a the bands which are visible 34 and 14kDa are not established to represent specific forms of IGFBP-4. Therefore the experiment was repeated with a control blot in which the IGFBP-4 antiserum was blocked by the addition of excess IGFBP-4 prior to using the antiserum for blotting.

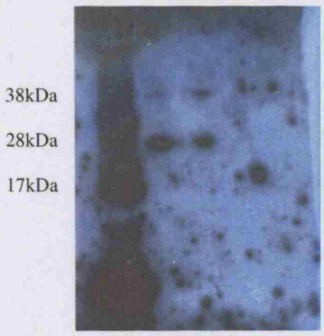


Fig 61b Western blot of IGFBP-4 incubated with conditioned medium from colo 205 cells using IGFBP-4 antiserum

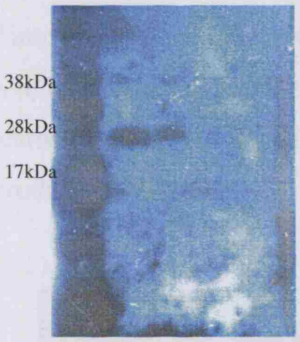


Fig 61c Western blot of IGFBP-4 incubated with conditioned medium from colo 205 cells using pre-absorbed IGFBP-4 antiserum

Lane	conditioned medium	IGFBP-4
1	no	10ng
2	yes	10ng
3	yes	0

When this experiment was performed, no smaller molecular weight bands were identified. Instead there are bands visible at approximately 28 and 38kDa. Pre-absorbing the antibody with excess IGFBP-4 has no effect on the bands visible. In figures 57-61a, bands were visible at 14 kDa which were postulated to represent degraded fragments of IGFBP-4, however the further experiments undertaken do not confirm this hypothesis.

5.5.5 The regulation of IGFBP-4 production

This experiment was performed to establish whether IGF-I has an effect on the expression of IGFBP-4.

It was performed using conditioned media from HT29, Colo 205 and WiDR cell lines.

Sample	CM
1	HT29
2	HT29(IGF-I)
3	Colo 205
4	Colo 205 (IGF-I)
5	WiDR
6	WiDR (IGF-I)

CM with (1) and without (2) IGF-I.

30µg of protein was loaded into each lane. At 5minutes, there was a very faint signal in lane 4, and strong signals in lanes 5 and 6. At 10 minutes there were bands in all 6 lanes. The strongest signal came from the WiDR cell line. There did not appear to be any effect of IGF-I on the production of IGFBP-4.

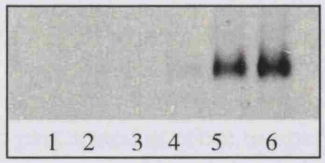


Figure 62 Western blot of conditioned media from cells grown either with or without IGF-I – film exposed for 5 minutes

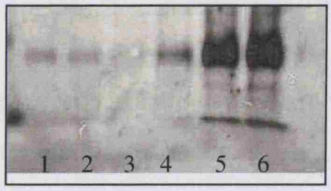


Figure 63 Western blot of conditioned media from cells grown either with or without IGF-I – film exposed for 10 minutes

This experiment was repeated with less primary antibody to decrease non-specific binding. In that instance lanes were only visible in the samples from WiDR.

5.6 Discussion

The first experiment to be performed was to see if conditioned medium from WiDR cells contained IGFBP-4 protease, by incubating the conditioned medium with IGFBP-4 and looking for proteolytic fragments. On Western blotting, no bands which would correspond to degraded IGFBP-4 were seen. Interestingly, as well as bands appearing in all lanes for IGFBP-4, another faint band was seen at a larger size which may represent IGFBP-4 bound to endogenous IGF-I.

The next set of experiments was designed to investigate the IGFBP-4 protease activity of Colo 205 cells using a similar method as above. The first experiment was prepared with the incubation period taking place on ice. Once again, no proteolytic fragments of IGFBP-4 were detected. In all 8 lanes of the Western blot, including those for conditioned medium alone, a band is detected at approximately 34kDa which is postulated to represent IGFBP-4 bound to endogenous IGF-I.

A similar experiment was then performed at room temperature to see if this would affect the stability of IGFBP-4 or the IGFBP-4 protease. On Western blotting, faint bands at 34kDa can be seen in the lanes containing the conditioned medium +/- IGFBP-4. However, in the lanes that contain only exogenous IGFBP-4, no signal is seen.

In the experiment on ice, there is a signal in the lane containing IGFBP-4 alone, but when this is repeated at room temperature, this disappears. This would suggest that IGFBP-4 is inherently unstable at room temperature and breaks down. It may be that when IGFBP-4 is incubated with conditioned medium, a substance is present in the conditioned medium that promotes its stability.

