Polypeptide Growth Factors
&
MDBK Cells

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This thesis is dedicated to my
parents and family

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Statement

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge it contains no material that has been previously published by any other person except where due reference is made in the text. The author consents to the thesis being made available for photocopying and loan.

David G. Mottershead

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Summary

The purpose of my thesis is to investigate the growth factors produced by the bovine kidney cell line, MDBK, and to determine which of the growth factors may be involved in autocrine stimulation. The MDBK cell line, cultured under serum-free conditions, was found to produce growth factors which stimulated protein synthesis in the rat L-6 myoblast cell line. As a result of characterizing these growth factors it was determined that the MDBK cell line produces, (1) an IGF binding protein, (2) IGF-2 related growth factors, (3) PDGF, and (4) two growth inhibitors which are related to TGF-β.

The IGF binding protein produced by MDBK cells was detected by the ability of fractions collected after acid gel filtration chromatography of conditioned medium to bind ^{125}I-IGF-1 and interfere in an IGF-1 radioimmunoassay. Two samples of partially purified growth factor from MDBK cell conditioned medium, exhibited IGF-2 receptor competing activity after removal of IGF binding protein by acid gel filtration chromatography. The IGF-2-related growth factors stimulated both protein and DNA synthesis in L-6 myoblasts and eluted from a HPLC gel filtration column with approximate molecular weights of 9,000 and 12,000.

The MDBK cell-derived PDGF was purified approximately 2,300-fold from serum-free conditioned medium by cation exchange chromatography and reverse phase HPLC. Growth factor activity was monitored by the stimulation of protein synthesis in L-6 myoblasts. The purified growth factor (referred to as bovine PDGF or bPDGF) appeared as a major and a minor band of approximately 35,000 molecular weight after SDS-polyacrylamide gel electrophoresis under non-reducing conditions. When the same technique was applied to bPDGF under reducing conditions a series of bands with molecular weights ranging from 14,000 to 30,000 appeared. Reduction of bPDGF was associated with the loss of biological activity.

Bovine PDGF, hPDGF-AB and hPDGF-BB all stimulated protein synthesis in the rat L-6 myoblast cell line to a similar maximum level, whereas the hPDGF-AA isoform had very little effect on protein synthesis in L-6 cells. Similar results were obtained when DNA synthesis in L-6 cells was investigated. In an ELISA assay specific for the A chain of PDGF, bPDGF competed with a similar potency to hPDGF-AA standard, whereas only a slight competition was observed between bPDGF and hPDGF-BB standard in a B chain specific ELISA assay. From the above results, and those from radioreceptor and antibody

neutralization assays specific for the different isoforms of PDGF (E.W. Raines, *pers. comm.*, 1989), bPDGF appears to be the bovine equivalent of hPDGF-AB, where the antigenicity of the bovine B chain differs significantly from that of the human form of the PDGF B chain.

Two growth inhibitors (referred to as GI-1 and GI-2) were partially purified from MDBK cell conditioned medium by cation exchange chromatography and reverse phase HPLC. Detection of growth inhibitor activity was by inhibition of DNA synthesis in L-6 myoblasts. Both growth inhibitors were determined to have a molecular weight of approximately 25,000 as determined by gel filtration HPLC. An anti-TGF-β antibody neutralized 70-75% of the inhibitory activity of GI-1 and GI-2 on L-6 myoblasts, indicating that both growth inhibitors are related to TGF-β.

Various growth factors were examined for their effects on protein synthesis, protein degradation and DNA synthesis in MDBK cells. The insulin-like growth factors (represented by IGF-1, des-(1-3)-IGF-1 (a more potent truncated derivative of IGF-1) and IGF-2) and insulin decreased protein degradation, and stimulated protein and DNA synthesis, whereas bPDGF, hPDGF-BB and hPDGF-AA had no significant effect in any of these assays on MDBK cells. The two MDBK cell derived growth inhibitors (GI-1 and GI-2) and TGF-β1, were found to inhibit DNA synthesis and to slightly decrease protein degradation in MDBK cells.

It is concluded from these studies that PDGF-AB produced by MDBK cells is not involved in an autocrine stimulation of this cell line. However, endogenously produced IGF-2 may be involved in autocrine stimulation, although it is considered unlikely due to the production by MDBK cells of an IGF binding protein that preferentially binds IGF-2. Hence, the relative serum independence of the MDBK cell line appears to be due to factors other than the production and secretion of autocrine growth factors.

CHAPTER ONE : Introduction and Literature Review

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Chapter 1: Introduction and Literature Review

1.1 Aims of the Project

The aim of my thesis is to identify the growth factors produced by the kidney cell line MDBK and to determine to which growth factors the cells are responsive. The project developed after it was discovered by the candidate that the MDBK cell line did not need serum to survive in culture, a characteristic often associated with the endogenous production of growth factors. The serum-free medium conditioned by MDBK cells was investigated for the presence of growth factor activity, particularly IGFs. It was discovered as a result of these experiments that MDBK cells were producing growth factors, at least some of which were not IGFs.

At the start of the work for my thesis the nature of the growth factors produced by MDBK cells was unknown, hence it was necessary to consider the various well known growth factors and compare them with the properties of those produced by MDBK cells. Therefore the current chapter consists largely of a literature review of the well characterized growth factors most likely to be produced by a cell of kidney origin and their mechanism of action. Other topics also considered include the synergistic interactions between growth factors and the autocrine growth of cells. Finally, I also review what is known about the MDBK cell line. The information in this chapter is current as at December 1988.

The term growth factor covers a very wide range of substances with different properties. Consequently, in order to restrict the scope of my thesis only polypeptide growth factors will be considered in relation to the stated aim.

1.2 Polypeptide Growth Factors

Polypeptide growth factors are proteins which regulate the growth state of a cell, resulting either in mitosis or differentiation of the cell. There is considerable overlap between the actions of growth factors and hormones, the former acting generally by autocrine/paracrine mechanisms (see **section 1.6**) and the latter operating in an endocrine manner via the circulatory system. Growth factors have pleiotropic effects on cell metabolism which may result in a stimulation of mitogenesis, an inhibition of mitogenesis or the expression of some differentiated function unrelated to mitogenesis (Sporn and

Roberts, 1988). Effects on cell metabolism include changes in nutrient uptake, ion transport, gene expression, protein synthesis and degradation, as well as RNA and DNA synthesis. The first step in producing these effects is the transduction of the growth factor signal across the plasma membrane, the mechanism of which is considered in the next section.

1.3 Transmembrane Signal Transduction

The response of a cell to a growth factor is initiated by the binding of the growth factor to its specific cell surface receptor. As a result of this interaction a signal transduction pathway is activated, which may ultimately lead to cell division. There are two well characterized transmembrane signal transduction pathways operating in eukaryotic cells (see **figure 1.1**). One pathway makes use of cAMP, produced by adenylate cyclase, as its second messenger (Levitzki, 1988). The effects of cAMP are thought to be mediated by cAMP-dependent protein kinase (PKA) which phosphorylates target proteins on specific Ser and Thr residues (Edelman *et al.*, 1987). The cell surface receptors which activate adenylate cyclase are coupled to this enzyme by guanine nucleotide-binding proteins or G proteins (reviewed in Stryer and Bourne, 1986; Casperson and Bourne, 1987; Gilman, 1987; Casey and Gilman, 1988; Neer and Clapham, 1988). The G proteins are heterotrimeric in structure, the three subunits of which are known as α, β and γ. There are two types of G protein which interact with adenylate cyclase, one of which stimulates enzymic activity, while the other inhibits adenylate cyclase activity. Upon receiving a signal from a receptor the G protein's α subunit binds GTP and disassociates from the β and γ subunits. It is the α-GTP complex which interacts with the adenylate cyclase resulting in either stimulation or inhibition of activity.

The second transmembrane signal transduction pathway is initiated by the activation of phosphatidylinositol 4,5-bisphosphate specific phospholipase C, which results in the production of inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and diacylglycerol (Hokin, 1985; Majerus *et al.*, 1986; Berridge, 1987). Both of these products can act as second messengers, Ins(1,4,5)P_3 causes the release of Ca^{2+} from the endoplasmic reticulum, thus increasing the intracellular Ca^{2+} concentration (Prentki *et al.*, 1984), and diacylglycerol activates the Ser/Thr-specific protein kinase C (PKC) (Bell, 1986; Kikkawa and Nishizuka,

1 The as yet undefined role of tyrosine kinase activity in the actions of IGF, insulin, EGF/TGF-α and FGF has been reviewed by Waterfield, M. D. (1989) Brit. Med. Bull. 45, 541-553

1986). This protein kinase is now known to consist of at least seven isozymes which differ in their activation requirements and tissue distribution (Nishizuka, 1988). The inositol phospholipids are not the only source of diacylglycerol since it is also formed by the action of phosphatidylcholine specific phospholipase C (Wolf and Gross, 1985). *In vitro* substrates of PKC, and hence potential *in vivo* sites of action include the EGF, insulin and IGF-1 receptors, Ca^{2+} transport ATPase, Na^+/K^+ ATPase, Na^+ channel protein, Na^+/H^+ antiport, guanylate cyclase and the S6 ribosomal protein (Kikkawa and Nishizuka, 1986).

After its formation, diacylglycerol is rapidly converted to either a phospholipid or metabolized to arachidonate by the sequential action of diglyceride and monoglyceride lipases (Majerus *et al.*, 1986). Arachidonate is also produced by the action of phospholipase A_2 on phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol (Burgoyne *et al.*, 1987). The effects of arachidonate, both direct and indirect, include stimulation of phospholipase C, PKC, guanylate cyclase and adenylate cyclase (Burgoyne *et al.*, 1987).

The involvement of G proteins in inositol phospholipid turnover is an area of active research and some controversy (Litosch and Fain, 1986; Cockcroft, 1987; Casey and Gilman, 1988). The most compelling evidence for an involvement is to be found for hormones such as vasopressin, epinephrine and angiotensin, the neurotransmitter acetylcholine and the growth factor/protease thrombin (Cockcroft, 1987). There is also some evidence for the involvement of a G protein in bombesin and bradykinin stimulated inositol phospholipid turnover (Wakelam *et al.*, 1986). However, no conclusive evidence has been published connecting the activity of polypeptide growth factor receptors with G proteins and the metabolism of inositol phospholipids.

It has been known for some time that growth factors cause both an increase in the Ca^{2+} concentration and the pH of the cytoplasm (Hesketh *et al.*, 1985). This increase in cytoplasmic pH (pH_i) is caused by the action of an amiloride sensitive Na^+/H^+ antiport located in the plasma membrane (Soltoff and Cantley, 1988). The Na^+/H^+ antiport acts by exchanging intracellular H^+ for extracellular Na^+. Evidence to date suggests that both PKC and Ca^{2+} play a role in activating the Na^+/H^+ antiport (Vicentini and Villereal, 1985; Villereal *et al.*, 1985) by increasing the affinity of the antiport for internal protons (Pouysségur, 1985).

Even though considerable information is now available about the involvement of cAMP and phospholipid turnover in transmembrane signalling, the mechanism of action of most polypeptide growth factors is still not well understood. What is known about each of the growth factors considered in this review will be discussed in the next section.

1.4 Mesodermal Growth Factors

This section consists of an overview of a number of well characterized growth factors which are active on cells and tissues of mesodermal origin, such as muscle, bone, connective tissue and also the kidney and associated blood vessels. The growth factors to be considered include platelet-derived growth factor (PDGF), the insulin-like growth factors (IGFs), insulin, epidermal growth factor (EGF), transforming growth factor α (TGF-α), the fibroblast growth factors (FGFs), transforming growth factor β (TGF-β) and the interferons. Those growth factors primarily produced by, or active on, hematopoietic tissues (predominantly the cytokines, see Harrison and Campbell, 1988), or bone and cartilage will not be discussed in this review.

1.4.1 PDGF

Platelet-derived growth factor (PDGF) is a major mitogen in serum, which the principal function of is thought to be wound healing (Ross *et al.*, 1986). The source of growth factor in most purification protocols for human PDGF has been outdated platelet-rich plasma, as PDGF is stored in the blood platelets and is released during clot formation (Raines and Ross, 1985). Human PDGF is a dimeric cationic protein which has a M_r of approximately 30k (Heldin *et al.*, 1979; Antoniades, 1981; Deuel *et al.*, 1981; Raines and Ross, 1982). Reduction of the protein's disulphide bonds destroys the mitogenic activity and results in the production of multiple protein species of 14-17k M_r. Two distinct but related sequences were revealed in these multiple species upon amino acid sequence analysis (Antoniades and Hunkapiller, 1983; Waterfield *et al.*, 1983), suggesting that PDGF isolated from human platelets consisted of a heterodimer, the two chains of which are known as A and B. This suggestion has recently been confirmed (Hammacher *et al.*, 1988b). The A and B chains are structurally related with approximately 60% amino acid

sequence identity between the finally processed forms (Betsholtz *et al.*, 1986a). In addition to the heterodimer, both of the homodimer isoforms, AA and BB are known to occur (Stroobant and Waterfield, 1984; Heldin *et al.*, 1986b).

The cDNAs and genes for the A and B chains of PDGF have been cloned (A chain cDNAs, Betsholtz *et al.*, 1986a; Collins *et al.*, 1987; Tong *et al.*, 1987; B chain cDNA, Collins *et al.*, 1985; Rao *et al.*, 1986; the A chain gene, Bonthron *et al.*, 1988; Rorsman *et al.*, 1988; and the B chain gene, Johnsson *et al.*, 1984). The studies on the A chain have revealed that the A chain gene can give rise to two structurally different A chain precursors, which differ by the presence or absence of a basic carboxy terminus. These two forms are generated by alternative splicing events.

Work to date on PDGF from orders other than primates has been minimal. Porcine PDGF has been purified from platelets and shown to be of the BB isoform (Stroobant and Waterfield, 1984). In their study Stroobant and Waterfield obtained amino acid sequence data for residues 1-15 from the amino terminus of porcine PDGF and showed 73% identity with that of the human B chain. Bovine PDGF has also been purified from platelets and was found to have a similar molecular weight and isoelectric point to human PDGF (Narczewska *et al.*, 1985). However, neither the amino acid sequence nor the subunit composition was reported. Hamster PDGF (referred to as fibroblast-derived growth factor or FDGF) has been purified from the medium conditioned by the SV40-transformed baby hamster kidney cell line SV28 and found to be closely related to human PDGF (Stroobant *et al.*, 1985). Finally, a report utilising a human placental membrane radio-receptor assay found no species specificity when comparing human PDGF with bovine and porcine PDGF (Czyrski and Gawlikowski, 1987).

Two different classes of receptor for PDGF have been shown to exist which differ in their ligand binding specificity (Hart *et al.*, 1988; Heldin *et al.*, 1988). The A type receptor binds all three isoforms of PDGF (AA, BB and AB) whereas the B type receptor binds only BB homodimers with high affinity (Claesson-Welsh *et al.*, 1988; Escobedo *et al.*, 1988; Gronwald *et al.*, 1988; Hart *et al.*, 1988; Heldin *et al.*, 1988). The cDNA encoding the B type of human receptor has been cloned and is functional when expressed in either BHK or CHO cells (Claesson-Welsh *et al.*, 1988; Escobedo *et al.*, 1988; Gronwald *et al.*, 1988). This receptor exhibits 85% overall amino acid sequence identity with the previously

cloned mouse PDGF receptor (Yarden *et al.*, 1986). Both receptors exhibit intrinsic tyrosine kinase activity, a feature of many polypeptide growth factor receptors (Yarden and Ullrich, 1988).

The effects elicited by the interaction of PDGF with its receptor(s) have been best characterized in mouse 3T3 fibroblasts (see Cochran, 1985 and Rozengurt, 1986). PDGF stimulates inositol phospholipid turnover and the associated appearance of diacylglycerol in quiescent Swiss 3T3 cells (Habenicht *et al.*, 1981). In the same cell line, PDGF also stimulates the release of arachidonate which is metabolized to form a number of prostaglandins (Shier and Durkin, 1982). The production of prostaglandins results in an increase in the intracellular concentration of cAMP via an autocrine/paracrine mechanism (Rozengurt, 1986). PDGF stimulates a number of ion fluxes in Swiss 3T3 cells including those catalyzed by the Na^+/H^+ antiport and the Na^+/K^+ ATPase (Rozengurt, 1986). The stimulation of the Na^+/K^+ ATPase is an indirect effect caused by the increase in intracellular Na^+ concentration due to the stimulation of the Na^+/H^+ antiport. PDGF causes an increase in the cytosolic Ca^{2+} concentration, however the mechanism of this increase does not appear to be via the action of Ins(1,4,5)P_3, since the rise in Ins(1,4,5)P_3 concentration occurs after that of cytosolic Ca^{2+} (Nånberg and Rozengurt, 1988). Nevertheless, evidence from Matvoka *et al.* (1988) indicates that phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) turnover is necessary for mitogenesis in NIH 3T3 fibroblasts initiated by PDGF. In this study a monoclonal antibody to PtdIns(4,5)P_2 completely abolished nuclear DNA labelling induced by PDGF after microinjection into the cytosol of NIH 3T3 cells.

PDGF stimulates the transcription of a number of genes in 3T3 fibroblasts (reviewed in Cochran, 1985). In particular, the proto-oncogenes c-*fos* and c-*myc* are induced, the stimulation of c-*fos* transcription preceding that of c-*myc* (Müller *et al.*, 1984). The c-*fos* proto-oncogene has recently been implicated in the transduction of the mitogenic signal initiated by PDGF. In the study by Mercola *et al.* (1987) antisense mRNA to c-*fos* was introduced into SSV-NIH cells, resulting in the cells reverting to a contact inhibited growth phenotype. The fos protein is thought to act as a *trans*-acting factor which modulates the expression of specific genes (Sassone-Corsi *et al.*, 1988), but how this regulation of gene expression is involved in mitogenesis is not known.

Cell types other than 3T3 fibroblasts are also susceptible to the effects of PDGF. For example, vascular smooth muscle cells (Kawahara *et al.*, 1988; Sjölund *et al.*, 1988), glial cells (Richardson *et al.*, 1988), mesangial cells (Shultz *et al.*, 1988), normal rat kidney cells (van Zoelen *et al.*, 1986), fetal rat pancreatic B cells (Swenne *et al.*, 1988) and fetal liver erythroid cells (Congote, 1987) are all mitogenically responsive to PDGF.

The AA homodimer isoform of PDGF has been compared with the AB heterodimer and found to exhibit different functional activities on human foreskin fibroblasts (Nistér *et al.*, 1988). The AA homodimer exhibits a much lower mitogenic activity on these cells, and a reduced ability to stimulate the autophosphorylation of a purified receptor preparation, however it does cause a decrease in the affinity of the human foreskin fibroblast EGF receptors, indicating that like the AB heterodimer, it activates protein kinase C. Recently, all three isoforms of PDGF have been tested for their effects on human fibroblasts and Swiss 3T3 cells (Kazlauskas *et al.*, 1988). In this study, all three isoforms had similar effects on Swiss 3T3 cells, leading Kazlauskas *et al.* to suggest that differences in the activity of the different PDGF isoforms may simply be due to differences in the number of A and B type receptors.

A number of cells, both transformed and normal, have been found to produce PDGF. These include cells which respond to the mitogen (autocrine growth) as well as those which do not (see **table 1.1**). The release of PDGF by platelets and the production of PDGF by endothelial cells, vascular smooth muscle cells and mononuclear phagocytes has lead to the suggestion that PDGF may be involved in wound healing and atherosclerosis (see Ross *et al.*, 1986 for further discussion). Two related areas in which PDGF is known to play a role are cellular transformation and autocrine growth of cells, topics which are discussed in **section 1.6.**

1.4.2 IGFs

The insulin-like growth factors (IGFs) (also known as somatomedins) are a group of structurally related polypeptide mitogens found in serum which have effects on a wide range of cells and tissues (Nissley and Rechler, 1984; Van Wyk, 1984). The IGFs are made up of two classes of polypeptide known as IGF-1 and IGF-2, both of which were first purified from human plasma (known as nonsuppressible insulin-like activity or NSILA

when originally purified, Rinderknecht and Humbel, 1976a). The name insulin-like growth factor has been given to these polypeptide mitogens due to their structural similarity to insulin (Rinderknecht and Humbel, 1976b). The complete amino acid sequences of human IGF-1 and IGF-2 show that they consist of single polypeptide chains of 70 and 67 residues respectively, which are constrained by three disulphide bonds (Rinderknecht and Humbel, 1978a,b). The overall sequence similarity between the two peptides is 62% and that with the A and B chains of insulin is approximately 47-49%.

A number of variant IGFs have been isolated and identified from human sources. Two larger molecular weight forms of IGF-2, possibly related to precursor forms, have been isolated from human plasma (Zumstein *et al.*, 1985; Gowan *et al.*, 1987). In both cases the data are consistent with the IGF-2 like molecules possessing carboxy terminal extensions. A variant form of IGF-1 with a truncated amino terminus has been isolated from both fetal and adult human brain (Carlsson-Skwirut *et al.*, 1986; Sara *et al.*, 1986).

IGFs have been detected in the serum of a range of species including mammalian and non-mammalian vertebrates (Daughaday *et al.*, 1985). Purification and amino acid sequence analysis has been carried out on rat IGF-2 (Marquardt *et al.*, 1981), rat IGF-1 (Rubin *et al.*, 1982), bovine IGF-1 and IGF-2 (Honegger and Humbel, 1986; Francis *et al.*, 1986, 1988) and chicken IGF-1 and IGF-2 (Dawe *et al.*, 1988). Variant forms of IGF-1 and IGF-2 have also been found in non-human species. For example, a family of IGF-2 related polypeptides (known as multiplication-stimulating activity or MSA) have been characterized in the conditioned medium of the rat liver cell line BRL-3A (Moses *et al.*, 1980), and an IGF-1 truncated at the amino terminus has been isolated from bovine colostrum (Francis *et al.*, 1988). The only difference between the truncated variant (des-(1-3)-IGF-1) and bovine IGF-1 isolated from colostrum (which is identical to human IGF-1) is the absence of the first three amino acid residues in the truncated form. This same study reported that the absence of the amino-terminal tripeptide caused approximately a 10-fold increase in the biological activity of the variant IGF in comparison to intact IGF-1.

The cDNA coding for IGF-1 has been cloned from a number of sources, including human liver (Jansen *et al.*, 1983; Le Bouc *et al.*, 1986), mouse liver (Bell *et al.*, 1986) and rat kidney (Murphy *et al.*, 1987a). Similarly, cDNAs encoding IGF-2 have been cloned

from the following sources, human liver (Bell *et al.*, 1984; Dull *et al.*, 1984; Jansen *et al.*, 1985), mouse placenta (Stempien *et al.*, 1986) and the rat liver cell line BRL-3A (Dull *et al.*, 1984; Whitfield *et al.*, 1984). The availability of these cDNAs has enabled the tissue distribution and developmental regulation of the IGF-1 and IGF-2 mRNAs to be investigated (Lund *et al.*, 1986; Murphy *et al.*, 1987b). These studies support the contention that the liver is the major source of circulating IGF-1 in the adult mammal (Schwander *et al.*, 1983), and suggest a role for IGF-2 in fetal development and in the adult central nervous system.

The receptors for IGF-1 and IGF-2 are structurally very different (Kasuga *et al.*, 1981; Massagué and Czech, 1982). The IGF-1 or type 1 receptor has a similar subunit composition to that of the insulin receptor (see **section 1.4.3**), consisting of α and β subunits held together by disulphide bonds into an $\alpha_2\beta_2$ structure. The intact receptor has a M_r of approximately 350,000 and binds IGF-1 with a higher affinity than IGF-2, as well as exhibiting a low affinity for insulin. The α and β subunits have molecular weights of approximately 130,000 and 90,000 respectively. The amino acid sequence of the human IGF-1 receptor has been deduced from the cDNA sequence and shows extensive similarity to the insulin receptor (Ullrich *et al.*, 1986). As in the case of the insulin receptor, the α subunits are extracellular and contain the ligand binding site, whereas the β subunits cross the plasma membrane and contain the cytoplasmic tyrosine kinase domain.

The IGF-2 or type 2 receptor consists of a single polypeptide chain with a M_r of approximately 250,000, which binds IGF-2 with a greater affinity than IGF-1 and shows no appreciable binding of insulin. Recently the human type 2 receptor cDNA was cloned and primary structure determined (Morgan *et al.*, 1987). The deduced amino acid sequence showed no similarity with either the insulin or type 1 IGF receptors. However, the type 2 receptor amino acid sequence exhibited considerable similarity (80%) to the bovine cation-independent mannose 6-phosphate receptor, suggesting that the two proteins are the human and bovine equivalents of the same protein. The functional significance of this similarity is unknown.

The IGFs circulate in the plasma bound to specific, high affinity binding proteins (Hintz and Liu, 1977; Furlanetto, 1980). Two forms of these binding proteins have been characterized; (1) a growth hormone-dependent form with a M_r of 150-200,000, which

dissociates under acid conditions to yield a 53,000 M_r subunit that binds IGF (Martin and Baxter, 1986), and (2) a 30-40,000 M_r acid stable form. The 30-40,000 M_r protein appears to be the fetal form of binding protein, since fetal and neonatal sera contain high concentrations of this form and do not contain the growth hormone-dependent 150-200,000 M_r form (White *et al.*, 1982; Drop *et al.*, 1984; Romanus *et al.*, 1986).

The role played by the IGF binding proteins in modulating the action of the IGFs is an area of active research. So far, the 53,000 M_r acid stable subunit (BP-53) of the 150-200,000 M_r IGF binding protein complex has been shown to inhibit IGF-1 stimulated ^{3}H-thymidine incorporation in neonatal human skin fibroblasts (De Mellow and Baxter, 1988). This result was obtained when simultaneously incubating IGF-1 and BP-53 with skin fibroblasts. However, preincubation of fibroblasts with BP-53 prior to the addition of IGF-1 resulted in a potentiation of the subsequent IGF-1 effect. Contrasting results have also been obtained with 30-40,000 M_r acid stable IGF binding proteins and their effects on cells in culture. Knauer and Smith (1980) have reported that purified MSA carrier protein inhibits the biological activity of MSA (rat IGF-2) on chicken embryo fibroblasts as measured by either a stimulation of DNA synthesis or glucose transport. Similarly, Ritvos *et al.* (1988) found that the 34,000 M_r binding protein produced by human decidua inhibited IGF-1 stimulated α-[^{3}H]aminoisobutyric acid uptake by JEG-3 cells, a human choriocarcinoma cell line. However, Elgin *et al.* (1987) reported that the IGF binding protein purified from human amniotic fluid potentiates the effect of IGF-1 on DNA synthesis in fibroblasts and aortic smooth muscle cells. The significance of these contradictory results to the function of the IGF binding proteins has yet to be established.

The IGFs have effects on a wide range of cells in which they may act as mitogens or differentiation factors or both. As mitogens they stimulate protein and DNA synthesis, and decrease protein degradation. These effects have been documented in the rat L-6 myoblast cell line (Ballard *et al.*, 1986) and in human skin fibroblasts (Conover *et al.*, 1985). In the L-6 myoblast cell line IGFs also act as stimulators or inhibitors of differentiation, depending on their concentration (Florini *et al.*, 1986a). Other cells in which the IGFs have a differentiation function include oligodendrocytes (McMorris *et al.*, 1986), osteoblasts (Schmid *et al.*, 1984) and granulosa cells (Veldhuis *et al.*, 1985). Cells, other than fibroblasts or myoblasts, which exhibit a proliferative response to IGFs include erythroid

progenitor cells (Kurtz *et al.*, 1985), skeletal muscle satellite cells (Dodson *et al.*, 1985), fetal and adult chondrocytes (Vetter *et al.*, 1986), fetal pancreatic B cells (Swenne *et al.*, 1988), keratinocytes (Ristow and Messmer, 1988) and the following transformed cells, F9 embryonal carcinoma cells (Nagarajan *et al.*, 1982), adrenergic neuroblastoma cells (Mattsson *et al.*, 1986), various small cell lung cancer cell lines (Nakanishi *et al.*, 1988) and T-47D breast cancer cells (Myal *et al.*, 1984).

The mechanism of signal transduction used by the IGFs is an area in which little research has been reported, certainly in comparison with growth factors such as PDGF, EGF and insulin. However, a recent report suggests that IGF-1 acts by activating a phosphatidylinositol-glycan (PtdIns-glycan) specific phospholipase C (Farese *et al.*, 1988), a mechanism thought to be involved in the action of insulin (see **section 1.4.3**). Similarly to PDGF, IGF action may involve the fos protein, since IGF-1 induces c-*fos* mRNA in the rat L-6 myoblast cell line (Ong *et al.*, 1987).

The evidence of production of IGFs by cells in culture covers a range of cell types, particularly fibroblasts, myoblasts, smooth muscle cells, hepatocytes and various transformed cells (see **table 1.2**). IGFs have also been detected in the extracts of various tissues and organs (Nissley and Rechler, 1984). However, *in situ* hybridization studies utilizing synthetic oligomers complementary to portions of the human IGF-1 and IGF-2 mRNAs indicated that the synthesis of these peptides is localized to connective tissues or cells of mesenchymal origin in the human fetus (Han *et al.*, 1987). Hence, the source of the circulating IGFs and the principal mode of action of these growth factors (i.e. autocrine, paracrine or endocrine, see **section 1.6**) is still an area of dispute.

1.4.3 Insulin

Insulin has traditionally been thought of as a hormone due to its endocrine mode of action and effects on cell metabolism. However, it also acts as a growth factor, stimulating the synthesis and inhibiting the degradation of proteins, and stimulating the synthesis of nucleic acids (Kahn, 1985). Insulin consists of two polypeptide chains known as A and B which are held together by disulphide bonds. The protein is initially synthesized as a single polypeptide chain, known as proinsulin, in the pancreatic B cells. Before secretion into the blood the majority of the proinsulin is proteolytically processed to its mature form.

The complete amino acid sequence of the insulin receptor has been deduced from the sequence of the receptor cDNA (Ebina *et al.*, 1985; Ullrich *et al.*, 1985). The sequence shows considerable similarity to that of the type 1 IGF receptor (Ullrich *et al.*, 1986), a similarity which is also present in the receptor's subunit structure. The insulin receptor is a glycoprotein with an $\alpha_2\beta_2$ structure. The α subunit is extracellular and contains the insulin binding site, whereas the β subunit contains the transmembrane and cytoplasmic tyrosine kinase domains.

It has been suggested that the "growth" effects of insulin are mediated primarily by the type 1 IGF receptor and the "metabolic" effects via the insulin receptor (Levine, 1982). Evidence from studies with a monoclonal antibody to the type 1 receptor suggests that insulin can stimulate DNA synthesis in human skin fibroblasts (adult forearm) via both the type 1 receptor and the insulin receptor (Flier *et al.*, 1986). However, other studies in human neonatal foreskin fibroblasts with the same antibody indicate that this effect of insulin is mediated by the type 1 IGF receptor (Van Wyk *et al.*, 1985). Therefore, it appears that the receptor that mediates the growth effects of insulin may vary depending on the cell type.

The mechanism of action of insulin has been an area of considerable research effort which has recently yielded some unexpected results (reviewed in Saltiel and Cuatrecasas, 1988; and Saltiel *et al.*, 1988). These results include the purification and characterization of two putative mediators of insulin action isolated from liver plasma membranes (Saltiel and Cuatrecasas, 1986). These mediators appear to be generated by the phosphodiesterase cleavage of a phosphatidylinositol-containing glycolipid, resulting in the production of inositolphosphate-glycans (Ins*P*-glycans) and diacylglycerol. A PtdIns-glycan specific phospholipase C has been purified from liver plasma membranes which shows a substrate specificity for PtdIns-glycan, the enzyme not catalyzing any hydrolysis of phosphatidylinositol or PtdIns(4,5)P_2 (Fox *et al.*, 1987). The Ins*P*-glycans produced by such a cleavage have been shown to modulate the activity of cAMP phosphodiesterase, adenylate cyclase and pyruvate dehydrogenase *in vitro* (Saltiel, 1987). The effects on these enzymes mimic those of insulin, suggesting that the release of Ins*P*-glycans may play a central role in the mechanism of insulin action. The diacylglycerol produced by the action of a PtdIns-glycan specific phospholipase C may well regulate the action of protein kinase

C. Therefore, the mechanism of insulin action may be analogous to that of peptide hormones which stimulate inositol phospholipid turnover with the concomitant production of Ins(1,4,5)P_3 and diacylglycerol (see **section 1.3**).

The observation that insulin action is associated with the phosphorylation or dephosphorylation of many cellular proteins, often key regulatory enzymes in various metabolic pathways, has lead to the suggestion that a phosphorylation cascade is central to the mechanism of insulin action (reviewed in Czech *et al.*, 1988). This second pathway may well operate in a co-ordinate manner with the PtdIns-glycan pathway. Indeed, both the PtdIns-glycan and phosphorylation pathways may well be integral components of the one pathway.

1.4.4 EGF/TGF-α

Epidermal growth factor (EGF) is a well characterized polypeptide mitogen with diverse biological effects (reviewed in Carpenter and Cohen, 1979). The source of its original isolation was the mouse submaxillary gland (Cohen, 1962). The amino acid sequence of EGF isolated from this source consists of a single polypeptide chain of 53 residues constrained by three disulphide bonds (Savage *et al.*, 1972; Savage *et al.*, 1973). Human EGF was first isolated from urine and found to consist of a single polypeptide chain of 53 amino acid residues which shares 70% sequence identity with mouse EGF (Cohen and Carpenter, 1975; Gregory, 1975). Rat EGF has also been purified from submaxillary glands and the proteins amino acid sequence determined (Simpson *et al.*, 1985). This form of EGF lacks the carboxy terminal five residues of mouse EGF, but exhibits identical mitogenic activity. The two polypeptides exhibit 77% sequence identity, while the identity between rat and human EGF is 69%.

Transforming growth factor α (TGF-α) is a polypeptide mitogen which exhibits structural similarity to EGF and is thought to act via the EGF receptor (reviewed in Derynck, 1988). The amino acid sequence of rat TGF-α, isolated from the conditioned medium of retrovirus-transformed fibroblasts, showed it to consist of a single polypeptide chain of 50 residues which displayed 30% sequence identity with mouse EGF, including the conservation of all six cysteine residues (Marquardt *et al.*, 1984). In another study, the same cell line was found to also produce a 17-19,000 M_r glycosylated precursor form of

TGF-α (Ignotz *et al.*, 1986). This precursor bound to EGF receptors and activated the receptor associated tyrosine kinase activity with the same biological potency as the fully processed form of TGF-α.

EGF cDNA clones have been isolated from male mouse submaxillary gland cDNA libraries (Gray *et al.*, 1983; Scott *et al.*, 1983). The predicted protein precursor consists of 1,217 amino acids with the fully processed EGF sequence (53 amino acids) being flanked by 976 and 188 amino acid segments of the precursor at the amino and carboxy termini, respectively. It has been suggested that the EGF precursor may be a membrane protein, due to the presence of an internal sequence of 20 hydrophobic amino acids, flanked by polar residues, which could anchor the precursor in a membrane (Pfeffer and Ullrich, 1985). If this is the case, the extracellular and cytoplasmic domains of this protein would consist of approximately 1,000 and 160 amino acid residues, respectively. The putative extracellular domain contains eight repeated units structurally similar to EGF, in addition to the actual EGF sequence. Each of these units consists of approximately 40 amino acids with a conserved pattern of cysteine residues, however no proteolytic cleavage sites are obvious between the repeating units. Evidence that the EGF precursor may encode a functional protein has been provided by Rall *et al.* (1985) who detected the unprocessed precursor, but not the 6,000 M_r form of EGF in mouse kidney.

The TGF-α cDNA clones isolated from human (Derynck *et al.*, 1984) and rat (Lee *et al.*, 1985) sources indicate, that like EGF, TGF-α is synthesized as a larger molecular weight precursor (160 and 159 amino acid residues for the human and rat precursors, respectively). This precursor also contains a putative transmembrane sequence which divides the precursor into an extracellular domain of about 100 amino acids, including the fully processed TGF-α sequence, and a 35 residue cytoplasmic domain (Derynck, 1988). The biological significance of the precursor structures of EGF and TGF-α have yet to be elucidated.

The receptor for EGF is one of the best characterized polypeptide growth factor receptors (reviewed in Carpenter, 1987). It consists of a single polypeptide chain with a M_r of approximately 170,000 which exhibits similar affinities for EGF and TGF-α. As is the

case for the receptors for PDGF, IGF-1 and insulin, the EGF receptor is an integral membrane glycoprotein with an extracellular ligand binding domain and a cytoplasmic tyrosine kinase domain.

EGF is active on many cell types including, fibroblasts, glial cells, keratinocytes, endothelial cells, hepatocytes, chondrocytes, mammary epithelial cells, and granulosa cells (Carpenter and Cohen, 1979). The effects of EGF on these cells include increased transport of ions and nutrients, stimulation of RNA, DNA and protein synthesis, and stimulation of cell proliferation. EGF has also been found to cause the inhibition of protein breakdown (Gunn *et al.*, 1983). The mechanism whereby these effects are achieved has been an area of dispute, in particular over the involvement of inositol phospholipid turnover in the action of EGF. For example, EGF has been found to stimulate PtdIns(4,5)P_2 hydrolysis in BALB/MK keratinocytes (Moscat *et al.*, 1988) and WB hepatic epithelial cells (Earp *et al.*, 1988), whereas EGF has no effect on phosphoinositide breakdown in Chinese hamster lung fibroblasts (L'Allemain and Pouysségur, 1986) and BALB/c 3T3 fibroblasts (Besterman *et al.*, 1986). A possible explanation for these contrasting results has been suggested by Paris *et al.* (1988) based on a synergistic interaction of EGF with an endogenous growth factor which stimulates inositol phospholipid turnover (see **section 1.5**). Recently, EGF was found to stimulate the hydrolysis of a PtdIns-glycan (Farese *et al.*, 1988), a process possibly involved in the mechanism of action of insulin (see **section 1.4.3**).

In most assays EGF and TGF-α have similar activities, although some differences have also been noted. This is an area which has recently been reviewed (Derynck, 1988) and will not be considered further here.

TGF-α-like mitogens are produced by a number of cells in culture, particularly transformed cells. Non-transformed cells which produce TGF-α include bovine anterior pituitary cells (Samsoondar *et al.*, 1986) and activated human alveolar macrophages (Madtes *et al.*, 1988). Transformed cells which produce TGF-α include the human MCF-7 breast carcinoma (Dickson *et al.*, 1986), human colon carcinomas (Coffey *et al.*, 1986) and Abelson murine leukemia virus transformed rat embryo fibroblasts (Twardzik *et al.*, 1982). TGF-α-like activity from conditioned medium has been purified and identified as TGF-α by amino acid sequence analysis for the following transformed cells, Snyder-Theilen feline sarcoma virus transformed rat embryo fibroblasts, Moloney murine sarcoma virus

transformed mouse 3T3 cells and two human metastatic melanoma cell lines (Marquardt *et al.*, 1983). In contrast, very few cells have been shown to produce EGF in culture, examples being WS-1 human foreskin fibroblasts (Kurobe *et al.*, 1985) and a human salivary gland adenocarcinoma (Sato *et al.*, 1985).

The *in vivo* functions of the EGF/TGF-α family of polypeptides have not been well characterized, although evidence to date suggests a possible involvement for these polypeptides in development (Twardzik, 1985) and wound healing (Schultz *et al.*, 1987). A role for EGF/TGF-α in carcinogenesis has also been suggested (Stoscheck and King, 1986).