To determine whether the stability of IGFBP-4 could be increased, an experiment was performed whereby IGFBP-4 was incubated with bovine serum albumin, conditioned media or conditioned media from cells grown with IGF-I. The Western blot showed non-specific binding to BSA.

Experiments were then performed to see if IGF-I could induce IGFBP-4 protease activity in the Colo 205 cell line. In the previous experiments, samples were incubated together for a period of 15 minutes and no proteolytic activity was detected. In these experiments it was decided to incubate the samples for periods of 24 and 48 hours. In the previous experiments, samples had been incubated at either room temperature or on ice. This time, samples were incubated at 37°C. At both time points the lane containing IGFBP-4 and IGF-I has no signal, however all other lanes have a band at approximately 34kDa and also a second band at approximately 14kDa. To identify whether these bands represent specific forms of IGFBP-4, the experiment was repeated with a control blot in which the IGFBP-4 antiserum is blocked by the addition of excess IGFBP-4 prior to using the antiserum for blotting. In the repeat experiment there is no difference in the blots when the pre-absorbed IGFBP-4 antiserum is used. In addition, the 14kDa band is not visible. Possible explanations are that the 14kDa band seen originally was due to non-specific binding of the antibody. Another explanation is that a different batch of the Colo 205 cell line was used to prepare the conditioned medium and this may have an effect on the result. In addition it is possible that the cells were not in the appropriate phase of growth at the time when the conditioned medium was taken and this may have an effect on the proteins that the cells produce.

An experiment was performed to see if IGF-I added to colon cancer cells would affect the expression of IGFBP-4. This was done with all three cell lines, and in none of them was a difference shown. Kuemmerle found that R3-IGF-I (an IGF-I analogue which has a 1000-fold reduced affinity for IGF binding proteins compared with IGF-I) increases the synthesis of IGFBP-4 in a concentration dependent manner[214]. They also found that inhibition of the IGF-I receptor by a monoclonal antibody inhibited IGFBP-4 synthesis, once again in a concentration dependent fashion.

Kuemmerle et al also investigated the regulation of IGFBP-4 protease[207]. They found that the co-incubation of IGF-I and IGFBP-4 did not cause significant degradation of IGFBP-4. However, when they were incubated together in the presence of conditioned medium from human intestinal smooth muscle cells, there was significant degradation of IGFBP-4 which was maximal at 24 hours. The effect of IGF-I on degradation of IGFBP-4 in the presence of conditioned medium was

concentration dependent. They also found that the cation-dependant protease inhibitor EDTA and the serine protease inhibitor benzamidine significantly inhibited the degradation of IGFBP-4. However, another protease inhibitor phenylmethylsulfonyl fluoride (PMSF) had no effect on IGFBP-4 degradation. They therefore concluded that the IGFBP-4 protease secreted in the conditioned medium is a cation-dependant serine protease.

Another important finding from their work is evidence that IGFBP-4 protease production is dependent on the phase of cellular growth. They found that IGFBP-4 protease activity was highest in proliferating cells, lower in confluent cells, and declined dramatically in post-confluent cells.

Using R3-IGF-I, they deduced that degradation of IGFBP-4 was dependent on its binding to IGF-I, as R3-IGF-I caused minimal degradation of IGFBP-4. They also found that an IGF-I receptor anagonist, also inhibits the activation of the IGFBP-4 protease.

Pregnancy-associated plasma protein-A(PAPP-A) has been shown to be the IGFBP-4 protease produced by human fibroblast cells[206]. They found that IGFBP-4 was inhibitory to human osteosarcoma MG63 cells treated with non-pregnancy serum, but that it failed to inhibit proliferation of these cells when they were treated with human pregnancy serum containing PAPP-A. However, a protease-resistant IGFBP-4 was inhibitory to cells treated with pregnancy serum. In the presence of PAPP-A antibody, IGFBP-4 was once again able to inhibit proliferation in cells treated with human pregnancy serum. These results show that PAPP-A present in human pregnancy serum, degrades IGFBP-4 and therefore increases the bioavailability of IGF-I.

Rees et al investigated the effect of a mutant IGFBP-4 on cellular proliferation[215]. They used porcine smooth muscle cells which produce a serine protease which has the capacity to cleave IGFBP-4 into 2 inactive fragments of approximately 18 and 14 kDa . By mutating the IGFBP-4 cleavage site which the serine protease in smooth muscle cells uses, they developed a protease-resistant IGFBP-4. In previous studies they found that IGFBP-4 lost its ability to inhibit the proliferative effects of IGF-I in

the presence of smooth muscle cell cultures that are producing the protease.They found that the protease-resistant IGFBP-4 inhibited the DNA synthesis response to IGF-I[215]. They concord with results from Kuemmerle that high density cell cultures have a lower IGFBP-4 proteolytic activity and therefore higher concentrations of active IGFBP-4 than low density cultures[207].