1.4.5 FGFs

The fibroblast growth factors (FGFs) are a group of structurally related polypeptide mitogens found principally in neural tissue, but also produced by some tumour cells (Folkman and Klagsbrun, 1987). These growth factors have been referred to under various names reflecting their tissue of origin, their *in vitro* activity or a property of the isolated mitogen (Thomas and Gimenez-Gallego, 1986). For example, the terms endothelial cell growth factor, eye-derived growth factor, brain-derived growth factor, retina-derived growth factor and chondrosarcoma-derived growth factor all refer to various FGF species. An alternative name for the FGFs is that of heparin-binding growth factors, since all these polypeptide mitogens show high affinity binding to heparin. In fact, heparin affinity chromatography is an essential step in the efficient purification of these growth factors (Lobb *et al.*, 1986).

The FGFs can be divided into two classes known as basic FGF (bFGF) and acidic FGF (aFGF) based on properties such as isoelectric point and amino acid sequence (Thomas and Gimenez-Gallego, 1986). The complete amino acid sequences are available for the bovine brain-derived aFGF (Gimenez-Gallego *et al.*, 1985), a 140 amino acid polypeptide, and the bovine pituitary-derived bFGF (Esch *et al.*, 1985), a 146 amino acid polypeptide. Comparison of the two sequences shows that aFGF and bFGF are structurally related, exhibiting 53% sequence identity. It appears from the nucleotide sequence data obtained from the cDNA clones of aFGF (Jaye *et al.*, 1986) and bFGF (Abraham *et al.*, 1986a,b) that both peptides are synthesized as 155 amino acid polypeptides with short

amino terminal extensions and no detectable signal sequence. Thus, the isolated 140 and 146 amino acid forms of aFGF and bFGF are likely to have been produced by protease action at the amino terminal end of the polypeptide.

Acidic FGF and bFGF differ in their tissue distribution. While aFGF is found mainly in neural tissue, bFGF has been isolated from a range of sources including tissues such as the pituitary, brain, hypothalamus, eye, cartilage, bone, corpus luteum, adrenal gland, kidney, placenta, and cells such as macrophages, a chondrosarcoma and a hepatoma cell line (Folkman and Klagsbrun, 1987).

The receptor(s) for the FGFs has not been purified or sequenced, characterization being limited to cross-linking of ^{125}I-FGF ligands to the receptor(s), and the analysis of glycosylation and its function in receptor action (Feige and Baird, 1988). The FGFs are active on a number of cell types including, fibroblasts, endothelial cells, chondrocytes, osteoblasts, and smooth muscle and glial cells (Thomas and Gimenez-Gallego, 1986). The specific activities of aFGF and bFGF are only identical in the presence of heparin, whereas in the absence of heparin, aFGF is much less active, displaying of the order of 1% the activity of bFGF (Gimenez-Gallego *et al.*, 1986). It has been suggested that this effect of heparin is due to it protecting aFGF from proteolytic inactivation (Rosengart *et al.*, 1988).

In BHK-21 cells the FGFs inhibited protein breakdown as well as stimulating DNA synthesis (Ross and Ballard, 1988). This effect on protein breakdown is similar to that caused by IGFs, insulin and EGF (see **sections 1.4.2** and **1.4.4**). The mechanism of action of the FGFs is being studied in the Chinese hamster lung fibroblast cell line by Pouysségur's laboratory. In their studies with this cell line Magnaldo *et al.* (1986) have found that bFGF initiates early events such as an increase in cytoplasmic pH, a rise in the cytoplasmic Ca^{2+} concentration and an increase in c-*myc* expression, followed by a 30-40 fold increase in the nuclei labelling index. However, bFGF failed to activate inositol phospholipid turnover, and the rise in cytoplasmic free Ca^{2+} was dependent on the presence of external Ca^{2+}. Hence, the increase in cytosolic Ca^{2+} does not seem to be mediated by the action of Ins(1,4,5)P_3, which is also the case for EGF and insulin in this cell line (Pouysségur *et al.*, 1988). It remains to be seen whether FGF will have any effect on PtdIns-glycan breakdown as is the case for IGF-1, EGF and insulin (Farese *et al.*, 1988; Saltiel and Cuatrecasas, 1988).

Evidence to date, has suggested the FGF family of polypeptides have roles in both blood vessel formation (angiogenesis) (Folkman and Klagsbrun, 1987), and developmental processes in the early vertebrate embryo such as mesoderm induction (Kimelman and Kirschner, 1987; Slack *et al.*, 1987; Kimelman *et al.*, 1988). A role for the FGF family of polypeptides in carcinogenesis has recently been highlighted by the realisation that the products of the oncogenes *hst* (Yoshida *et al.*, 1987), *int*-2 (Smith, R. *et al.*, 1988; Mansour and Martin, 1988) and the FGF-5 gene (Zhan *et al.*, 1988) all code for FGF related proteins.

1.4.6 TGF-β

The type β transforming growth factors (TGF-β) form a family of structurally related proteins which act on a wide variety of cells to alter their proliferative and phenotypic properties (reviewed in Sporn *et al.*, 1987; and Roberts and Sporn, 1988). Human platelets were one of the original sources for the purification of TGF-β (Assoian *et al.*, 1983), along with human placenta (Frolik *et al.*, 1983) and bovine kidney (Roberts *et al.*, 1983). TGF-β, as isolated from human platelets, is a homodimeric protein of 25,000 M_r, consisting of two polypeptide chains of 12.5k M_r held together by disulphide bonds (Derynck *et al.*, 1985). The biological activity of TGF-β is destroyed upon reduction (Roberts *et al.*, 1983).

Since the original purification of TGF-β, the protein has been isolated from a number of sources including, bovine bone (Seyedin *et al.*, 1985, 1986, 1987), porcine platelets (Cheifetz *et al.*, 1987) and the conditioned medium of the human prostatic adenocarcinoma PC-3 (Ikeda *et al.*, 1987). The purification and characterization of TGF-β from these sources indicated that there existed at least two different forms of the polypeptide. Both these forms are homodimers and are known as TGF-β1 and TGF-β2. A heterodimer consisting of one TGF-β1 chain and one TGF-β2 chain has also been isolated from porcine platelets, and denoted TGF-β1.2 (Cheifetz *et al.*, 1987).

The complete amino acid sequence of the human TGF-β1 chain has been deduced from a cDNA clone (Derynck *et al.*, 1985) and that of the human TGF-β2 chain by amino acid sequence analysis (Marquardt *et al.*, 1987). The two sequences show approximately 70% identity. A third TGF-β chain has been cloned from human (Derynck *et al.*, 1988; Dijke *et al.*, 1988), porcine (Derynck *et al.*, 1988) and chicken (Jakowlew *et al.*, 1988a)

sources. The deduced amino acid sequence of this polypeptide (termed TGF-β3) exhibits approximately 80% sequence identity with the sequences of TGF-β1 and TGF-β2. A fourth TGF-β sequence has recently been cloned from a chicken embryo chondrocyte cDNA library (Jakowlew *et al.*, 1988b). This sequence, known as TGF-β4, exhibits 60-80% amino acid sequence identity with the sequences of processed TGF-β1, 2 and 3.

The TGF-β released by cells and platelets is in a biologically inactive form (Lawrence *et al.*, 1985; Pircher *et al.*, 1986). This latent TGF-β is activated *in vitro* by treatments such as acidification, alkalinization or exposure to urea. The latent form of TGF-β has been purified from human platelets, and found to consist of TGF-β1, the amino-terminal part of the TGF-β1 precursor lacking the signal peptide, and a component of 125-160,000 M_r distinct from any previously described proteins (Miyazono *et al.*, 1988). As found previously, the latent TGF-β was activated by exposure to extremes of pH or to urea. The reason for the latent TGF-β's inactivity is likely to be the inability of this form of the polypeptide to bind to a receptor (Wakefield *et al.*, 1987 and 1988).

Cells of a wide variety of types and origins express receptors for TGF-β, including those of fibroblastic, epithelial, endothelial, chondrogenic, myogenic, adipogenic, hematopoietic and neural origin (Wakefield *et al.*, 1987; Cheifetz *et al.*, 1988). These receptors consist of at least three types, a 280,000 M_r, a 85,000 M_r and a 65,000 M_r receptor, as determined by affinity cross-linking techniques (Cheifetz *et al.*, 1986). It is not known if these receptors are related in any way. The 280,000 M_r form is a subunit of a larger molecular weight disulphide-linked TGF-β receptor complex (Massagué, 1985). The various forms of TGF-β receptor appear to differ in their affinities for TGF-β1 and TGF-β2 (Segarini *et al.*, 1987; Cheifetz *et al.*, 1988). The study by Cheifetz *et al.* (1988) found that all three forms of TGF-β (β1, β2 and β1.2) were equipotent in competing for binding to the 280,000 M_r receptor. However, TGF-β1 was more potent than TGF-β2 in competing for binding to the 65,000 and 85,000 M_r receptors, with TGF-β1.2 exhibiting an intermediate potency. The study by Segarini *et al.* (1987) found evidence for a TGF-β receptor which preferentially binds TGF-β2, whereas Cheifetz *et al.* (1988) found no evidence of such a receptor.

1.4.6 TGF-β

Transforming growth factor β is a multifunctional protein, either stimulating cell proliferation, inhibiting cell proliferation, or exerting effects on numerous processes unrelated to proliferation (Sporn *et al.*, 1987; Roberts and Sporn, 1988). A proliferation assay, consisting of the stimulation of NRK fibroblast growth in soft agar, was used in the original purification of TGF-β (Assoian *et al.*, 1983). Although TGF-β was initially found to stimulate fibroblast proliferation, it is now realised that the protein can also inhibit fibroblast proliferation depending on the culture conditions and what other growth factors are also present (see table III, Roberts *et al.*, 1988). For example, TGF-β stimulates the growth of NRK cells in soft agar, but inhibits their growth in monolayer culture. Recently TGF-β was found to stimulate the growth of osteoblasts in monolayer culture (Centrella *et al.*, 1987; Robey *et al.*, 1987). These cells were also found to produce high levels of TGF-β . These results along with the finding of high levels of TGF-β in bone (Seyedin *et al.*, 1986) suggest a role for the polypeptide in bone regeneration and repair.

Antiproliferative effects of TGF-β have been observed on various cell types, particularly epithelial cells, but also on vascular endothelial and tumour cells. Of the epithelial cells investigated, one of the most widely studied has been hepatocytes, in which TGF-β inhibits the DNA synthesis caused by EGF and insulin (Nakamura *et al.*, 1985; Carr *et al.*, 1986). TGF-β is also active in inhibiting DNA synthesis in hepatocytes *in vivo* after partial hepatectomy, suggesting a role for the polypeptide in the growth and repair of the liver (Russell *et al.*, 1988). Since the identification of a second TGF-β species (TGF-β2) experiments have been undertaken to compare TGF-β1 and TGF-β2 for any differences in the responses they evoke. In some systems, such as mink lung epithelial cells and chicken embryonic calvarial bone cells, the two polypeptides have identical effects (inhibition of cell proliferation in mink lung epithelial cells and the stimulation of DNA synthesis in chicken embryonic calvarial bone cells) (Jennings *et al.*, 1988). In other systems, such as aortic and adrenal capillary endothelial cells (Jennings *et al.*, 1988), and hematopoietic progenitor cells (Ohta *et al.*, 1987) TGF-β2 is much less potent than TGF-β1 in inhibiting cell proliferation.

Numerous effects of TGF-β have been discovered which appear not to be related to cell proliferation, including effects on the production of extracellular matrix components (Ignotz and Massagué, 1986; Bassols and Massagué, 1988), myoblast differentiation

(Florini *et al.*, 1986b; Massagué *et al.*, 1986; Olson *et al.*, 1986), adipogenic differentiation (Ignotz and Massagué, 1985), the activation of macrophages (Tsunawaki *et al.*, 1988), FSH release from pituitary cells (Ying *et al.*, 1986) and bronchial epithelial cell differentiation (Masui *et al.*, 1986). These effects and those on cellular proliferation, suggest that TGF-β is of importance in the regulation of many developmental and tissue repair processes. A number of recent studies have also suggested a role for TGF-β or a TGF-β-like factor in early vertebrate embryogenesis (Kimelman and Kirschner, 1987; Weeks and Melton, 1987; Rappolee *et al.*, 1988; Rosa *et al.*, 1988).

Many cell types produce TGF-β or a TGF-β-like polypeptide including, the African green monkey kidney cell line BSC-1 (Tucker *et al.*, 1984), the Buffalo rat liver cell line BRL-3A (Florini *et al.*, 1986b), the *Xenopus* XTC cell line (Smith, J.C. *et al.*, 1988), activated human macrophages (Assoian *et al.*, 1987), and the following transformed cells, a human glioblastoma (Wrann *et al.*, 1987), a human salivary gland adenocarcinoma (Sato *et al.*, 1985), the human breast carcinoma MCF-7 (Knabbe *et al.*, 1987), mouse L-929 cells (Fernandez-Pol *et al.*, 1986), retrovirally transformed rat embryo fibroblasts (Massagué, 1984) and the human prostatic adenocarcinoma PC-3 (Ikeda *et al.*, 1987). The production of TGF-β by various tumour cells has lead to the suggestion of an involvement of TGF-β in carcinogenesis, a topic reviewed in Roberts *et al.* (1988).

1.4.7 Interferons

The interferons (IFNs) are a group of proteins with the properties of inhibiting viral and cellular growth (reviewed in Mannering and Deloria, 1986; and Pestka *et al.*, 1987). On the basis of antigenic differences the interferons have been divided into three classes, IFN-α (leukocyte), IFN-β (fibroblast), and IFN-γ (immune). The major sites of production of the different IFN classes are cells of the following types: IFN-α in macrophages and certain types of lymphocytes, IFN-β in fibroblasts and epithelial cells and IFN-γ in T lymphocytes with the support of macrophages.

The human IFN-α class is made up of at least 14 structurally related polypeptides, whereas the human IFN-β and IFN-γ classes consist of one member each (Pestka, 1986).

Human IFN-β displays approximately 30% identity at the amino acid level with human IFN-α1 (Taniguchi *et al.*, 1980), whereas IFN-γ exhibits very little amino acid sequence similarity to any other class of interferon (Gray and Goeddel, 1982).

Members of all three classes of human interferon have been purified and characterized (Pestka *et al.*, 1987). Multiple forms of human IFN-α have been purified from the conditioned medium of various cell lines and peripheral blood leukocytes. The purified proteins range in M_r from 16-27,000, generally consisting of 165-166 amino acid residues which vary in their extent of glycosylation. Both IFN-β and IFN-γ purified from human sources are glycoproteins. Interferon-β was found to have a M_r of 20,000, whereas IFN-γ was isolated as three different forms of 15.5-17,000, 20,000 and 25,000 M_r, due to variable glycosylation.

The levels of interferon in the blood of normal healthy individuals are generally below detectable levels, and become detectable only upon induction by a number of endogenous and exogenous agents (Mannering and Deloria, 1986). For IFN-α and IFN-β these agents include viruses and double stranded RNAs, both strong inducers, as well as bacteria and bacterial products, which tend to have a weaker effect. Inducers of IFN-γ include various mitogens as well as bacterial and viral antigens.

The biological effects of the interferons are mediated by specific cell surface receptors located on a number of different cell types. Two types of interferon receptor appear to exist, one which binds the α and β interferons and one specific for IFN-γ (Pestka *et al.*, 1987). The IFN-α/β receptor appears to be an integral membrane glycoprotein with a M_r of approximately 130,000, which is not disulphide-linked to any other subunits (Joshi *et al.*, 1982). The human IFN-γ receptor has been purified and the corresponding cDNA cloned (Aguet *et al.*, 1988). The deduced amino acid sequence of the receptor encodes a protein of 54,000 M_r which has no resemblance to any known proteins in terms of its amino acid sequence. Glycosylation may account for a substantial portion of the 90,000 M_r of the native receptor as the putative extracellular domain of the receptor contains five potential glycosylation sites.

Apart from their well established antiviral activities the interferons exhibit effects on cell proliferation and differentiation as well as numerous other cellular functions (Mannering and Deloria, 1986). The antiviral effects of interferon are thought to be

mediated by an inhibition of transcription and translation in virus infected cells. The inhibition of transcription is the result of the interferon induced production of (2′-5′)-oligoadenylate synthetase (Pestka *et al.*, 1987). The (2′-5′)-oligoadenylates produced by this enzyme activate a latent endoribonuclease (known as RNase L or RNase F) which catalyses the cleavage of both viral and cellular RNA. The inhibition of translation is the result of the interferon induced production of eIF-2α protein kinase, which phosphorylates the α subunit of initiation factor eIF-2 (Pestka *et al.*, 1987).

In contrast to the antiviral effects of the interferons, the effects on cell proliferation and differentiation are not well characterized (reviewed in Clemens and McNurlan, 1985). The major effect on cell proliferation is its inhibition, via an inhibition of DNA synthesis, whereas the effects on cellular differentiation may be either inhibitory or stimulatory depending on the cell type involved. Yap *et al.* (1986) have obtained evidence that diacylglycerol production can be correlated to IFN-β induced inhibition of cell proliferation in Daudi cells. Diacylglycerol is thought to act by activating protein kinase C (see **section 1.3**), however Mehmet *et al.* (1987) have shown that IFN-β does not activate protein kinase C in Swiss 3T3 cells, while still causing an inhibition of their proliferation. In BALB/c 3T3 cells, Zagari *et al.* (1988) have suggested that α/β interferons inhibit serum stimulated cell growth by inhibiting the PDGF-induced release of intracellular Ca^{2+}. In this study IFN-α/β inhibited PDGF-stimulated increases in the cytosolic Ca^{2+} concentration and DNA synthesis, but had no effect on the PDGF-induced increase in inositol phosphate levels.

It has been suggested that the inhibition of Daudi cell growth by IFN-α is caused by an inhibition of protein synthesis, based on results showing an inhibition of amino acid incorporation after IFN-α treatment (McNurlan and Clemens, 1986). These results were obtained in the absence of virus infection, and no effect on the rate of protein degradation was observed. In a separate study, Clemens and Tilleray (1986) found no evidence of the involvement of the eIF-2α protein kinase or the (2′-5′)-oligoadenylate synthetase pathways in this inhibition of protein synthesis. Instead, the authors suggested impairment at the level of [80S ribosome.mRNA] initiation complex formation.

It has been suggested that IFN-α may inhibit cell growth by down-regulating the receptors for various polypeptide growth factors (Zoon *et al.*, 1986a). This suggestion was based on results demonstrating both an inhibition of cell growth and a down-regulation of EGF receptors by IFN-α in MDBK cells. If this is the mechanism of IFN-α action in this case, it certainly is not in all cases of IFN induced inhibition of cell growth. For example, Hosang (1988) has reported that interferons α, β and γ all inhibited PDGF-induced DNA synthesis in normal human dermal fibroblasts, however none of the interferons inhibited the binding of ^{125}I-PDGF to the same fibroblasts.

Finally, a recent study has obtained evidence that IFN-γ causes growth inhibition by a mechanism involving tryptophan depletion in the growth inhibited cells culture medium (Takikawa *et al.*, 1988). This depletion is caused by the induction of the enzyme indoleamine 2,3-dioxygenase (a tryptophan degradation enzyme) by IFN-γ in cells in which this interferon causes growth inhibition.

1.5 Synergistic Actions of Growth Factors

The interactions of various growth factors with their target cells is often synergistic, that is the combined effect on the cell is greater than the sum of the individual effects. For example, EGF, IGF-1 and dexamethasone (a synthetic glucocorticoid) act synergistically to stimulate DNA synthesis in WI-38 human fibroblasts to a level equivalent to that produced by 10% serum (Phillips and Cristofalo, 1988). The basis for this synergism may well lie in the pluralistic nature of the transmembrane signal transduction pathways (see **section 1.3**). For instance, in order to achieve the maximal rate of mitogenesis it may be necessary to stimulate more than one signal transduction pathway. This could occur by the interaction of a growth factor with a single receptor type, provided this receptor type acts on more than one signal transduction pathway, or may require the activation of more than one growth factor receptor.