Conover et al also constructed wild type IGFBP-4 proteins by single amino acid substitutions around the cleavage site[216]. The mutant IGFBP-4s had equal affinity for IGF-I and IGF-II as the wild-type IGFBP-4. They were all equally effective in the inhibition of the effects of IGF-I. Conditioned medium from human fibroblasts was prepared containing IGFBP-4 protease. Incubation of wild-type IGFBP-4 and IGF-II for 5-6 hours in the presence of the conditioned medium caused almost complete hydrolysis of wild type IGFBP-4, but only minimal hydrolysis of the mutant IGFBP-4s. At 24 hours, the mutant IGFBP-4s had undergone extensive hydrolysis.

Myers et al found that IGF-I enhanced IGFBP-4 proteolytic activity[217]. The concentration of IGFBP-4 increases in the medium of human decidual cells over a period of 5 days. However, the addition of IGF-I to the growth medium decreases the concentration of IGFBP-4 in the medium. Experiments comparing the effect of IGF-I and the IGF-I analogue [Leu^{24}, 1-62]IGF-I which has a 100-fold decreased affinity for IGF-IR showed that this effect was independent of the IGF-I receptor. Both IGF-I and its analogue caused proteolysis of IGFBP-4. In support of this, when cells were exposed to IGF-I in the presence and absence of α-IR_3 (a monoclonal antibody which blocks IGF-IR), the degree of IGFBP-4 proteolysis was similar.

The role of IGFBP-4 protease in human osteoblast-like (hOB) cells has also been investigated[154]. Although these cells lines all had normal IGFBP-4 mRNA expression and protein secretion, IGFBP-4 could only be detected in the conditioned medium from some of them. They deduced that the regulation of IGFBP-4 was an extracellular phenomenon involving IGFBP-4 protease which is dependent on activation by endogenous IGFs. They found that in those cells where IGFBP-4 completely degraded in the conditioned medium, the cells had an 8-fold increased IGF-I mRNA compared with those cells which were not able to degrade IGFBP-4. They also showed that the addition of antibodies to neutralize IGF-II in the

conditioned medium decreased proteolysis of IGFBP-4. This shows that both endogenous IGF-I and IGF-II have a role in activation of IGFBP-4 protease and subsequent bioactivity of IGFBP-4.

IGFBP-4 protease has been shown to be secreted by a variety of cells including intestinal smooth muscle cells, vascular smooth muscle cells, fibroblasts, human decidual cells, glial cells and osteoblasts[215,216]. Whether or not these all represent an identical protease is unclear.

5.7 Conclusion

The presence of IGFBP-4 proteolytic activity was investigated in colon cancer cell lines *in vitro*. Using a cell-free system, IGFBP-4 proteolytic activity was not detected in the WiDR cell line. In the initial experiments with the Colo 205 cell line, IGFBP-4 protease activity was thought to be detected, as conditioned medium incubated with exogenous IGFBP-4 produced a 14kDa fragment. However subsequent experiments did not corroborate this result.

rhIGFBP-4 is unstable at room temperature, however the addition of conditioned medium prevents its breakdown.

Experiments were performed to see if the expression of IGFBP-4 is IGF-I dependent. In all three of the cell lines tested, IGF-I was shown to have no effect on the expression of IGFBP-4.

Chapter 6 Discussion

6.1 Discussion

This thesis provides further evidence as to the importance of the IGF system in the development of carcinoma of the colon.

The cell culture experiments demonstrate the potent effect of IGF-I on cellular proliferation. This has been confirmed by other groups[43]. IGF-I has been shown to be important in the development of different malignancies.

A Swedish study investigated the relationship between plasma IGF-I and colorectal cancer risk. They found that the risk of developing colon cancer increased with increasing levels of IGF-I[218]. This correlates with findings from the Physicians Health Study which found that men in the highest quintile for IGF-I levels, had a relative risk of 2.51 of developing colorectal cancer compared with men in the lowest quintile[41].

High levels of IGF-I predispose not only to the development of colon cancer, but to other malignancies as well[58,78,85]. A study comparing the plasma IGF-I levels in different ethnic groups in Hawaii and Los Angeles found that Latino-American women who had the lowest breast cancer rates among the different ethnic groups also had statistically significant lower IGF-I levels than the other ethnic groups[219].

This thesis has also shown the role of endogenous IGFBP-4 in controlling cellular proliferation. In addition, the administration of high concentrations of exogenous IGFBP-4 has been demonstrated to decrease the proliferation of colon cancer cells and exogenous IGFBP-4 has been shown to decrease the proliferative effect of IGF-I.

Radioimmunoassays have now been developed which enable the quantification of IGFBP-4 in rat[220] as well as in human serum[221,222]. The concentration of IGFBP-4 has been shown to increase with age. IGFBP-4 in individuals aged 61-87 was found to be 35% higher than in individuals aged 23-40. There was no significant difference in mean plasma IGFBP-4 levels for males and females, although there was a slight

increase in concentration in females with age. The development of a specific IGFBP-4 radioimmunoassay will allow the measurement of IGFBP-4 levels in the serum of patients with colon cancer and allow a comparison to be made with matched controls. More information will also be gained as to the mechanisms that control IGFBP-4 concentration.

IGF-II also has an important role in the regulation of IGFBP-4[223]. In fibroblasts it has been shown that the addition of 10nM IGF-II led to an 85% reduction of IGFBP-4 in the conditioned medium. They concluded that this post-translational decrease in IGFBP-4 was mediated by a protease in the conditioned medium. This could be inhibited by protease inhibitors or by incubation at 4°C. The protein was shown to be cleaved into 2 fragments of 18 and 14 kDa in size. They postulate that part of the role that IGF-I and IGF-II have in controlling cellular growth is by the post-translational regulation of IGFBP-4 by its protease.

IGFBP-4 has been demonstrated to be a powerful inhibitor of IGF-I as shown by the development of a protease-resistant mutant form of IGFBP-4. When this mutant was added to smooth muscle cell cultures, there was a decrease in DNA and cell migration responses to IGF-I[224]. This shows how important not only the factors are in controlling IGFBP-4 expression, but also the factors which regulate the IGFBP-4 protease. As further information is gained as to these regulatory mechanisms, it may be possible to put this knowledge into clinical practice.

In this research we have not been able to positively detect IGFBP-4 protease activity in the Colo 205 cell line. However, further work is required to look in more depth at this area and to do experimental work in other colorectal cancer cell lines as well as in human tissue.

Smith et al investigated the regulation of IGFBP-4 and IGFBP-4 protease in vascular smooth muscle cells. They found that following arterial injury, IGFBP-4 mRNA increased but that this was accompanied by an increase in IGFBP-4 protease activity[225]. Therefore there is a constant level of IGFBP-4 and the expression of IGFBP-4 and the protease may be co-regulated.

IGFBP-4 protease has been investigated in human osteoblasts. It has been found that IGF-II interacting with IGFBP-4 is needed for optimal proteolysis of IGFBP-4[226] in this cell line.

The IGF system has potential uses in the management of colon cancer. There are possibilities for its use as a predictor of people with a higher risk of developing colon cancer, as a target for chemoprevention, and most importantly for treatment.

Higher levels of serum IGF-I are associated with a significantly increased risk of colon cancer[41]. Work needs to be done to investigate if a target population had their IGF-I levels measured whether it would be cost-effective and beneficial to select those with high levels to go onto colorectal cancer screening programmes as they are at a higher risk of developing the disease than those with normal IGF-I levels.

IGF-I is regulated by a number of factors including growth hormone, thyroid hormones, sex hormones and nutritional status.Various dietary factors are associated with higher levels of IGF-I. In those people who have high levels of IGF-I, dietary modification may help them to decrease their IGF-I levels and potentially decrease their risk of developing colon cancer.

By modulating the IGF system in humans it may be possible to decrease the rate of growth of colon cancer and also to decrease its metastatic potential. There are various targets within the IGF system that could be investigated. These include methods to decrease IGF-I, IGF-IR or IGFBP-4 protease, or to increase IGFBP-4 levels.

Gene therapy is one technique that could be used to try to modify the IGF system *in vivo*. Another possibility is the administration either systemically or locally of rhIGFBP-4 to try to decrease tumour growth. The use of recombinant proteins in clinical medicine is becoming increasingly common. The use of recombinant IGF-I as a treatment for a patient with a homozygous partial deletion of the IGF-I gene has been described. As a result of this abnormality the patient suffered from IGF-I deficiency, insulin resistance and short stature. After 1 year of treatment with rhIGF-I, the patient increased his body mass index, his insulin sensitivity reached the normal range and there was an increase in bone mineral density as well as in linear growth[227].

This shows that treatment with recombinant proteins is a reality and that if their scope was widened to include IGFBP-4, this may be beneficial for patients with colon cancer.