Synergism has also been reported at the level of inositol phospholipid turnover. For instance, FGF, which has no effect by itself on inositol phospholipid turnover in Chinese hamster lung fibroblasts, stimulates the thrombin induced production of inositol phosphates (Paris *et al.*, 1988). These results are particularly important as they suggest an explanation for the contradictory results in the literature on the subject of inositol

phospholipid turnover caused by growth factors of the tyrosine kinase family (Paris *et al.*, 1988). The confusion arises because growth factors such as EGF have been shown to stimulate inositol phospholipid turnover in some cells, but not in others (Besterman *et al.*, 1986; L'Allemain and Pouysségur, 1986; Earp *et al.*, 1988; Moscat *et al.*, 1988). However, the explanation may lie in a synergistic interaction between EGF and an endogenous growth factor which initiates the inositol phospholipid turnover.

Synergism has important consequences for the *in vivo* action of growth factors as has been shown for wound healing (Lynch *et al.*, 1987). In this study, neither PDGF nor IGF-1 alone had significant effects on the healing of skin wounds in young White Yorkshire pigs. However, the combination of PDGF and IGF-1 caused significant hyperplasia of both connective and epithelial tissues.

1.6 Polypeptide Growth Factors and Autocrine Growth

The term "autocrine secretion" was first proposed by Sporn and Todaro (1980) to describe the situation whereby a cell produces a growth factor for which the same cell expresses functional receptors. The more general term autocrine growth can be defined as the growth of a cell which is not dependent upon growth factors originating from other cells. A dependency on growth factors must be distinguished from a dependency on the availability of nutrients, which all cells share to varying degrees. Paracrine growth of a cell is that which is dependent on the production of growth factors by other cells in the growing cell's immediate environment, whereas endocrine growth is mediated by growth factors derived from the plasma. A cell may undergo autocrine growth by, (a) producing its own growth factors to which it is responsive, (b) producing a growth factor receptor which is independent of its agonist for activation, and (c) the activation of a post-receptor pathway which negates the necessity for receptor activation. These mechanisms apply to stimulatory growth factors. The involvement of inhibitory growth factors, such as TGF-β, in autocrine growth amounts to the disruption of an autocrine loop. This may be achieved by, (1) the failure to produce a growth factor to which a cell is responsive, (2) an inability to activate the latent form of a growth factor, (3) the failure to express the receptor for a growth factor or, (4) the desensitization of a post-receptor pathway. In the case of TGF-β, examples of

mechanisms (2) and (3) have been reported, e.g. A-549 human lung carcinoma cells fail to activate the latent TGF-β which they secrete (Wakefield *et al.*, 1987) and various retinoblastoma cell lines do not express TGF-β receptors (Kimchi *et al.*, 1988).

In the remainder of this section I shall briefly consider PDGF as an example of a stimulatory growth factor involved in the process of autocrine growth. This topic and the closely related topic of the involvement of polypeptide growth factors in carcinogenesis, have recently been the subject of a number of reviews, including Goustin *et al.* (1986), Heldin *et al.* (1986a), Deuel (1987), Heldin *et al.* (1987), and Westermark and Heldin (1988).

The most convincing evidence of an involvement of PDGF in autocrine growth is to be found in the transformation event caused by the simian sarcoma virus (SSV). An association between PDGF and transformation by SSV was established with the realisation that the SSV transforming protein ($p28^{v\text{-}sis}$) shared considerable sequence similarity with human PDGF (Doolittle *et al.*, 1983; Waterfield *et al.*, 1983). The cloning and sequencing of the human c-*sis* gene further confirmed this relationship, indicating that c-*sis* encodes the B chain of PDGF (Chiu *et al.*, 1984; Johnsson *et al.*, 1984). Following these studies it was found that cells transformed by SSV secrete a PDGF-like growth factor into their culture medium (Huang *et al.*, 1984; Johnsson *et al.*, 1985a). However, the actual evidence that an autocrine loop operates in establishing SSV transformation of cells came with the results of antibody inhibition experiments. Johnsson *et al.* (1985b) found that antibodies against PDGF inhibited transformation caused by SSV and Huang *et al.* (1984) showed that anti-PDGF antisera blocked ^{3}H-thymidine incorporation into SSV transformed cells. Furthermore, Betsholtz *et al.* (1986b) have shown that suramin, a chemical which displaces PDGF from its receptor, causes the reversion of the SSV-induced transformed phenotype.

There has been some controversy as to the cellular location in which the interaction of the v-*sis* oncogene product and the PDGF receptor take place. Inhibition of SSV transformation by anti-PDGF antibodies is consistent with this interaction occurring at the cell surface. However, in some cases this interaction may well be occurring at intracellular sites (Huang and Huang, 1988; Keating and Williams, 1988). Where ever the location of the interaction between the PDGF receptor and the v-*sis* oncogene product, it has been well established that an autocrine loop is operative in SSV-induced transformation.

1.7 MDBK Cells

MDBK cells (ATCC CCL 22) are an immortalized cell line which was derived from the kidney of a normal adult steer (Madin and Darby, 1958). This cell line is epithelial in morphology and forms a monolayer which develops fluid filled domes or towers. These structures (also known as hemicysts) are caused by the build up of hydrostatic pressure between the cell monolayer and the culture dish, resulting from the vectorial transport of fluid across the cell monolayer. Dome formation and transepithelial fluid and ion transport are well characterized processes in the canine kidney cell line MDCK (see **figure 1.2**) (Leighton *et al.*, 1970; Lever, 1979 and 1981; Cereijido *et al.*, 1981). Apart from these structural and functional similarities, the two cell lines MDBK and MDCK exhibit similar responses to some hormones. For example, parathyroid hormone, calcitonin and prolactin induced a similar response in both cell lines in terms of cAMP production (Rindler *et al.*, 1979). However, in the same study, substantial differences were noted in the effects of vasopressin (stimulatory for MDCK cells), prostaglandin E1 (stimulatory for MDBK cells) and catecholamines (stimulatory for MDBK cells).

The MDBK cell line has been used extensively in the field of interferon research (Yonehara *et al.*, 1983; Lefkowitz and Reyes Luna, 1984; Zoon *et al.*, 1986a,b), as this cell line is quite sensitive to the antiviral effects of α-interferon. Other uses of the cell line have included the production of cDNA libraries (Kristensen *et al.*, 1986; Shaper *et al.*, 1986), the investigation of the enzymes ribonucleotide reductase (Engstöm *et al.*, 1985) and cAMP-dependent protein kinase type II (Nigg *et al.*, 1985), and the response of cells to heat shock (Chousterman *et al.*, 1987). However, very little work to date has been carried out on polypeptide growth factors and their effects on MDBK cells, and no studies have been reported as to which, if any, polypeptide growth factors these cells produce.

1.8 Thesis Outline

In my thesis the purification and characterization of the growth factors produced by the MDBK cell line is reported, as well as the sensitivity of the cell line to a range of growth factors. The results are presented in Chapters 2-5 and consist of the initial characterization of the MDBK cell conditioned medium (**Chapter 2**), including the evidence of IGF binding protein and IGF-2 production by the cells, the purification and

identification of PDGF-AB (**Chapter 3**), and the partial purification of two growth inhibitors related to TGF-β from MDBK cell conditioned medium (**Chapter 4**). The characterization of the responsiveness of MDBK cells to various growth factors is presented in **Chapter 5**.

Chapters 3-5 are presented in the form of manuscripts for publication, and therefore some repetition is evident, particularly in the **Materials and Methods** sections which appear in each chapter. As the development of the ELISA assays utilized in **Chapter 3** is not discussed in this manuscript it is presented as an appendix at the end of the thesis (**Appendix A1**).

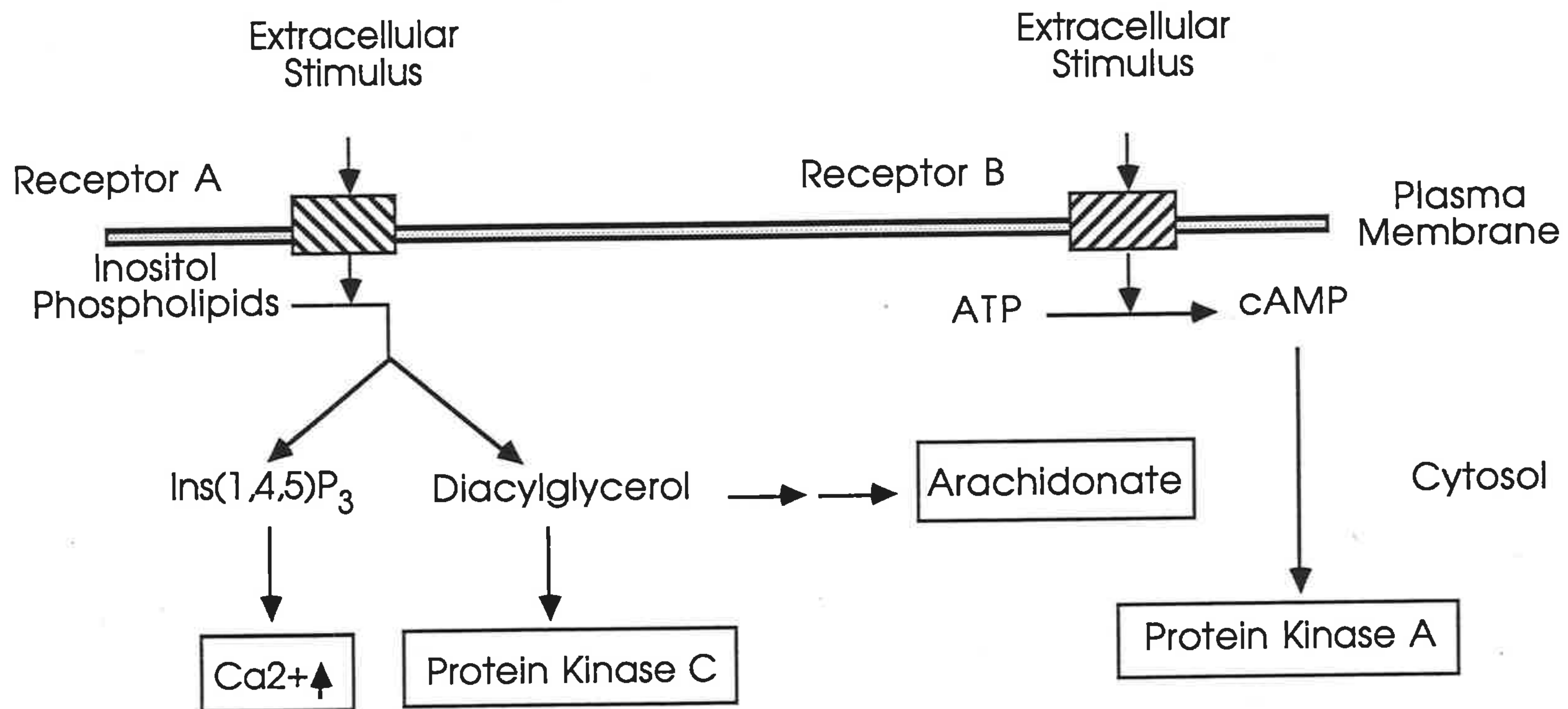


Figure 1.1 : Transmembrane Signaling Pathways

(a schematic representation adapted from Nishizuka, 1984)

Boxes : central mediators of physiological responses

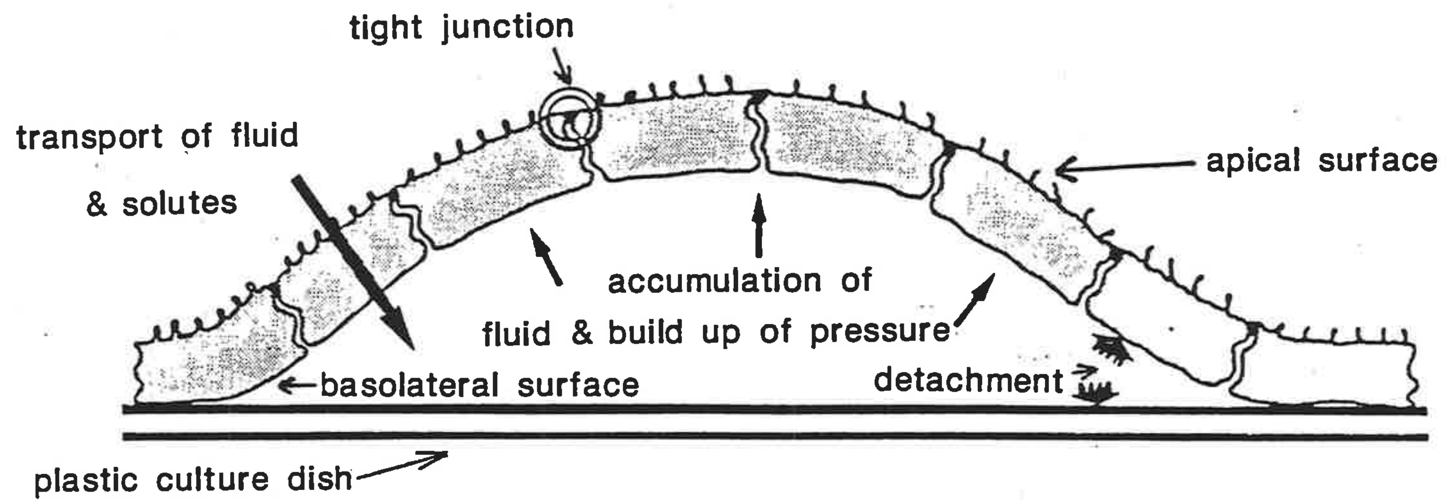


Figure 1.2: Diagramatic representation of the process of hemicyst formation (adapted from Cereijido et al., 1981)

Table 1.1 : PDGF Production by Cells

Cell Type[1]	Trans-formed	Respon-sive[2]	Evidence of Production[3]	Reference
1) adult human arthero-sclerotic plaque SMC	no	NR	RRA, Ab NB, A mRNA.	Libby *et al.*, 1988.
2) adult rat aortic SMC.	no	yes	RRA, Ab NB, A mRNA.	Sjölund *et al.*, 1988.
3) rat skeletal myoblasts L6J1.	no	NR	RRA, Ab Ppte, A mRNA.	Sejersen *et al.*, 1986.
4) human iliac artery endothelial cells.	no	NR	RRA, Ab Ppte, A&B mRNA.	Sitaras *et al.*, 1987.
5) human umbilical vein endothelial cells.	no	NR	RRA.	Di-Corleto Bowen-Pope, 1983
6) human umbilical vein endothelial cells.	no	NR	B chain cDNA.	Collins *et al.*, 1985.
7) human umbilical vein endothelial cells.	no	NR	A chain cDNA.	Collins *et al.*, 1987.
8) bovine aortic endothelial cells.	no	NR	RRA.	Di-Corleto Bowen-Pope, 1983
9) human peritoneal and alveolar macrophages	no	NR	RRA, Ab NB, Ab Ppte, B mRNA.	Shimokado *et al.*, 1985.
10) human fetal kidney cells[4].	no	yes[5]	RRA.	Fraizer *et al.*, 1987.
11) BSC-1 kidney epithelial cells.	no	no	Ab NB, Ab Blot, B mRNA.	Kartha *et al.*, 1988.
12) human mesangial cells.	no	yes	RRA, Ab NRRA, Ab NB, A&B mRNA.	Shultz *et al.*, 1988.

Cont.

Table 1.1 (cont.) : PDGF Production by Cells

Cell Type[1]	Trans-formed	Respon-sive[2]	Evidence of Production[3]	Reference
13) human rhabdomyo-sarcoma RD.	yes	NR	RRA, Ab NRRA, Ab Ppte.	Betsholtz *et al.*, 1983.
14) human hepatoma Hep G2.	yes	NR	RRA, Ab NRRA, Ab NB.	Bowen-Pope *et al.*, 1984.
15) human glioma U-343 MGa Cl 2.	yes	NR	RRA, RIA, Ab Ppte.	Nistér *et al.*, 1984.
16) human glioblastoma A172.	yes	NR	Ab Ppte, B mRNA.	Pantazis *et al.*, 1985.
17) human fibrosarcoma HT-1080.	yes	NR	Ab Ppte, B mRNA.	Pantazis *et al.*, 1985.
18) human osteosarcoma U-2 OS.	yes	NR	A chain partial amino acid sequence.	Heldin *et al.*, 1986.
19) human melanoma WM 266-4.	yes	NR	RRA, Ab NB, Ab Ppte, A mRNA.	Westermark *et al.*, 1986.
20) human mesothelioma HUT28.	yes	NR	Ab NB, A&B mRNA.	Gerwin *et al.*, 1987.
21) human breast carcinoma MCF-7 & MDA-MB-231.	yes	NR	RRA, Ab NRRA, Ab Ppte, A&B mRNA.	Bronzert *et al.*, 1987.
22) human prostate carcinoma cells, PC-3 & DU-145.	yes	no	RRA, Ab NB, Ab Ppte, A&B mRNA	Sitaras *et al.*, 1988.
23) various malignant human epithelial cells.	yes	no	RRA, Ab NB, Ab Ppte, A&B mRNA.	Sariban *et al.*, 1988.
24) murine neuroblastoma Neuro-2A.	yes	NR	Ab NB, Ab Blot, B mRNA.	van Zoelen *et al.*, 1985.
25) SV-40 transformed BHK cells (SV28).	yes	NR	Ab Blot.	Stroobant *et al.*, 1985.
26) Dif 5 endoderm cells.	yes	yes[6]	RRA, Ab NB.	Grotendorst *et al.*, 1988.

1. SMC :smooth muscle cells. 2. Responsive refers to whether the cells producing PDGF are also responsive to PDGF. NR :not reported. 3. RRA :positive for activity in a radio-receptor assay. RIA :positive for activity in a radio-immune assay. Ab NRRA :antibody neutralization of RRA activity. Ab NB :antibody neutralization of biological activity. Ab Ppte :immunoprecipitation. Ab Blot :Western blot. A mRNA :A chain mRNA detected. B mRNA :B chain mRNA detected. 4. Report did not state whether the cells were fibroblastic or epithelial in morphology. 5. Low level, low affinity binding only. 6. Dif 5 cells express a PDGF receptor, but it was not determined whether the receptor was functional.

Table 1.2 : IGF Production by Cells

Cell Type[1]	Trans-formed	Respon-sive[2]	Evidence of Production[3]	IGF[4]	Reference
1) human skin fibroblasts.	no	NR	RRA, RIA.	1&2	Adams *et al.*, 1984.
2) human skin and fetal lung fibroblasts.	no	yes	Ab NB.	1	Clemmons and Van Wyk, 1985.
3) rat embryo fibroblasts.	no	yes	RRA, RIA, BP.	2	Adams *et al.*, 1983.
4) porcine aortic SMC.	no	yes	Ab NB.	1	Clemmons and Van Wyk, 1985.
5) fetal rat myoblasts.	no	yes	RIA, Ab NB.	1&2	Hill *et al.*, 1985.
6) adult rat hepatocytes.	no	NR	RIA.	1	Scott *et al.*, 1985.
7) BRL-3A rat liver cells.	no	NR	amino acid sequence analysis	2	Marquardt *et al.*, 1981.
8) rat pancreatic B cells.	no	yes	RRA, RIA.	1	Romanus *et al.*, 1985.
9) porcine granulosa cells.	no	NR	RRA, RIA.	NR	Hammond *et al.*, 1985.
10) rat osteoblast-like cells.	no	yes	Ab NB.	1	Ernst and Froesch, 1988.
11) a human fibrosarcoma.	yes	NR	RRA.	NR	De Larco and Todaro, 1978a.
12) various human breast carcinoma cell lines	yes	yes	RIA, IGF-1 mRNA.	1	Huff *et al.*, 1986.
13) primary human lung carcinoma.	yes	yes	RIA, Ab NB.	1	Minuto *et al.*, 1988.
14) a murine mammary carcinoma.	yes	NR	RRA.	NR	Knauer *et al.*, 1980.
15) Dif 5 endoderm cells.	yes	NR	RRA, RIA.	2	Nagarajan *et al.*, 1985.