Techniques that lead to decreased expression of the IGF-IR or which render it non-functional could have an effect on decreasing tumour growth and metastasis. The transcription of the IGF-IR gene is under the control of tumour suppressor genes such as WT1, p53 and BRCA1, as well as oncogenes such as mutant p53 and c-myb. The interactions between these different factors play a role in determining the concentration of the IGF-IR and subsequent malignant transformation and proliferation[228].

The IGF-I receptor is known to be imperative in the development and progression of colon cancer. Using RT-PCR, IGF-IR mRNA has been shown to be 5-fold higher in colon cancer tissue than in adjacent normal mucosa. The actual increase in IGF-IR mRNA may in fact be higher as one could postulate that as colon cancer comes about as a field change that the adjacent 'normal' mucosa may in fact have some pre-malignant change. Immunohistochemistry comfirmed that the increase in IGF-I mRNA translated into increased protein concentration. They found that 91% of colon cancers stained positive for IGF-IR, whereas there was only faint or no significant staining in the adjacent normal tissue[229].

As well as being involved in tumour development and growth, the IGF system is involved in cellular migration and tumour metastasis. Using the C10 cell line, Playford et al found that IGF-I increased tyrosine phosphorylation of β-catenin and IRS-1[230]. These form a complex with E-cadherin. Tyrosine phosphorylation leads to the dissociation of β-catenin from E-cadherin at the plasma membrane. This may have an effect on increasing cellular motility and metastatic potential.

The effect of the IGF-IR on colon cancer growth and metastasis has also been investigated[231]. The human colon cancer cell line KM12L4 was transfected with a truncated dominant-negative IGF-IR. These cells showed a decreased expression of VEGF mRNA and protein. When this cell line was injected into nude mice there was decreased tumour growth. This was associated with decreased cellular proliferation,

increased apoptosis, as well as decreased VEGF and vessel count. In contrast to control cells, injection of the transfected cell line into the spleen did not produce subsequent liver metastases. Direct injection of the transfected cell line into the liver also failed to produce tumours. These results show the importance of IGF-IR in promoting growth of the tumour at the primary site as well as promoting metastasis.

In another study, a mouse colon cancer model was developed in control and liver-specific IGF-I deficient (LID) mice, whereby colon cancer tissue was orthotopically transplanted onto the caecum. The mice were divided into two groups, one of which received twice daily IGF-I injections for six weeks, and the other group received saline injections. In the second group, there was a higher incidence of tumour growth and of hepatic metastases in the control mice compared to the LID mice. In the group receiving IGF-I injections, there was a higher rate of tumour development and of hepatic metastases compared to the group receiving saline. In the IGF-I group, the LID mice had fewer hepatic metastatic lesions than the control mice. This research shows that IGF-I plays an important role in tumour growth and metastasis in colon cancer[232].

Methods which can modify the IGF system, may help not only with decreasing the rate of tumour growth, but may also help to prevent tumour metastasis.

6.2 Conclusions

Colon cancer is an extremely common condition and in many cases has a poor prognosis with current treatment. For this reason, new approaches to the management of colon cancer need to be explored, which include diagnosis and treatment. One potential direction could be to look at the Insulin-like growth factor system. Within this system, there are many different possible targets.

The cell culture experiments demonstrate the powerful effect of IGF-I on promoting cellular proliferation and of IGFBP-4 in counteracting this. There is a fine balance between these two competing factors and it would appear that in malignancy of the colon, this balance is tilted in favour of IGF-I. One area to investigate would be to see if redressing this balance in favour of IGFBP-4 would either decrease or stop the rate

of growth of colon cancer *in vivo*. This could potentially be done by increasing available IGFBP-4, by methods such as intra-venous injections, direct intra-tumoural injections or gene therapy. Alternatively methods could be developed to down-regulate IGF-I production.

IGFBP-4 protease activity has a role in the bioavailability of IGFBP-4. Protease activity is another target that should be further investigated. If IGFBP-4 protease could be down-regulated or halted completely, this would lead to an accumulation of IGFBP-4 which would remain biologically active and would potentially slow down malignant cell proliferation. Alternatively, work could be done to investigate what effect an antibody against IGFBP-4 protease had on cellular proliferation. More work needs to be done on the mechanisms that regulate IGFBP-4 protease activity in the colon, as well as if protease activity is stopped, what degree of effect this has on IGFBP-4 activity.

Another area which should be investigated further is the IGF-I receptor, as it is this which mediates the effect of IGF-I. If its expression can be down-regulated or altered so that it loses its ligand binding site, or its tyrosine kinase activity, this would be another target for therapy.

6.3 Future Experiments

The role of IGFBP-4 protease should be investigated further. A specific antibody against the protease could be used to directly look at its production by different colon cancer cell lines and compare this to its production by the normal colon to see if there is any difference in expression.

The regulation of IGFBP-4 production could be further explored. Quantitative PCR could be used to look at the effect of IGF-I on IGFBP-4 expression in the colon.

The effect of IGFBP-4 on tumour growth in a nude mouse model of colon cancer should be investigated.

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Thomas, for his constant encouragement and support

Appendix

7.1 Sources of reagents and media used for cell culture

Media and antibiotics were purchased from Gibco.

Medium for Colo 205 cells:
RPMI 1640 + 2mM glutamine

10% foetal bovine serum

1% penicillin/streptomycin

Medium for HT29 cells:
McCoys 5a + 2mM glutamine

10% foetal bovine serum

1% penicillin/streptomycin

Medium for WiDR cells:
EMEM + 2mM glutamine

1% non-essential amino acids

10% foetal bovine serum

1% penicillin/streptomycin

rhIGF-I was purchased from GroPep

rhIGFBP-4 was purchased from GroPep and dsl

7.2 Sequence of oligonucleotide primers

IGF-I
Forward GGATGAGTGCTGCTTCC

Reverse AATGTTATCAAACTTAT

IGFBP-4
Forward TTCAGCCCCTGTAGCGCCCATGACC

Reverse GGCTGAAAACTAGAGCTGGCACGTAG

IGF-IR
Forward GAAGTATGCCGACGGCACCATCGAC

Reverse CTCGCTGATCCTCAACTTGTGATCC

dNTP Master Mix purchased from Bioline

AmpliTaq Gold 5U/µl purchased from Perkin Elmer

Platimum Pfx DNA Polymerase 2.5U/µl purchased from GIBCOBRL

7.3 PCR protocol for IGFBP-4

cDNA	1µl
Fwd(25pm/µl)	1µl
Rev(25pm/µl)	1µl
10x Buffer	5µl
dNTP 100µM	1µl
$MgCl_2$(25mM)	4µl
dH_2O	37.5µl
AmpliTaq gold	0.5µl
Total	50µl

10x buffer:

100mM Tris-HCl pH 8.3

500mM KCl

The enzyme is the last component to be added.

The enzyme is activated at 94°C for 7 minutes. Then there are 35 cycles of 94°C (30 seconds), 62°C (1 minute) and 72°C (1 minute), followed by a 10 minute extension at 72°C.

7.4 PCR protocol for IGF-I using gold Taq

cDNA	1µl
Fwd(25pm/µl)	1µl
Rev(25pm/µl)	1µl
10x Buffer	5µl
dNTP 100µM	1µl
$MgCl_2$	0,3,5,7µl
dH_2O	40.5,37.5,35.5,33.5µl
AmpliTaq gold	0.5µl
Total	50µl

The magnesium concentration was optimised by using 3 different concentrations of 1.5mM, 2.5mM and 3.5mM $MgCl_2$

7.5 PCR protocol for IGF-I using platinum Taq

cDNA	1µl
Fwd(25pm/µl)	1µl
Rev(25pm/µl)	1µl
10x Buffer	5µl
dNTP 100µM	1µl
$MgSO_4$	1µl
Enhancer	0,5µl
dH_2O	39.5,34.5µl
Platinum Pfx	0.5µl
Total	50µl

Platinum Pfx requires $MgSO_4$ rather than $MgCl_2$.

7.6 PCR protocol for IGF-II

cDNA	1µl
Fwd(25pm/µl)	1µl
Rev(25pm/µl)	1µl
10x Buffer	5µl
DNTP 100µM	1µl
$MgCl_2$	3,6,9µl
dH_2O	37.5,34.5,31.5µl
AmpliTaq gold	0.5µl
Total	50µl

7.7 PCR protocol for IGF-IR

CDNA	1µl
Fwd(25pm/µl)	1µl
Rev(25pm/µl)	1µl
10x Buffer	5µl
DNTP 100µM	1µl
$MgCl_2$	3,6,9µl
dH_2O	37.5,34.5,31.5µl
AmpliTaq gold	0.5µl
Total	50µl

The cycle was run with an initial 7 minutes at 94°C, followed by 30 cycles of 94°C (30 seconds), 68°C (30 seconds) and 72°C (1 minute), then 72°C for 10 minutes.

7.8 LB agar plates

2g tryptone

1g yeast extract

1g sodium chloride

200µl 1M sodium hydroxide

3g agar

This was made up to 200mls and then autoclaved and poured into plates. Before use the following were added to each plate: 50µl 50µg/ml ampicillin, 20µl X-Gal and 100µl IPTG and allowed to dry before use.