1. SMC :smooth muscle cells. 2. Responsive refers to whether the cells producing IGFs are also responsive to IGFs. NR :not reported. 3. RRA :positive for activity in a radio-receptor assay. RIA :positive for activity in a radio-immune assay. Ab NB :antibody neutralization of biological activity. BP :positive for activity in a competitive binding assay utilizing IGF binding protein. 4. Type of IGF detected, either IGF-1, IGF-2, both IGF-1 and IGF-2 or NR :not reported.

CHAPTER TWO : Initial Characterization of MDBK Cell Conditioned Medium

Chapter 2: Introduction

As part of a study of the production of insulin-like growth factors (IGFs) by cells in culture, serum-free medium conditioned by the MDBK cell line was collected and assayed for the presence of IGFs. During the collection of this conditioned medium it was observed that MDBK cells survived well in serum-free medium provided the conditioned medium was replaced regularly by fresh medium. The survival and growth of cells in serum-free conditions has previously been associated with the endogenous production of growth factors by such cells (Dulak and Temin, 1973; De Larco and Todaro, 1978a,b; Kaplan *et al.*, 1982; Messing *et al.*, 1984). In a number of cases, including examples involving the growth factors TGF-α, PDGF, bombesin and TGF-β, an autocrine loop operates where the externalized growth factor acts on the cell which produces it enabling it to grow without exogenous growth factors (see **section 1.5** and Sporn and Roberts, 1985). In the light of this knowledge the medium conditioned by MDBK cells was investigated to determine what, if any, growth factors were present.

The primary assay used to detect growth factor activity was the stimulation of protein synthesis in the rat L-6 myoblast cell line. This assay has been well characterized in this laboratory and is routinely used during the purification of IGFs from bovine colostrum (Ballard *et al.*, 1986; Francis *et al.*, 1986, 1988). To identify any growth factors that are present in MDBK cell conditioned medium it was necessary to at least partially purify the activities. The procedures used to achieve this end were chosen such that each procedure would,

1) leave the growth factor in a form which could conveniently be assayed, and

2) provide information about the growth factors properties and therefore help reveal its identity.

Protein separation procedures are based on differences in the properties of the protein of interest and any contaminating proteins present. These properties are most commonly based on differences in, molecular weight (e.g. gel filtration or size exclusion chromatography), charge (e.g. ion exchange chromatography), hydrophobicity (e.g. hydrophobic interaction and reverse phase chromatography) and affinities for ligands or

specific recognition molecules (affinity chromatography). All of these various methods have been utilized in an attempt to characterize any growth factors produced by MDBK cells, the results of which are presented in this chapter.

2.1 Materials and Methods

Materials

Fetal-bovine serum (batch 29106909) was purchased from Flow Laboratories; bovine serum albumin (BSA) (fraction V), protein molecular weight standards, amino acids and vitamins for the preparation of media were obtained from Sigma Chemical Co.; L-[4,5-^{3}H]leucine (40-60 Ci/mmol) and [*methyl*-^{3}H]thymidine (20 Ci/mmol) were from New England Nuclear. Sources for the antibiotics used in media are given by Ballard *et al.* (1986) and for ^{125}I-labelled growth factors by Read *et al.* (1986). All dilutions of growth factors were made in the presence of 0.1% (w/v) BSA. Albumin used for this purpose and in the binding media was treated as described by Chen (1967).

Cell cultures and the collection of conditioned medium

L-6 myoblasts, obtained from Dr. J. M. Gunn, Texas A&M University, College Station TX, U.S.A., were grown by serial passaging of prefused cultures in Dulbecco-modified Eagle's Minimal Essential Medium containing 5% (v/v) fetal-bovine serum, together with 50 mg of gentamycin, 100 mg of streptomycin, 60 mg penicillin and 1 mg of fungizone/litre of growth medium. The cells were grown and used for experiments as monolayers at 37°C under an atmosphere of CO_2/humidified air (1:19). For experimental purposes the cells were subcultured into Linbro 24-place multiwell dishes in growth medium and used during the 4 days after the monolayers first became confluent. At this stage myotubes were not evident. MDBK cells (Flow Laboratories ATCC CCL22) were grown as for L-6 cells except the growth medium contained 10% (v/v) fetal-bovine serum. For the production of conditioned medium, MDBK cells were grown in roller bottles in growth medium until confluent. Subsequently, cells were transferred to growth medium without serum supplementation. After 24 hours, conditioned medium was replaced with fresh growth medium without serum. This collection was discarded. Conditioned medium was henceforth collected every third day while the cells remained viable (about 8 weeks) and stored at -15°C until used.

Dialysis

Dialysis, unless otherwise stated, was carried out using Spectrapor #3 dialysis tubing (3,500 molecular weight cut off) against at least a 100-fold excess of 1% (v/v) acetic acid at 4°C.

Protein synthesis determination

The measurement of protein synthesis in L-6 myoblasts has been described previously (Francis *et al.*, 1986). Briefly, the measurement involves the incorporation of [^{3}H]leucine into total cell protein during an 18 hour incubation of confluent cell monolayers in 24-place multiwell dishes. Activity is expressed as the percent stimulation of protein labelling over that occurring in monolayers incubated without growth factors in DMEM.

DNA synthesis determination

Confluent monolayers of L-6 cells in 24-place multiwell dishes were washed with DMEM for 2 hours and exposed to growth factors for 18 hours in 1 ml of DMEM. At the end of this period, 5 nmol of thymidine containing 1 µCi of [^{3}H]thymidine was added for a further 6 hours. Harvesting of the monolayers was carried out as described previously (Ballard *et al.*, 1986). Activity is expressed as the percent stimulation of DNA labelling over that occurring in monolayers incubated without growth factors in DMEM.

IGF-1 radioimmunoassay

The IGF-1 radioimmunoassay was carried out as described by Read *et al.* (1986) utilizing the mouse monoclonal antibody 3D1 and bovine ^{125}I-IGF-1 (6,000 c.p.m. per tube) as radioligand. The antibody 3D1 was prepared in the Immunology unit, Department of Medicine, University of Sydney, N.S.W., Australia, as described by Baxter *et al.* (1982).

IGF-2 radioreceptor assay

Binding of growth factors to sheep placental membranes was carried out as described by Read *et al.* (1986), utilizing bovine ^{125}I-IGF-2 (12,000 c.p.m. per tube) as radioligand.

IGF binding protein assay

The IGF binding protein assay was based on the procedure described by Moses *et al.* (1979). Aliquots from the fractions collected after Sephadex G-75 chromatography were lyophilized and resuspended in 20 µl of 10 mM HCl. To these 100 µl was added of assay buffer (10 mM sodium phosphate pH 7.4, 150 mM NaCl, 2 mg/ml BSA) containing bovine

^{125}I-IGF-1 (4,000 c.p.m.). After 18 hours incubation at 4°C, 100 µl of a 5% (w/v) charcoal solution (prepared in assay buffer after washing the charcoal with assay buffer until the pH of the supernatant was 7.0-7.5) was added and the samples incubated at 4°C for a further 15 min. Samples were then centrifuged at 10,000 rpm for 5 min and the radioactivity of the supernatant determined.

Sephadex G-75 chromatography

MDBK cell conditioned medium (350 ml) was concentrated approximately 100-fold by lyophilization, dialysed against 1% acetic acid, lyophilized and resuspended in 3.5 ml of 1 M acetic acid. The clear solution was chromatographed at a flow rate of 0.35 ml/min on a 2.6 cm diam. x 80 cm column of Sephadex G-75 (Pharmacia) equilibrated with 1 M acetic acid.

Fractogel TSK chromatography

MDBK cell conditioned medium (4 litres) was concentrated by lyophilization, dialysed against 1% acetic acid, lyophilized and resuspended in 45 ml of 1 M acetic acid. The clear solution was chromatographed at a flow rate of 2 ml/min on a 5 cm diam. x 90 cm column of Fractogel TSK HW-55 (S) (Merck) equilibrated with 1 M acetic acid.

HPLC gel filtration chromatography

Pooled fractions from reverse phase HPLC (rpHPLC) were adjusted to 40% (v/v) acetonitrile and applied in a volume not greater than 400 µl to a TSK G3000 SW column (7.5 mm diam. x 60 cm) equilibrated with 0.1% (v/v) trifluroacetic acid (TFA)/40% (v/v) acetonitrile. The flow rate was 0.5 ml/min, with absorbance measured at either 280 or 215 nm.

Reverse phase HPLC

Following acid gel filtration chromatography on Fractogel TSK, pooled fractions were adjusted to 0.1% (v/v) TFA and applied to a Vydac C_{18} Radial-Pak cartridge (8 mm diam. x 10 cm, 15-20 µm particle size) equilibrated with 0.1% TFA. This concentration of TFA was maintained throughout. Growth factor activity was eluted at a flow rate of 2 ml/min by a two-step linear gradient, from 0 to 10% (v/v) acetonitrile in 5 min and then to 45% acetonitrile over 20 min.

Cation exchange chromatography

The pooled fractions from rpHPLC were lyophilized, dissolved in 50 mM sodium phosphate pH 7.4 (buffer A) and injected onto a Mono S HR 5/5 (Pharmacia) column equilibrated with buffer A. Protein, monitored by absorbance at 280 nm, was eluted at a flow rate of 1 ml/min with a gradient of 0 to 650 mM NaCl in buffer A over 30 min.

Protein concentration

Throughout this study protein content was monitored by absorbance at 280 or 215 nm. Protein concentrations were calculated by assuming that A_{280}=1.0 for a 1 mg/ml solution in a 1 cm light path and an A_{280}/A_{215} ratio of 0.1.

Reduction by dithiothreitol

The MDBK-derived growth factors were lyophilized (post Fractogel TSK gel filtration), resuspended in 200 µl of 6 M guanidine.HCl, 100 mM Tris Cl pH 8.5, ± 50 mM dithiothreitol (DTT) and incubated at 40°C for 4 hours. After this time 2 ml of 1 mg/ml BSA in phosphate buffered saline was added and dialysis carried out against 1,000-fold excess of water for 18 hours. Samples were then lyophilized and assayed in the L-6 protein synthesis assay.

SDS-gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed (Laemmli, 1970) using a 15% separating gel and a 4.5% stacking gel of 1.5 mm thickness. Lyophilized samples (non-reduced) were boiled for 5 min in SDS sample buffer before loading. After electrophoresis the tracks containing material to be eluted were sliced into 3 mm fractions and activity eluted as described by Raines and Ross (1982).

2.2 Results

To determine if growth factor activity was present in the serum-free medium conditioned by MDBK cells, concentrated acid dialyzed conditioned medium was tested for its effect on protein synthesis in L-6 myoblasts. As the results in **table 2.1** show, this conditioned medium is active in stimulating protein synthesis in this cell line. The insulin-like growth factors (IGFs) and insulin are known to stimulate protein synthesis in L-6 cells, with IGF-1-like growth factors being the most potent (Ballard *et al.*, 1986; Ballard *et al.*, 1987). Therefore, MDBK cell conditioned medium was investigated for the

presence of IGF-1, by the use of an IGF-1 RIA. As IGF binding proteins are commonly produced by cells in culture and their presence in an IGF RIA causes interference, it is necessary to take measures to separate any IGF present in the sample from any binding protein present. Therefore a sample of MDBK cell conditioned medium (equivalent to 350 ml) was chromatographed on a Sephadex G-75 gel filtration column in 1M acetic acid (conditions under which the IGFs and their binding proteins separate), fractions collected and assayed in an IGF-1 RIA and a binding protein assay (see **figure 2.1**). From this data it can be seen that a peak of binding protein activity coincides with a peak of RIA competing activity at fraction number 25. The molecular weight of a protein eluting at this position corresponds to that of the acid stable IGF binding protein (as shown by the elution position of the BRL-3A rat liver IGF binding protein) and is approximately 35,000 M_r. No IGF-1 RIA competing activity was observed at the elution position of an IGF nor that of any IGF precursor. A peak of IGF binding protein activity also co-elutes with the peak of protein (i.e. A_{280} absorbing material). This is likely to be due to non-specific interference by the large amount of protein in these fractions (relative to neighbouring fractions) in the binding protein assay.

To characterize the growth factors in MDBK cell conditioned medium which stimulate protein synthesis in L-6 myoblasts, the equivalent of 4 litres of conditioned medium was chromatographed on a Fractogel TSK HW-55 (S) gel filtration column in 1 M acetic acid, fractions collected and assayed in both protein and DNA synthesis assays on L-6 myoblasts. The results obtained (see **figure 2.2**) indicated the presence of three different growth factors which stimulated protein synthesis in L-6 cells (labelled peaks A, B and C in **figure 2.2**). The DNA synthesis results indicated that peak A was also associated with DNA synthesis stimulating activity, whereas peaks B and C were not. On the contrary, the region of the chromatogram between peak B and C showed evidence of an activity(s) which inhibited DNA synthesis in L-6 cells (see **figure 2.2**). The peaks of activity A, B and C were pooled as designated (see **figure 2.2**) and subjected individually to reverse phase HPLC (rpHPLC) on a C_{18} column with 0.1% TFA as the mobile phase and acetonitrile as the organic modifier. Pool A eluted as a broad region of both protein and DNA synthesis stimulating activity from 26-29% acetonitrile (**figure 2.3**). Contrary to **figure 2.2**, the growth factors represented by pools B and C eluted as peaks of both protein

and DNA synthesis stimulating activity at 33% and 35% acetonitrile respectively (**figure 2.3**). Pool C also contained some protein synthesis stimulating activity which eluted between 37% and 42% acetonitrile, which was not associated with any DNA synthesis stimulating activity (**figure 2.3**).

In order to determine the molecular weights of the growth factors represented by pools A, B and C, aliquots of each of the peak fractions of coincident protein and DNA synthesis stimulating activity from rpHPLC were chromatographed on a TSK G3000 SW HPLC gel filtration column in 0.1% TFA/40% acetonitrile. Chromatography under these conditions with a TSK G3000 PW column has previously been shown to resolve peptides and proteins on the basis of their molecular weight (Swergold and Rubin, 1983). This was also shown to be true for the TSK G3000 SW column used in this study (see inset **figure 2.4**). The three pools A, B and C each eluted from the HPLC gel filtration column as a peak of protein synthesis stimulating activity with approximate molecular weights of 35,000, 12,000 and 9,000 respectively (**figure 2.4**).

To investigate whether more than one activity was present in the broad region of activity which constituted pool A after rpHPLC (**figure 2.3**), the fractions pooled as indicated were lyophilized and resuspended in 50 mM sodium phosphate pH 7.4 prior to injecting onto a Pharmacia Mono S HR 5/5 cation exchange column. Protein synthesis stimulating activity was eluted as a broad peak of activity from this column by a linear gradient of sodium chloride (**figure 2.5**). Therefore pool A was not resolvable into more than one peak of protein synthesis stimulating activity utilizing the techniques of rpHPLC, gel filtration HPLC or cation exchange FPLC.

The growth factors represented by pool A (post Mono S), B and C (both post rpHPLC) were assayed for their ability to compete with ^{125}I-IGF-2 for binding to sheep placental membranes (see **table 2.2**). Both pools B and C competed in this assay. Since these growth factors exhibit both protein and DNA synthesis stimulating activity on L-6 myoblasts (**figure 2.3**) and elute from a HPLC gel filtration column in an elution volume corresponding to molecular weights of 12,000 and 9,000 respectively, it is concluded that they are related to IGF-2. The growth factor represented by pool B may also be

contaminated by some other activity which causes the combination of the two to have a higher biological/RRA activity ratio compared to pool C. The growth factor represented by pool A did not compete in the IGF-2 RRA and therefore is unlikely to be related to IGF-2.

A number of chromatography matrices and conditions were investigated to determine which would be of use in the purification of the growth factor represented by pool A. Hydrophobic interaction chromatography was investigated utilizing a HPLC butyl cartridge and initial buffer conditions of 2.5 M ammonium sulphate in 50 mM sodium phosphate pH 7.4 (similar to Kato *et al.*, 1983). Elution was attempted with a linear gradient of ammonium sulphate (2.5 to 0 M). Under these conditions the growth factor bound to the butyl cartridge but did not elute (an acetonitrile gradient in 0.1% TFA caused elution of bioactivity). A phenyl-Superose column was injected with pool A in 2.5 M ammonium sulphate pH 7.4, a hydrophobic interaction chromatography based step used in the purification of PDGF from platelets (see Raines and Ross, 1985). However, under these conditions the growth factor did not bind to phenyl-Superose.

As rpHPLC had produced good results in the characterization of pool A (see **figure 2.3**) the method was further investigated by altering the mobile phase composition and pH (similar to Bennett, 1983). A butyl cartridge was used with a mobile phase of 10 mM triethylamine acetate pH 5.5 and acetonitrile as the gradient. Under these conditions the growth factor represented by pool A binds to the butyl cartridge but fails to elute. An acetonitrile gradient in 0.1% TFA caused the elution of protein synthesis stimulating activity.

Since a number of growth factors have a high affinity for heparin (Lobb *et al.*, 1986), the binding of pool A growth factor activity to heparin-Sepharose was investigated. The growth factor activity bound to heparin-Sepharose in 150 mM NaCl/10 mM sodium phosphate buffer pH 7.4, but eluted at approximately 300 mM NaCl. This is the behaviour of a ligand interacting with the heparin-Sepharose in an ion-exchange mode, as affinity interactions generally require a NaCl concentration of greater than 1 M for disruption and elution of the ligand (Lobb *et al.*, 1986). Finally, the binding of pool A to Concanavalin A-Sepharose was investigated in case the growth factor displayed α-D-glucosyl or α-D-mannosyl residues on its surface, however pool A growth factor activity did not bind to this matrix.

The susceptibility of the pool A growth factor activity to the effects of the reducing agent dithiothreitol (DTT) was investigated. The two MDBK-derived IGF-2 related growth factors were also tested as positive controls, since reduction destroys the activity of IGF-2. The activity of all three growth factors was destroyed by reduction (**table 2.3**), indicating that disulphide bonds are also essential for the activity of the growth factor represented by pool A.

In order to determine the molecular weight of the growth factor represented by pool A under conditions of SDS-polyacrylamide gel electrophoresis, and to determine the growth factors stability to exposure to sodium dodecyl sulphate (SDS), an aliquot of pool A was electrophoresed on a 15% SDS-polyacrylamide gel under non-reducing conditions. After electrophoresis the gel was sliced into segments and bioactivity eluted and assayed in the L-6 protein synthesis assay (**figure 2.6**). The results from these assays indicate the molecular weight of the pool A growth factor is approximately 35,000, which is in agreement with the result obtained by HPLC gel filtration chromatography.

2.3 Discussion

Central to the approach used in this study is the use of serum-free medium as the starting material to investigate the growth factors produced by MDBK cells. Other workers have acknowledged the benefits of this approach when attempting to purify cell-derived products (Alderman *et al.*, 1985). The MDBK cell line is similar to the human colon tumour cell line, HT-29, utilized by Alderman *et al.*, as MDBK cells remain viable in serum-free medium for periods > 3 months while cell proliferation is minimal and subculturing unnecessary. To obtain proliferation of cells without the presence of serum, other factors often present in serum are frequently necessary, the result being a serum-free defined medium which usually needs to be adjusted to the particular cell type being cultured (Taub *et al.*, 1979; Barnes and Sato, 1980; Phillips and Cristofalo, 1981; Kawamoto *et al.*, 1983). This was not necessary in the current study as proliferation was not desirable.