LB:

2g Tryptone

1g yeast extract

1g sodium chloride

200µl 1M sodium hydroxide

This was made up to 200mls with water and autoclaved. Ampicillin was then added to a final concentration of 50µg/ml.

7.9 IGF-I from the Colo 205 nude mouse model

The IGF-I PCR product was gel purified and ligated into the pGEM-easy vector and used to transform competent cells. Positive colonies were grown and the DNA purified. This was then used to perform a digest with the enzyme EcoR1. This was run on an agarose gel to confirm the correct insert.

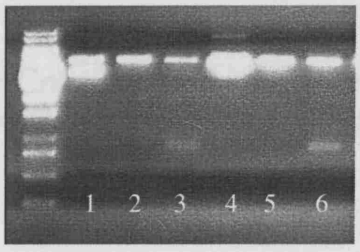

Figure 56 EcoR1 digest of vector containing insert of IGF-I from Colo 205

Inserts for IGF-I can be seen in lanes 3 and 6 at 622bp.

The EcoR1 digest was repeated after using a different mini-prep protocol, and as can

be seen, inserts can be seen in all colonies tested.

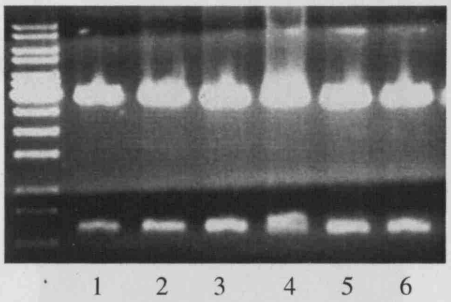

Figure 57 EcoR1 digest of vector containing insert of IGF-I from Colo 205

A Not1 digest was also performed for Colo 205 IGF-I, and inserts were seen in all three colonies tested.

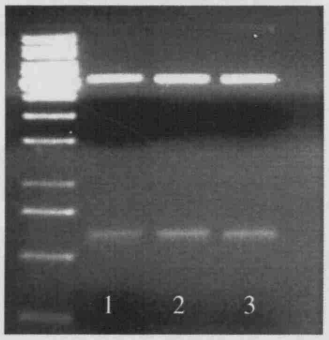

Figure 58 Not1 digest of vector containing insert of IGF-I from Colo 205

7.10 IGFBP-4 from the Colo 205 nude mouse model

EcoR1 was used to perform a digest on the purified IGFBP-4 PCR product. Inserts can be seen in lanes 1,3 and 5.

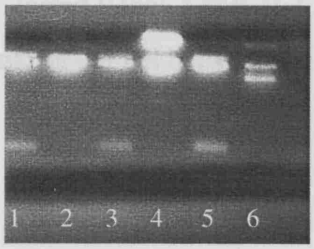

Figure 59 EcoR1 digest of vector containing insert of IGFBP-4 from Colo 205 at 492bp

7.11 IGF-IR from the Colo 205 nude mouse model

Not1 was used to perform a digest on the purified rhIGFBP-4 PCR product. As can be seen there are inserts for IGF-IR in all 6 lanes at 600 bp.

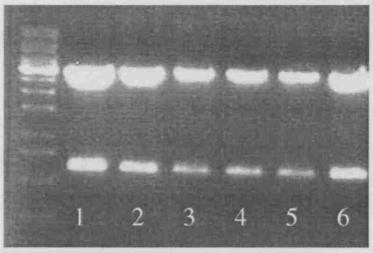

Figure 60 Not1 digest of vector containing insert of IGF-IR from Colo 205

7.12 IGF-I from the HT29 nude mouse model

The IGF-I PCR product from HT29 underwent a digest with Not1. Inserts can be seen in lanes 1, 2, 4 and 5.

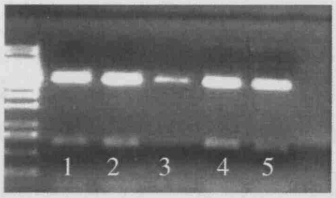

Figure 61 Not1 digest of vector containing insert of IGF-I from HT29

7.13 IGFBP-4 from the HT29 nude mouse model

Instead of performing a digest to confirm the presence of an insert, the plasmids that had undergone a mini-prep were used for PCR. This clearly demonstrates that inserts are present in 2 out of the 3 colonies tested (lanes 1 and 2). Lane 4 shows the positive control. A lane has been left blank on the gel between lanes 3 and 4.

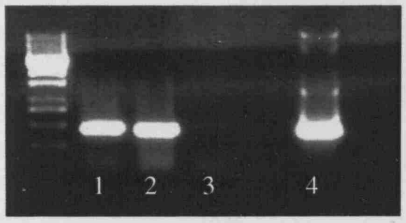

Figure 62 PCR amplification of Vector containing IGFBP-4 from HT29

7.14 IGF-IR from the HT29 nude mouse model

A Not1 digest was performed and inserts were seen in lanes 1, 2, 3 and 5.

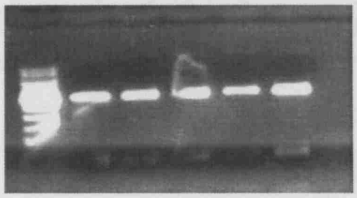

Figure 63 Not1 digest of vector containing insert of IGF-IR from HT29

7.15 Elution of IGFBP-4

The column was clamped vertically and the protein eluted by the application of increasing concentrations of Imidazole buffers. 5mls of the following were applied consecutively:

50mM Imidazole elution buffer

200mM Imidazole elution buffer

350mM Imidazole elution buffer

500mM Imidazole elution buffer

The OD_{280} was measured throughout the elution process to determine the protein concentration in each aliquot.

Native binding buffer:
20 mM Sodium Phosphate

500 mM Sodium Chloride

pH 7.8

Native wash buffer:
20 mM Sodium Phosphate

500mM Sodium Chloride

pH 6.0

7.16 Equilibriation of the purification column

Denaturing binding buffer:
8M Urea

20 mM Sodium Phosphate

500 mM Sodium Chloride

pH 7.8

7.17 Column Washing

Denaturing wash buffer:
8M Urea

20 mM Sodium Phosphate

500 mM Sodium Chloride

pH 6.0

7.18 Elution of rhIGFBP-4

Denaturing elution buffer:
8M Urea

20 mM Sodium Phosphate

500 mM Sodium Chloride

pH 4.0

7.19 Preparation of the immunoaffinity column

Antibody coupling mixture:
200µg IGFBP-4 antibody(1.02ml)

1.48ml coupling buffer

Coupling Buffer:
0.2M Na HCO_3

0.5M NaCl

pH 8.3

Buffer A:
0.5M ethanolamine

0.5M NaCl

pH 8.3

Buffer B:
0.1M acetate

0.5M NaCl

pH 4

Start Buffer:
PBS pH 7.4

Elution Buffer:
100mM glycine

7.20 BCA Protein assay

Working reagent:
49mls bicinchonic acid

1ml 4% copper sulphate

7.21 Preparation of 12% SDS-acrylamide gels

Solution for the resolving gel:

30% protogel	6mls
Tris-SDS pH 8.8 (4x)	3.8mls
water	4.9mls
10% ammonium persulfate	150µl
TEMED	7.5µl

Solution for the stacking gel:

30% protogel	1.3mls
Tris-SDS pH 6.8 (4x)	1mls
water	5.5mls
10% ammonium persulfate	80µl
TEMED	8µl

Tris-SDS pH 8.8 (4x):
91g Tris base

made upto 500mls and pH 8.8

Filter through a 0.4µm filter

Add 2g SDS

Tris-SDS pH 6.8 (4x):
6.05g Tris base

made upto 100mls and pH 6.8

Add 0.4g SDS

10x running buffer for SDS-PAGE:
30.3g tris base

144.2g glycine

10g SDS

made up to 1l in water and pH 8.3

7.22 Protein transfer to nitrocellulose

Transfer buffer:
5.8g tris base

2.9g glycine

0.37g SDS

200mls methanol

made up to 1l with water

7.23 Coomassie brilliant blue staining

50% methanol

10% acetic acid

0.25% (w/v) coomassie brilliant blue

40% deionised water

Destain buffer:
45% methanol

50% deionised water

5% acetic acid

7.24 Antibody staining (ECL method)

TBS:
20ml 0.5M tris-HCL pH 8

8.8g sodium chloride

made up to 1l with water pH 8

0.5M Tris-HCL :
60.55g tris-base

made up to 1l with water pH 8

IGFBP-4 antibody:
Primary antibody Anti-hIGFBP-4 antibody, polyclonal affinity purified, goat

7.25 Antibody staining (Western Breeze method)

Blocking solution:
14mls water

4 mls concentrated buffered saline containing detergent

2 mls concentrated Hammersten casein solution

Antibody wash:
150mls water

10mls concentrated buffered saline solution containing detergent

Chemiluminescent substrate:
2.375 mls CDP-Star chemiluminescent substrate for alkaline phosphatase

0.125 mls chemiluminescent substrate enhance

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