The MDBK cell line shares a number of properties with the rat liver cell line, BRL-3A, which also is a producer of growth factors (Dulak and Temin, 1973). Both cell lines are epithelial in morphology, remain viable in serum-free medium, produce an IGF

binding protein (Moses *et al.*, 1979; Mottola *et al.*, 1986; this study and Szabo *et al.*, 1988, see **Appendix A2**) and produce IGF-2-related polypeptides (Moses *et al.*, 1980; Marquardt *et al.*, 1981; and this study). Other cells which have been found to produce IGF binding proteins include the human hepatoma cell line HEP G2 (Povoa *et al.*, 1985), the rat hepatoma H-35 (Mottola *et al.*, 1986), vascular endothelial cells (Bar *et al.*, 1987), adult rat hepatocytes (Scott *et al.*, 1985), human dermal fibroblasts (Adams *et al.*, 1984) and various muscle cells, including rat L-6 myoblasts (Hill *et al.*, 1985; McCusker and Clemmons, 1988). The IGF binding proteins produced by the cell lines MDBK, BRL-3A and HEP G2 have been isolated and partial amino acid sequence analysis undertaken (Povoa *et al.*, 1985; Mottola *et al.*, 1986; Szabo *et al.*, 1988). The results indicate that the MDBK and BRL-3A binding proteins are closely related, exhibiting identity in 20 of the first 23 residues. No similarity is evident between the MDBK and HEP G2 binding proteins.

Apart from MDBK and BRL-3A cells, other cells which have been found to produce IGF-2-related polypeptides include rat embryo fibroblasts (Adams *et al.*, 1983), human dermal fibroblasts (Adams *et al.*, 1984), fetal rat myoblasts (Hill *et al.*, 1985) and Dif 5 endoderm cells (Nagarajan *et al.*, 1985). It is quite common for the IGF-2 produced by cells to be present in more than one molecular weight form (Moses *et al.*, 1980; Adams *et al.*, 1983; Nagarajan *et al.*, 1985). This is also true of the IGF-2 produced by MDBK cells, which was found to be present in two forms of molecular weight 12,000 and 9,000 as determined by gel filtration HPLC in 0.1% TFA/40% acetonitrile. These IGF-2 forms may well be the same polypeptides as MSA I ($M_r \approx 16,000$) and MSA II/III ($M_r \approx 7\text{-}9,000$) characterized by Moses *et al.* (1980) using gel filtration in guanidine.HCl.

MDBK cells also produce a third growth factor, referred to as pool A, which stimulates protein synthesis in L-6 myoblasts. Pool A is an acid stable, cationic growth factor of approximately 35,000 M_r which stimulates both protein and DNA synthesis in L-6 cells and which is inactivated upon reduction. These properties are very similar to those exhibited by platelet-derived growth factor (PDGF, see **section 1.3.1**) a growth factor that is produced by a number of cells in culture (see **table 1.1**). One difference between pool A and PDGF is their behaviour on phenyl bonded matrices. Pool A did not bind at all to a phenyl-Superose column under conditions (i.e. high salt, neutral pH) in which PDGF would be expected to bind (Raines and Ross, 1985). However, the binding properties of

PDGF to phenyl-Sepharose varies depending on the source of the PDGF, and therefore the contaminating proteins present (E.W. Raines, *pers. comm.*, 1988). The production of a PDGF-like growth factor by MDBK cells may be a similar situation to the production of osteosarcoma-derived growth factor (ODGF) by U-2 OS cells (Heldin *et al.*, 1980) and fibroblast-derived growth factor (FDGF) by SV40 transformed BHK cells (Dicker *et al.*, 1981). Both of these growth factors were recognized as exhibiting similarities to PDGF and have since been purified and identified as PDGF-related growth factors (Stroobant *et al.*, 1985; Heldin *et al.*, 1986b).

The assay used in this study for the detection of growth factor activity (the stimulation of protein synthesis in L-6 myoblasts), is an unusual one for the purification of growth factors. The most common assays utilized in the purification of growth factors are the stimulation of DNA synthesis in a cell monolayer or colony formation in soft agar. If the stimulation of DNA synthesis was followed as the sole indication of growth factor activity in this study, the two IGF-2-related growth factors produced by MDBK cells would not have been discovered, since upon gel filtration of MDBK cell conditioned medium only protein synthesis stimulating activity is associated with these two growth factors (**figure 2.2**). The DNA synthesis assays of the fractions containing these two growth factors result either in no effect or an inhibition of DNA synthesis, suggesting the presence of growth inhibitors in MDBK cell conditioned medium. The results from rpHPLC of the MDBK-derived IGF-2 related growth factors (**figure 2.3**) support this conclusion, as upon elution from the C_{18} matrix both growth factors were associated with protein and DNA synthesis stimulating activity.

The production of growth inhibitors is yet another property that MDBK cells have in common with the cell line BRL-3A (Golub and Straus, 1981). Other cells and cell lines have also been reported to produce growth inhibitors that have been characterized to varying degrees (Wang and Hsu, 1986; Zarling *et al.*, 1986; Hilton *et al.*, 1988; Chen *et al.*, 1988). The characterization of the growth inhibitors produced by MDBK cells is considered in **chapter 4.**

In conclusion, the approach taken in this study has provided evidence that MDBK cells produce both growth factors (IGF-2 and pool A) and growth inhibitors (or negative

growth factors). Further chapters of this thesis will deal with the purification and characterization of the growth factor represented by pool A and MDBK cell-derived growth inhibitors, as well as the responsiveness of MDBK cells to growth factors.

Figure 2.1

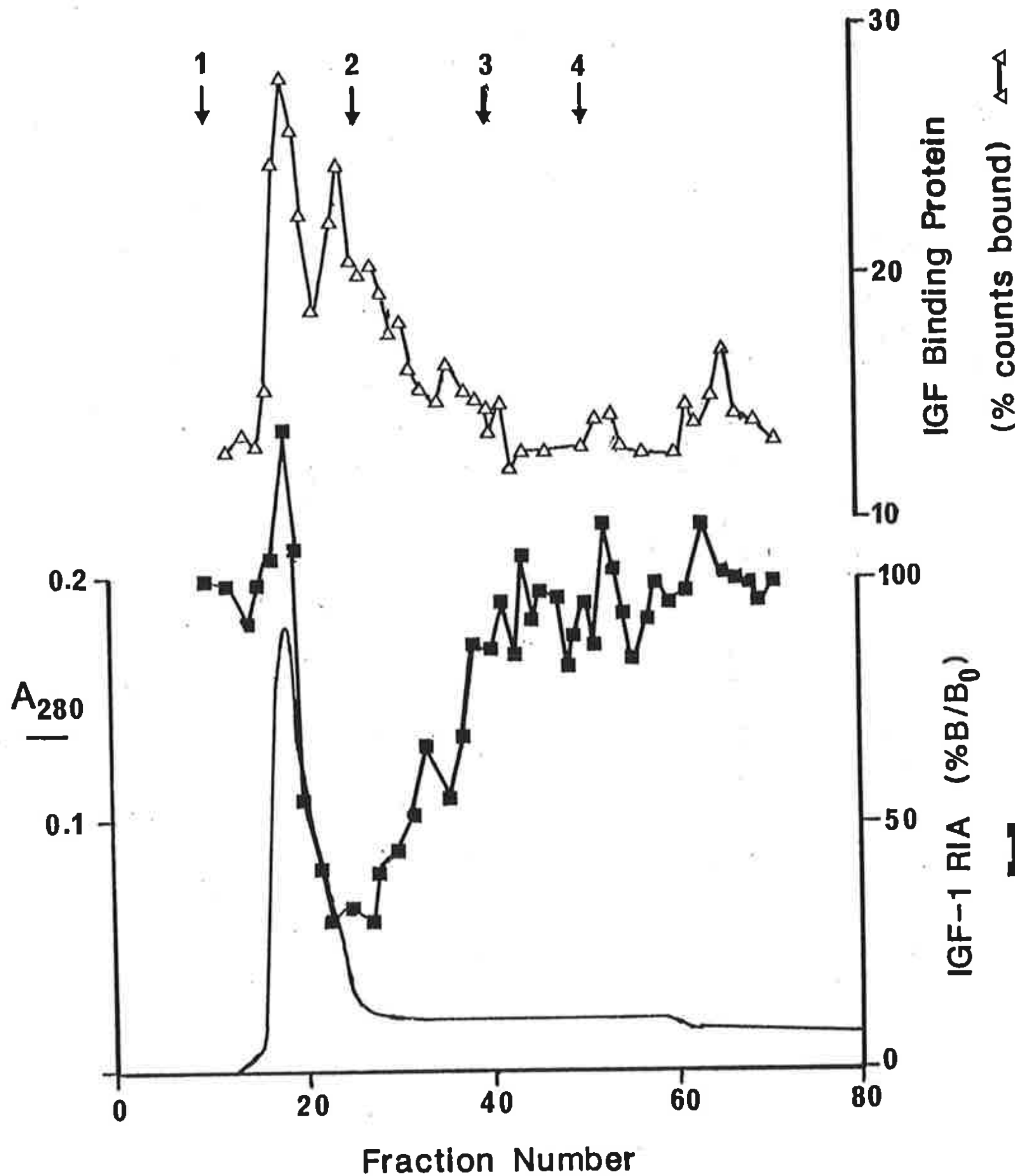


Figure 2.1 Gel filtration chromatography of MDBK cell conditioned medium. Elution profile of the equivalent of 350 ml of MDBK cell conditioned medium applied in 3.5 ml of 1 M acetic acid to a Sephadex G-75 column equilibrated and eluted with the same solution at a flow rate of 0.35 ml/min. Fractions were collected (3.8 ml) and assayed in an IGF-1 RIA (25 µl per fraction) and an IGF binding protein assay (250 µl per fraction). Arrows indicate, (1) the void volume, and the elution positions of, (2) BRL-3A IGF binding protein (31-33,000 M_r), (3) α-chymotrypsinogen A (25,000 M_r) and (4) MSA (multiplication stimulating activity or IGF-2, produced by BRL-3A cells, 8-16,000 M_r). The elution positions of BRL-3A IGF binding protein and MSA were determined by an IGF-2 radioreceptor performed on the fractions collected after acid gel filtration chromatography of BRL-3A cell conditioned medium.

Figure 2.2

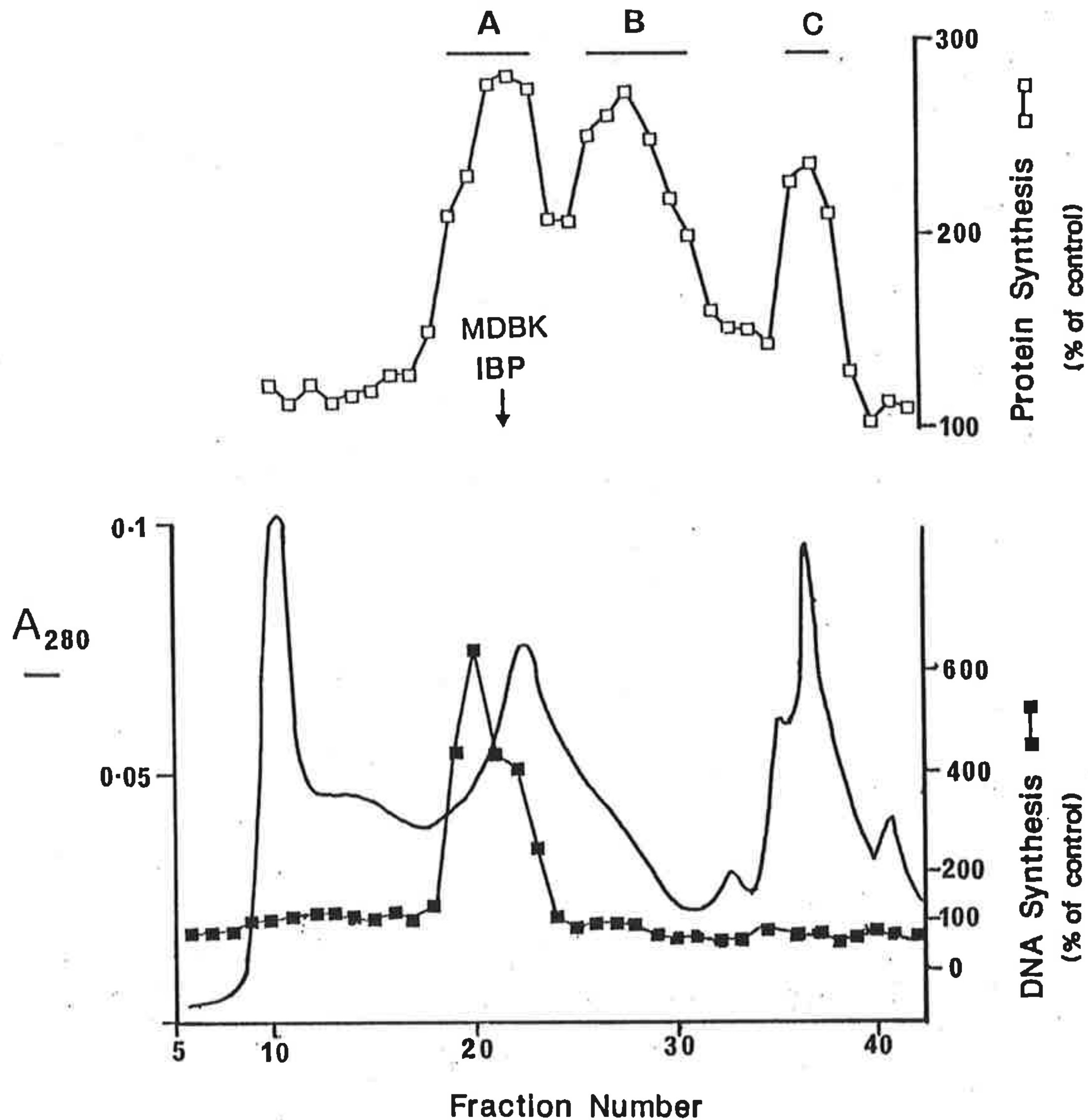


Figure 2.2 Preparative gel filtration chromatography of MDBK cell conditioned medium. Elution profile of the equivalent of 4 litres of MDBK cell conditioned medium applied in 45 ml of 1 M acetic acid to a Fractogel TSK HW-55 (S) column equilibrated and eluted with the same solution at a flow rate of 2 ml/min. Fractions were collected (30 ml) and assayed in protein synthesis (400 µl per fraction) and DNA synthesis (200 µl per fraction) assays. The elution position of the MDBK IGF binding protein (MDBK IBP) is indicated. Fractions pooled for subsequent characterization steps are indicated by solid bars (A, B and C).

Figure 2.3

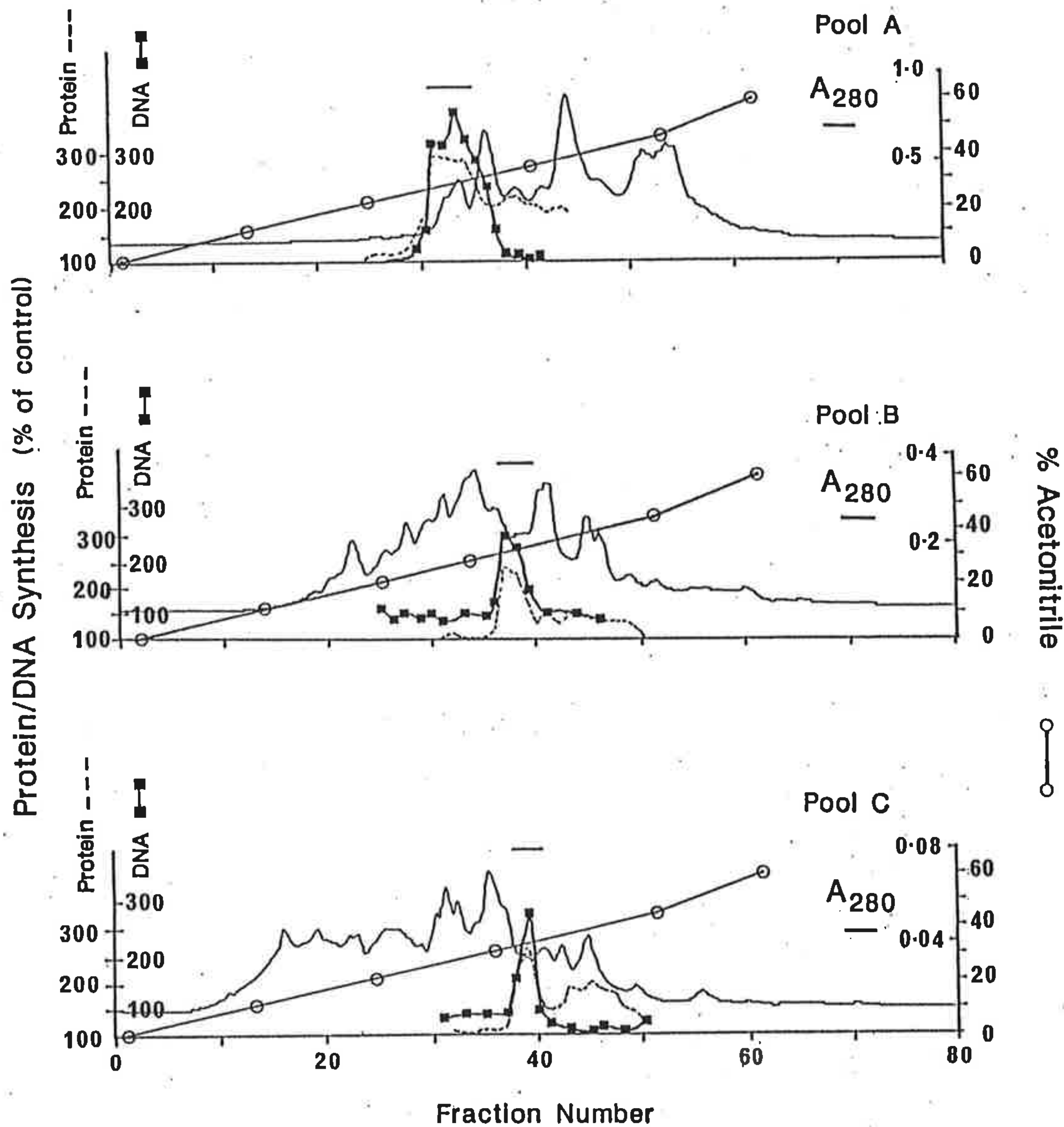


Figure 2.3 Reverse phase HPLC of pools A, B and C from preparative gel filtration chromatography. Pooled fractions were adjusted to 0.1% (v/v) TFA and pumped onto a Vydac C_{18} Radial-Pak cartridge equilibrated with 0.1% TFA. Growth factor activity was eluted with a two step linear gradient, 0 to 10% (v/v) acetonitrile in 5 min and then to 45% (v/v) acetonitrile over 20 min at a flow rate of 2 ml/min; fractions of 1 ml were collected. Aliquots of each fraction were lyophilized and assayed for protein synthesis (20, 15 and 50 µl for pools A, B and C respectively) and DNA synthesis (10, 10 and 50 µl for pools A, B and C respectively) activity. The horizontal bars represent the fractions pooled for further characterization.

Figure 2.4

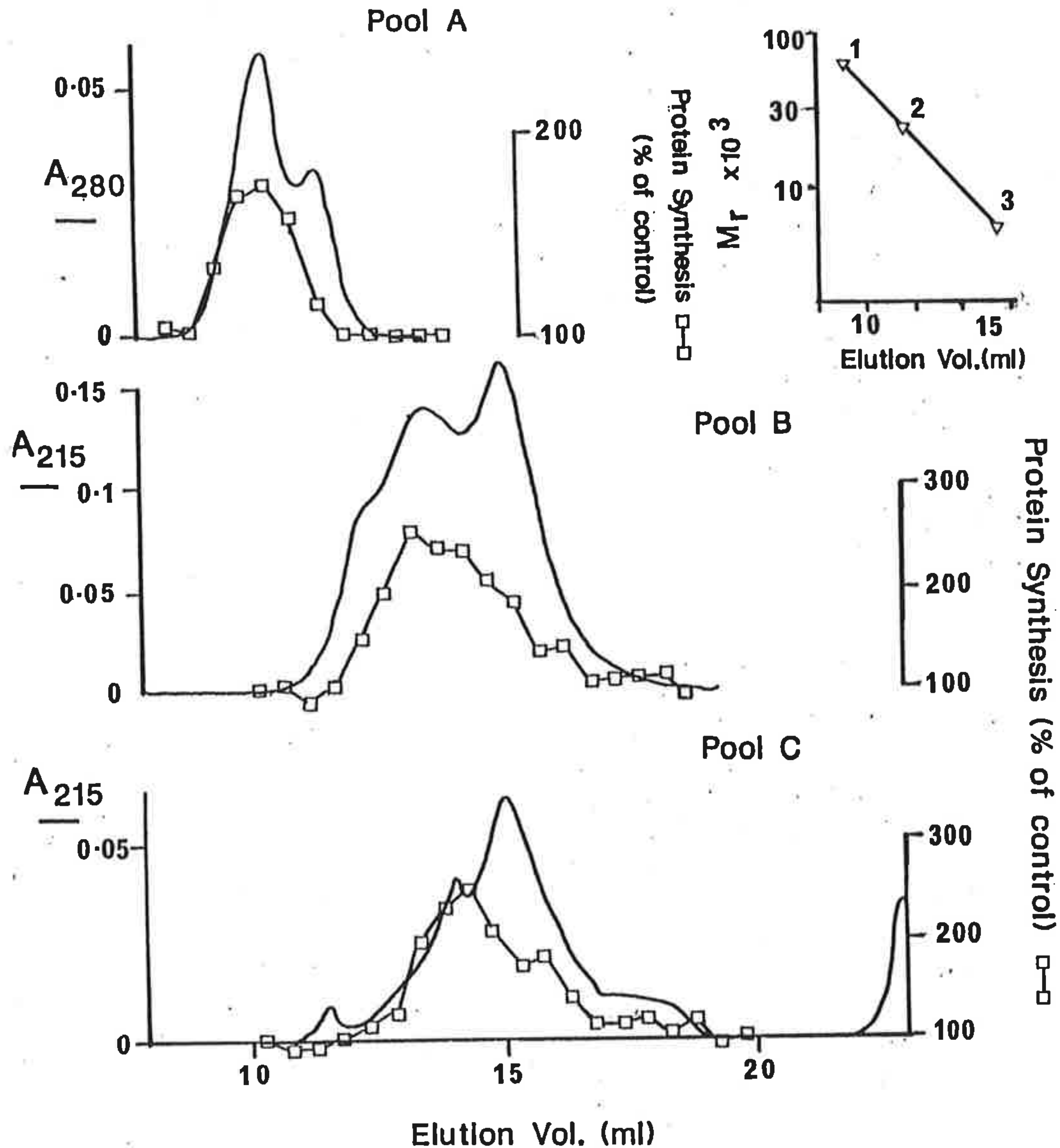


Figure 2.4 HPLC gel filtration chromatography of pools A, B and C from reverse phase HPLC. A proportion of each of the pooled fractions of growth factor activity (A, 10%; B, 5% and C, 20%) from reverse phase HPLC was adjusted to 40% acetonitrile and applied in a volume not greater than 400 µl to a TSK G 3000 SW gel filtration column (7.5 mm diam. x 60 cm) equilibrated with 0.1% TFA/40% acetonitrile and eluted at a flow rate of 0.5 ml/min. Fractions were collected (0.5 ml), lyophilized and assayed for stimulation of protein synthesis on L-6 myoblasts (for pool A, 50 µl of each fraction was assayed, for pools B and C, the entire fraction was lyophilized and assayed). The elution volumes of (1) bovine serum albumin, (2) α-chymotrypsinogen A and (3) ^{125}I-hEGF with M_r values respectively of 68,000, 25,000 and 6,000 are plotted as an inset.

Figure 2.5

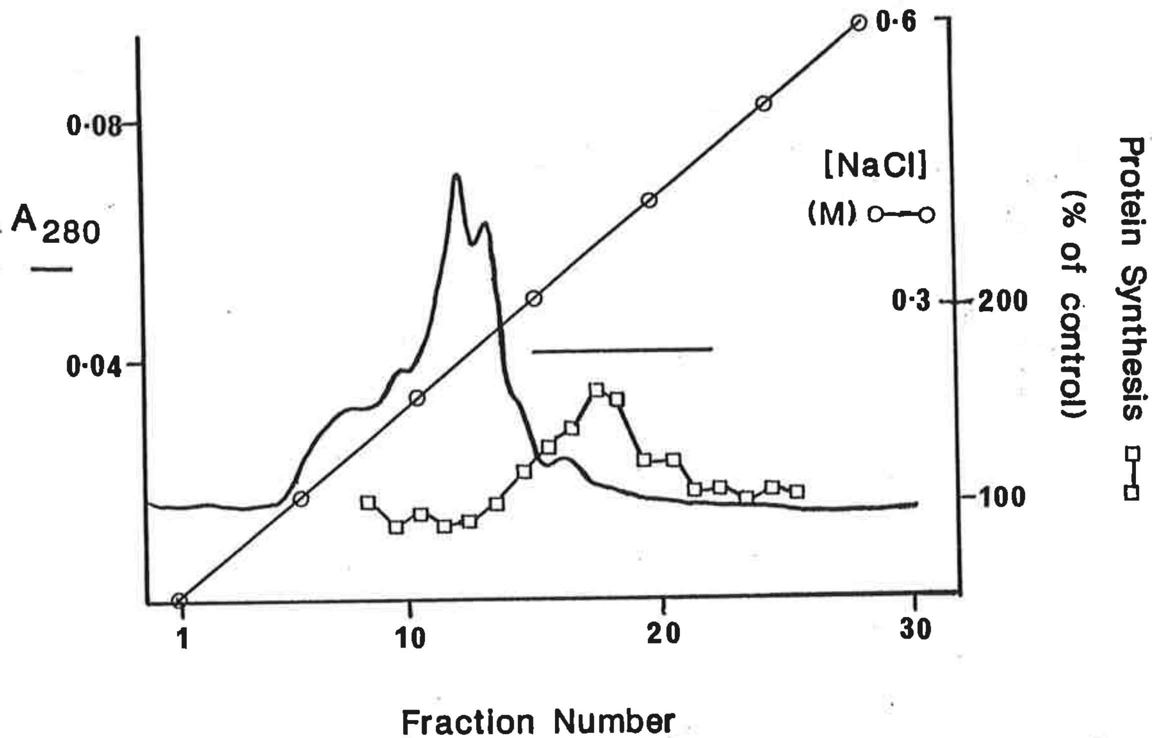


Figure 2.5 Cation exchange chromatography of pool A from reverse phase HPLC. The pooled fractions of pool A from reverse phase HPLC were lyophilized, dissolved in 50 mM sodium phosphate pH 7.4 (buffer A) and applied to a Mono S HR 5/5 column equilibrated with buffer A. Protein was eluted with a linear gradient of 0 to 650 mM NaCl in buffer A over 30 min at a flow rate of 1 ml/min. Fractions were collected (1 ml) and 1 μl of each assayed for protein synthesis activity. Fractions pooled for subsequent characterization are indicated by the solid bar.

Figure 2.6

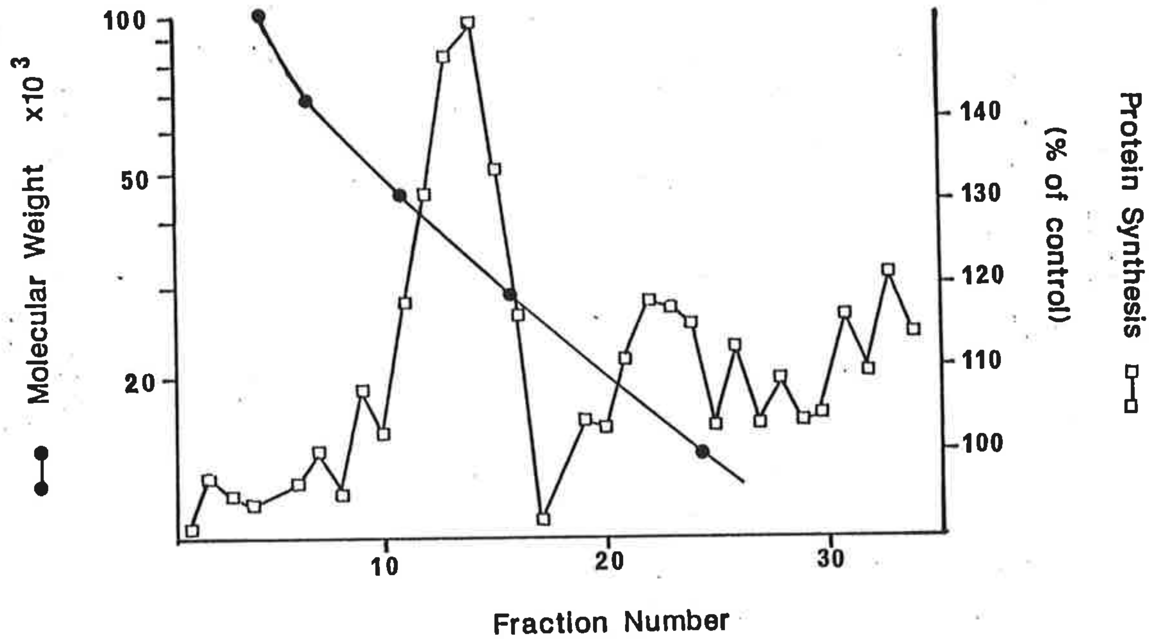


Figure 2.6 SDS-polyacrylamide gel electrophoresis of pool A and elution of bioactivity. An aliquot of pool A (1 μg of protein, equivalent to 5% of the total bioactivity after cation exchange chromatography) was lyophilized, dissolved in SDS loading buffer and run on a 15% SDS-polyacrylamide gel (Laemmli, 1970). After electrophoresis the gel track containing growth factor activity was cut into 3 mm slices and the protein eluted by homogenization in 1 M acetic acid/ 0.5% (w/v) SDS (as described by Raines and Ross, 1982). Fractions were assayed for bioactivity after acetone precipitation to remove SDS. Molecular weight standards were, phosphorylase b (97,000), bovine serum albumin (68,000), ovalbumin (45,000), carbonic anhydrase (29,000) and lysozyme (14,000).

Table 2.1[1]

Volume of Conditioned Medium	Protein Synthesis (% of control)
0.9 ml	245%
2.1 ml	275%
4.2 ml	320%
5% FBS	360%

1. Effect of MDBK cell conditioned medium on protein synthesis in L-6 myoblasts. Conditioned medium was lyophilized, dialyzed and assayed in the protein synthesis assay as stated in the Materials and Methods.

Table 2.2 : IGF-2 RRA of MDBK-derived Growth Factors

Growth Factor[1]	Protein Synthesis[2,4] (% of control)	IGF-2 RRA[3] (B/B_o)
Pool A (1 µl)		101 ± 5.0%
(5 µl)	172%	103 ± 4.6%
(10 µl)	253%	99 ± 0.4%
Pool B (0.2 µl)		127 ± 1.6%
(0.4 µl)		112 ± 4.4%
(2 µl)		80 ± 0.2%
(10 µl)	271%	47 ± 1.5%
Pool C (0.4 µl)		105 ± 4.5%
(1 µl)		85 ± 4.3%
(5 µl)		36 ± 2.5%
(10 µl)	160%	38 ± 1.2%

1. Pool A consisted of the fractions pooled from cation exchange chromatography, Pools B and C consisted of the fractions pooled from rpHPLC. 2. Results are the mean of duplicate determinations and are expressed as a percent of the negative control (i.e. no growth factor added). 3. Results are the mean of triplicate determinations ± SEM, and are expressed as percent radioligand bound. The EDequ "sub 50" type=text for IGF-2 in this assay is 5ng/ml. 4. See table 5.3 for typical protein synthesis responses of MDBK ana L-6 cells to the insulin-like growth factors.

Table 2.3 : Effect of Reduction on the Activity of the MDBK-derived Growth Factors[1]

Growth Factor (µg of protein)	Protein Synthesis in L-6 myoblasts (% of control)	
	-DTT	+DTT
Pool A (20 µg)	326%	124%
Pool B (17 µg)	273%	125%
Pool C (13 µg)	246%	116%

1. Pools A, B and C (pooled fractions from preparative gel filtration chromatography) were lyophilized and dissolved in 6 M guanidine.HCl, 100 mM Tris Cl pH 8.5, with or without 50 mM dithiothreitol (DTT) and incubated at 40°C for 4 hours. After dialysis, samples were assayed for protein synthesis stimulating activity in L-6 myoblasts.

CHAPTER THREE : Production of PDGF-AB heterodimer by MDBK Cells

Chapter 3: Introduction

The endogenous production of growth factors is a common feature of cells which survive or grow in culture without serum supplementation (Dulak and Temin, 1973; De Larco and Todaro, 1978a,b; Marquardt and Todaro, 1982; Massagué, 1983; Nagarajan *et al.*, 1985; Minuto *et al.*, 1988). One growth factor commonly produced by such cells is platelet-derived growth factor (PDGF) (Stroobant *et al.*, 1985; Van Zoelen *et al.*, 1985; Heldin *et al.*, 1986b; Grotendorst *et al.*, 1988; Hammacher *et al.*, 1988a). Human PDGF was originally purified from outdated platelet-rich plasma (Heldin *et al.*, 1979; Antoniades, 1981; Deuel *et al.*, 1981; Raines and Ross, 1982) and was found to be a dimeric cationic glycoprotein with a molecular weight of approximately 30,000. Reduction of the disulphide bonds in the protein destroys mitogenic activity and results in the production of multiple protein species of 14-17,000 M_r. Two distinct but related sequences were revealed in these multiple species upon amino acid sequence analysis (Antoniades and Hunkapiller, 1983; Waterfield *et al.*, 1983), suggesting that PDGF isolated from human platelets consisted of a heterodimer, the two chains of which are known as A and B. This suggestion has recently been confirmed (Hammacher *et al.*, 1988b). The A and B chains are structurally related with approximately 45% amino acid sequence identity between the finally processed forms (Betsholtz *et al.*, 1986a). In addition to the heterodimer, both of the homodimer isoforms, AA and BB, are known to occur (Stroobant and Waterfield, 1984; Heldin *et al.*, 1986b).

A range of cell types have been found to produce PDGF, including smooth and skeletal muscle cells (Sejersen *et al.*, 1986; Sjölund *et al.*, 1988), endothelial cells (Di Corleto and Bowen-Pope, 1983; Sitaras *et al.*, 1988), peritoneal and alveolar macrophages (Shimokado *et al.*, 1985), mesangial cells (Shultz *et al.*, 1988), BSC-1 kidney epithelial cells (Kartha *et al.*, 1988) and various transformed cells (see Heldin *et al.*, 1986a; and **table 1.1**). The evidence of PDGF production by these cell types has predominantly relied upon radioreceptor and antibody neutralization assays performed on conditioned medium. In only a few instances has a PDGF-like growth factor been purified and characterized from conditioned medium. Such examples include SV-40 transformed BHK cells (Stroobant *et al.*, 1985), the U-2 OS osteosarcoma (Heldin *et al.*, 1986b), WM 266-4

melanoma cells (Westermark *et al.*, 1986), the glioma cell line, U-343 MGa Cl 2:6 (Hammacher *et al.*, 1988a) and Neuro 2A neuroblastoma cells (Van den Eijnden-Van Raaij *et al.*, 1989).

The bovine kidney cell line MDBK was found to produce a cationic growth factor of 35,000 M_r that stimulates both protein and DNA synthesis in L-6 myoblasts and is inactivated upon reduction (see **Chapter 2**). Here I report the purification and characterization of this growth factor and demonstrate its structural and functional similarity to PDGF. Evidence is presented that this growth factor is the bovine equivalent of the PDGF-AB heterodimer.

3.1 Materials and Methods

Materials

Fetal-bovine serum was purchased from Flow Laboratories; bovine serum albumin (RIA grade), protein molecular weight standards, amino acids and vitamins for the preparation of media were obtained from Sigma Chemical Co.; L-[4,5-^{3}H]leucine (40-60 Ci/mmol) and [*methyl*-^{3}H]thymidine (20 Ci/mmol) were from New England Nuclear. Sources for the antibiotics used in media are given by Ballard *et al.* (1986). All dilutions of growth factors were made in the presence of 0.1% (w/v) BSA.

Rabbit anti-human PDGF-AB serum was a generous gift of Prof. Carl-Henrik Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden. Recombinant human PDGF-AA and PDGF-BB were kindly provided by Chiron Corporation, Emeryville, California, and purified human PDGF-AB by Dr. Elaine Raines, Department of Pathology, University of Washington, Seattle.

Cell cultures and the collection of conditioned medium

L-6 myoblasts, obtained from Dr. J. M. Gunn, Texas A&M University, College Station TX, U.S.A., were grown by serial passaging of prefused cultures in Dulbecco-modified Eagle's Minimal Essential Medium containing 5% (v/v) fetal-bovine serum, together with 50 mg of gentamycin, 100 mg of streptomycin, 60 mg penicillin and 1 mg of fungizone/litre of growth medium. The cells were grown and used for experiments as monolayers at 37°C under an atmosphere of CO_2/humidified air (1:19). For experimental purposes the cells were subcultured into Linbro 24-place multiwell dishes in growth

medium and used during the 4 days after the monolayers first became confluent. At this stage myotubes were not evident. MDBK cells (Flow Laboratories ATCC CCL22) were grown as for L-6 cells except the growth medium contained 10% (v/v) fetal-bovine serum. For the production of conditioned medium, MDBK cells were grown in a Nunc cell factory in growth medium until confluent. Subsequently, cells were transferred to growth medium without serum supplementation. After 24 hours, conditioned medium was replaced with fresh growth medium without serum. This collection was discarded. Conditioned medium was henceforth collected every third day while the cells remained viable (about 8 weeks) and stored at -15°C until used.

Assay for growth factor activity

The determination of growth factor activity was based on the stimulation of protein synthesis in L-6 rat myoblasts as described by Francis *et al.* (1986). Briefly, the measurement involves the incorporation of [^{3}H]leucine into total cell protein during an 18 hour incubation of confluent cell monolayers in 24-place multiwell dishes. Activity is expressed as the percent stimulation of protein labelling over that occurring in monolayers incubated without growth factors in DMEM.

Purification of growth factor.

Conditioned medium (40 litres) collected from MDBK cells was thawed and filtered through Whatman No. 1 paper. All further steps were carried out at room temperature. The filtrate was acidified to 50 mM acetic acid with glacial acetic acid and the pH adjusted to 3.0 with concentrated HCl. After a second filtration through Whatman No. 1 paper, batches of 8 to 13 litres of clear filtrate were pumped onto a S-Sepharose Fast Flow column (3.2 cm diam. x 25 cm, Pharmacia) at a flow rate of 10 ml/min. The column was washed with 1 litre of 50 mM acetic acid followed by 1 litre of 50 mM ammonium acetate pH 6.5. Growth factor activity was eluted with a 1.2 litre linear gradient of 50 mM ammonium acetate pH 6.5 to 2.0 M ammonium acetate pH 8.0 at a flow rate of 3 ml/min. Fractions were collected and an aliquot (150 µl) of each assayed for the stimulation of ^{3}H-leucine incorporation into L-6 myoblasts. Fractions of the highest specific activity were pooled as indicated (**figure 3.1**).

Following S-Sepharose chromatography, trifluoroacetic acid (TFA) was added to the pooled fractions to achieve a final concentration of 0.1% (v/v), and the pH adjusted to 2.1 with concentrated HCl. The resulting solution was filtered through a 0.45 µm filter (Minisart NML, Sartorius) and pumped onto an Aquapore Prep 10 RP-300 HPLC cartridge (1 cm diam. x 10 cm, Brownlee Labs) equilibrated with 0.1% TFA. This concentration of TFA was maintained throughout. The cartridge was washed with 0.1% TFA/15% acetonitrile until the absorbance at 280 nm returned to baseline. Growth factor activity was eluted with a linear gradient from 15% to 40% acetonitrile over 50 min at a flow rate of 1 ml/min. Growth factor activity eluted as two broad peaks which were pooled as indicated (**figure 3.2**).

Pool A and B were diluted with 0.1% (v/v) heptafluorobutyric acid (HFBA) to adjust the acetonitrile concentration to approximately 10%, and separately loaded onto an Aquapore RP-300 HPLC cartridge (4.6 mm diam. x 3 cm, Brownlee Labs) equilibrated with 0.1% HFBA. This concentration of HFBA was maintained throughout. The cartridge was washed with 0.1% HFBA/10% propan-1-ol until the absorbance at 280 nm had returned to baseline. Growth factor activity was eluted with a two step linear gradient of 10 to 25% propan-1-ol in 10 min and then to 40% propan-1-ol in 60 min at a flow rate of 1 ml/min. Growth factor activity was pooled as indicated (**figure 3.3**), diluted with 0.1% TFA to adjust the propan-1-ol concentration to approximately 5%, and pools A and B separately re-applied to the Aquapore RP-300 HPLC cartridge (4.6 mm diam. x 3 cm) which was equilibrated with 0.1% TFA. The cartridge was washed with 0.1% TFA/13% propan-1-ol in the case of pool A and 0.1% TFA/9% propan-1-ol in the case of pool B. Growth promoting activity was eluted with a linear gradient of propan-1-ol (from 13 to 28% in 60 min in the case of pool A, and from 9 to 24% in 60 min in the case of pool B) at a flow rate of 1 ml/min. Growth promoting activity which eluted between 14-16% propan-1-ol in the case of pool A and B was pooled as indicated in **figure 3.4**.

Pools A and B from the third rpHPLC step (**figure 3.4**) were combined and diluted with 0.1% TFA to adjust the propan-1-ol concentration to approximately 5%. The resulting solution was loaded onto an Aquapore RP-300 microbore HPLC cartridge (2.1 mm diam. x 3 cm, Brownlee Labs) equilibrated with 0.1% TFA. The cartridge was washed with 0.1% TFA/10% acetonitrile until the absorbance at 215 nm returned to baseline. Growth factor

activity was eluted with a two step linear gradient from 10 to 15% acetonitrile in 5 min and then to 40% acetonitrile in 100 min at a flow rate of 0.5 ml/min. Growth factor activity eluted as a single peak coincident with the peak of A_{215} absorbing material (**figure 3.5**). The fractions with growth factor activity were pooled and used for all further characterization steps. To minimize losses, growth factor was stored in 0.02% (v/v) Tween 20/0.1% TFA/acetonitrile (acetonitrile concentration was as eluted from the final rpHPLC) at -20°C (Simpson *et al.*, 1987).

Protein determination

Throughout this study protein content was monitored by absorbance at 280 or 215 nm. Protein concentrations were calculated by assuming that A_{280}=1.0 for a 1 mg/ml solution in a 1 cm light path and an A_{280}/A_{215} ratio of 0.1.

DNA synthesis determination

Confluent monolayers of L-6 cells in 24-place multiwell dishes were washed with DMEM for 2 hours and exposed to growth factors for 18 hours in 1 ml of DMEM. At the end of this period, 5 nmol of thymidine containing 1 µCi of [^{3}H]thymidine was added for a further 6 hours. Harvesting of the monolayers was carried out as described previously (Ballard *et al.*, 1986). Activity is expressed as the percent stimulation of DNA labelling over that occurring in monolayers incubated without growth factors in DMEM.

PDGF ELISA assays

PDGF-BB or PDGF-AA, at a concentration of 10 ng/ml or 500 ng/ml respectively, in phosphate-buffered saline (PBS, 10 mM sodium phosphate, pH 7.4, 150 mM NaCl) was used to coat Nunc Immuno Plate 1 96-well microtitre plates (100 µl/well) for 18 hours at 4°C. The plates were blocked with 1 mg/ml BSA in PBS for 1 hour at 37°C. All dilutions of antiserum and samples were made in PBS containing 0.1% (v/v) Tween 20 and 1 mg/ml BSA. The PDGF-AB antiserum was used at a dilution of 1/800, a dilution which resulted in a A_{450} absorbance of approximately 0.8 without the addition of competing ligand. PDGF standard was diluted to concentrations of 0.06 to 10 ng/ml for the PDGF-BB specific assay, and to 1.0 ng/ml to 2.0 µg/ml for the PDGF-AA specific assay. PDGF antiserum and test samples or PDGF standards were incubated for 18 hours at 4°C prior to addition to the blocked microtitre plates. The blocked plates were washed 4 times with PBS/0.1% Tween 20 and 100 µl of each test/standard solution was added to appropriate wells of the coated

and blocked microtitre plates and incubated for 4 hours at 37°C. The plates were washed 4 times with PBS/0.1% Tween 20. Subsequently 100 µl of peroxidase conjugated goat anti-rabbit IgG (1/2000 dilution), diluted in PBS/0.1% Tween 20/ 1 mg/ml BSA was added to each well which was then incubated for 2 hours at 37°C. The plates were washed as before, drained, and 100 µl of activated substrate solution (2.5 mM O-tolidine dihydrochloride dissolved in 10 mM sodium citrate, pH 4.5, 0.025 mM EDTA, activated just before use by adding 10 µl of a 3% solution of hydrogen peroxide to 10 ml of substrate solution) added to each well. After 10 min at room temperature the reaction was stopped by the addition of 50 µl of 3 M HCl per well. The optical density of each well was measured with a microtitre plate scanner at 450 nm.

SDS-gel electrophoresis and silver staining

SDS-polyacrylamide gel electrophoresis was performed (Laemmli, 1970) using a 15% separating gel and a 4.5% stacking gel. Gels were silver-stained using the method described by Heukeshoven and Derynck (1985).

3.2 Results

Studies on the serum-free conditioned medium of MDBK cells indicated that these cells produced a cationic, acid stable growth factor, of 35,000 M_r (see **Chapter 2**). Attempts to purify this growth factor have concentrated on cation exchange and rpHPLC chromatography steps with growth factor activity monitored by the effect of each column fraction on the incorporation of ^{3}H-leucine (as a measure of protein synthesis) in L-6 myoblasts (Francis *et al.*, 1986). The purification procedure resulted in approximately a 2,300-fold purification of growth factor activity and a yield of 4% relative to acidified MDBK cell conditioned medium (**table 3.1**). Dose response curves for the stimulation of protein synthesis by the MDBK cell-derived growth factor at various stages of purification are shown in **figure 3.6** and a summary of the purification is presented in **table 3.1**.

The purified growth factor was analysed by SDS-polyacrylamide gel electrophoresis and silver staining (**figure 3.7**). Under non-reducing conditions the growth factor appeared as a major and a minor band of approximately 35,000 M_r. When the same technique was

applied to a sample of growth factor under reducing conditions a series of bands with molecular weights ranging from 14-30,000 appeared, indicating that the sample is not a homogenous preparation.

As the MDBK cell-derived growth factor exhibited a number of properties in common with platelet-derived growth factor (PDGF), the purified growth factor was tested in a radioreceptor assay specific for PDGF. The growth factor competed in parallel with hPDGF-AB standard in the assay, indicating the presence of PDGF in the growth factor sample (E.W. Raines, *pers. comm.*, 1989). This radioreceptor assay utilized monolayers of human dermal fibroblasts as the source of PDGF receptors and ^{125}I-hPDGF-AB as the radioligand. The MDBK-derived PDGF (which I shall refer to as bovine or bPDGF) was also assayed in a radioreceptor assay in which ^{125}I-hPDGF-BB was the radioligand. The level of competition indicated that less than 10% of the competition in the AB radioreceptor assay was due to the PDGF-BB isoform (E.W. Raines, *pers. comm.*, 1989).

A comparison was made between the different isoforms of PDGF (AB, BB and AA) and bPDGF for their effects on L-6 myoblasts. In protein synthesis assays on L-6 cells the growth factors hPDGF-AB, hPDGF-BB and bPDGF exhibited a similar maximum response (approximately 70% of the effect of 10% fetal-bovine serum (FBS)), whereas hPDGF-AA had no effect (**figure 3.8**). Of the various forms of PDGF, the BB isoform was the most potent exhibiting an ED_{50} of ≈ 0.5 ng/ml, the ED_{50} of the AB isoform being ≈ 6 ng/ml. Similarly in DNA synthesis assays on L-6 myoblasts hPDGF-BB and bPDGF exhibited similar maximum responses (80-90% of the effect of 10% fetal-bovine serum (FBS)) whereas hPDGF-AA had no effect (**figure 3.8**). The ED_{50} of hPDGF-BB on DNA synthesis in L-6 myoblasts was 0.35 ng/ml. Bovine PDGF exhibited similar potencies in both the protein and DNA synthesis assays. As the AA homodimer had no effect on L-6 cells, the effects of hPDGF-AA, hPDGF-BB and bPDGF on DNA synthesis in He[39]L fibroblasts was investigated. The results (**table 3.2**) indicated that all three growth factors are active on He[39]L cells, the BB homodimer being the most potent.

Bovine PDGF was further investigated for its structural similarity to human PDGF by the utilization of antibodies raised against hPDGF-AB. For this purpose, ELISA assays specific for the A and B chains of PDGF were developed (see **Appendix A1**). These assays utilized a polyclonal antiserum raised against human PDGF-AB, and either recombinant

human PDGF-AA or PDGF-BB as the antigen. In the ELISA assay specific for the A chain of PDGF, bPDGF and hPDGF-AA competed with similar potencies, whereas hPDGF-BB, IGF-1 and insulin did not compete to any significant extent in this assay (**figure 3.9**). In the B chain ELISA assay bPDGF appears to be approximately 1,000-fold less potent than hPDGF-BB, whereas IGF-1 and insulin do not compete even at high concentrations (**figure 3.9**).

Monoclonal antibody neutralization assays also supported a structural similarity between bPDGF and hPDGF (E.W. Raines, *pers. comm.*, 1989). In these assays the effect of various antibodies on the mitogenic activity of bPDGF or the various forms of hPDGF on human dermal fibroblasts was investigated. The antibodies used were monoclonals specific for either the AA homodimer or the AB/BB PDGF isoforms. The results (see **table 3.3**) show that the AB/BB specific monoclonal antibody neutralized the mitogenic activity of bPDGF, hPDGF-AB and hPDGF-BB to a similar extent, whereas the monoclonal antibody specific for the AA homodimer had no effect on any of these growth factors. It is concluded from the above results that bPDGF represents the bovine equivalent of hPDGF-AB.

3.3 Discussion

In this report I have described the purification and characterization of a form of PDGF isolated from the conditioned medium of the MDBK cell line. The results presented indicate that the majority of this form of PDGF consists of the AB heterodimer. To the best of my knowledge, this is the first report of a kidney epithelial cell line producing the AB heterodimer form of PDGF. A previous report concluded that BSC-1 monkey kidney cells produce a PDGF B-chain homodimer (Kartha *et al.*, 1988). Evidence of such production consisted of the neutralization of 80-90% of the activity of BSC-1 conditioned medium on fibroblasts and smooth muscle cells by an antibody against human PDGF, and the detection of the mRNA for the B chain of PDGF, but not the A chain, in mRNA obtained from BSC-1 cells. Human fetal kidney cells have also been reported to produce PDGF, however the type of PDGF and the morphology of the cells was not reported (Fraizer *et al.*, 1987).

3.3 Discussion

That PDGF exists as a heterodimer was first suggested from the protein sequence data obtained for PDGF isolated from human platelets (Antoniades and Hunkapiller, 1983; Waterfield *et al.*, 1983). This has since been confirmed by immunoprecipitation experiments with antisera specific for the A and B chains of PDGF (Hammacher *et al.*, 1988b). Recently a human glioma cell line, U-343 MGa Cl 2:6, was reported to produce all three forms of PDGF (Hammacher *et al.*, 1988a). This was the only report of a cell line producing the AB heterodimer of PDGF until the current report (except for the engineered situation of CHO cells transfected with A and B chain cDNA clones, Östman *et al.*, 1988).

Since the homodimer isoforms (AA and BB) of PDGF have become available, it has become obvious that different cell types respond differently to the two isoforms. The first report of a difference in the biological activities of the A and B chains of PDGF was that of Nistér *et al.* (1988). The differences reported were between the AA homodimer and the AB heterodimer when tested on human dermal fibroblasts in mitogenic, receptor autophosphorylation and chemotatic activity assays. In all these assays PDGF-AB is active, whereas PDGF-AA has either limited or no effect. Similar results have now been reported by other workers (Hammacher *et al.*, 1988a; Kazlauskas *et al.*, 1988; Raines *et al.*, 1989). These differences between the PDGF isoforms also appears to extend to differences in the transforming potential of AA and BB homodimers, transforming ability being confined to the BB homodimer (Beckmann *et al.*, 1988; Bywater *et al.*, 1988).

The above results are suggestive of the existence of more than one receptor for PDGF. Indeed, binding experiments with the different isoforms also indicate the presence of more than one receptor (Hart *et al.*, 1988; Heldin *et al.*, 1988). The existence of two receptor types for PDGF is now well established following the cloning and expression of the human PDGF-BB and PDGF-AA specific receptors (Claesson-Welsh *et al.*, 1988; Escobedo *et al.*, 1988; Gronwald *et al.*, 1988; Matsui *et al.*, 1989). Our results, as reported here, indicate that the rat L-6 myoblast cell line predominantly expresses the PDGF-BB specific form of the PDGF receptor, as hPDGF-AA is not active on protein or DNA synthesis in this cell line. This is in contrast to the human lung derived fibroblast cell line, He[39]L, upon which hPDGF-AA is active, and human aortic smooth muscle cells, in which the numbers of PDGF-AA and PDGF-BB binding sites are approximately equal (Raines *et al.*, 1989).

Little work to date has been reported on PDGF from non-primate sources. Porcine PDGF has been purified from platelets and identified as the BB isoform (Stroobant and Waterfield, 1984). Bovine PDGF has also been purified from platelets and was found to have a similar molecular weight and isoelectric point to human PDGF (Narczewska *et al.*, 1985). Further evidence of the similarity between bovine, porcine and human PDGF was provided by Czyrski and Gawlikowski (1987) who reported that all three isolates of PDGF competed to a similar extent in a human placental membrane radioreceptor assay. However, our results indicate that the form of PDGF produced by the bovine kidney cell line, MDBK (bPDGF, an AB heterodimer), is a 1,000-fold less potent in a B chain specific ELISA assay than hPDGF-BB standard. In a similar A chain specific ELISA assay, bPDGF was equipotent with human PDGF-AA. These results suggest that the sequence of the bovine PDGF B chain is different enough from the human counter-part that antibodies raised against the human B chain do not recognize the bovine B chain. This does not appear to be true in the case of the A chain, and therefore the sequence divergence between the human and bovine A chains may be less than that between the B chains.

The production by kidney epithelial cells of PDGF raises questions as to the *in vivo* role of PDGF in terms of kidney function. Kartha *et al.* (1988) have reported that the monkey kidney epithelial cell line BSC-1 produces a B chain homodimer form of PDGF. I have reported evidence here that the MDBK bovine kidney cell line produces the AB heterodimer form of PDGF. Neither of these cell lines appear to respond to PDGF (Kartha *et al.*, 1988; and **Chapter 5**), therefore if the mitogen is produced *in vivo* in the kidney it may well have a paracrine role, with smooth muscle, mesangial and fibroblastic cells being likely targets. Another possibility is that the production of growth factors such as PDGF by cell lines such as MDBK and BSC-1 is purely a by-product of the immortalization process. An investigation of what growth factors are produced by the kidney *in vivo* in different growth states (e.g. during development and compensatory growth) may well distinguish between these possibilities.

Figure 3.1

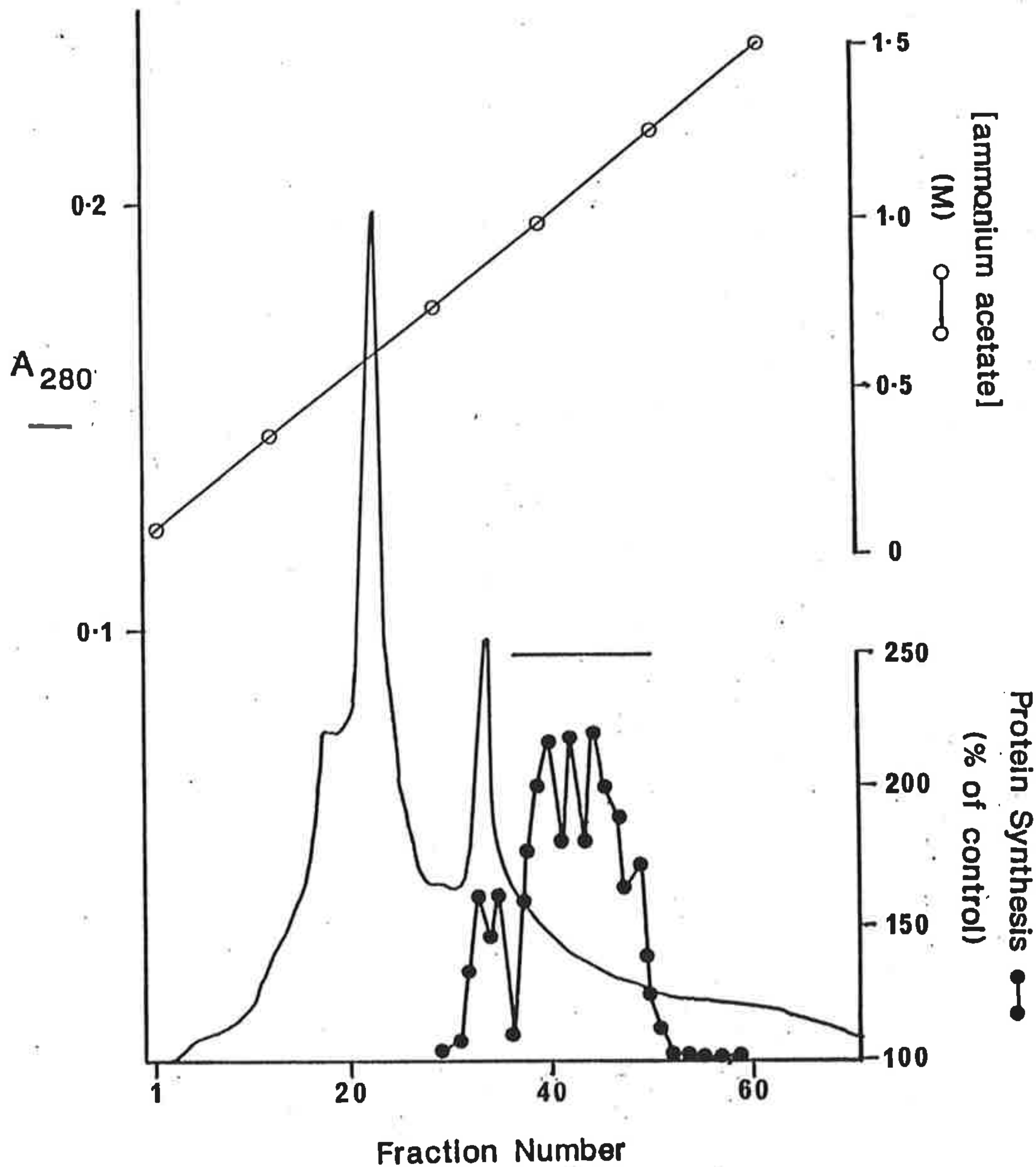


Figure 3.1 Cation exchange chromatography of MDBK cell conditioned medium. Acidified MDBK cell conditioned medium (11.5 litres) was pumped onto a S-Sepharose Fast Flow column equilibrated with 50 mM acetic acid. The column was washed with 50 mM ammonium acetate pH 6.5 and growth factor activity eluted with a 1.2 litre linear gradient of 50 mM ammonium acetate pH 6.5 to 2.0 M ammonium acetate pH 8.0 at a flow rate of 3 ml/min. Fractions (13.5 ml) were collected and 150 µl of each fraction lyophilized and assayed for growth factor activity. The horizontal bar represents those fractions pooled for subsequent purification steps.

Figure 3.2

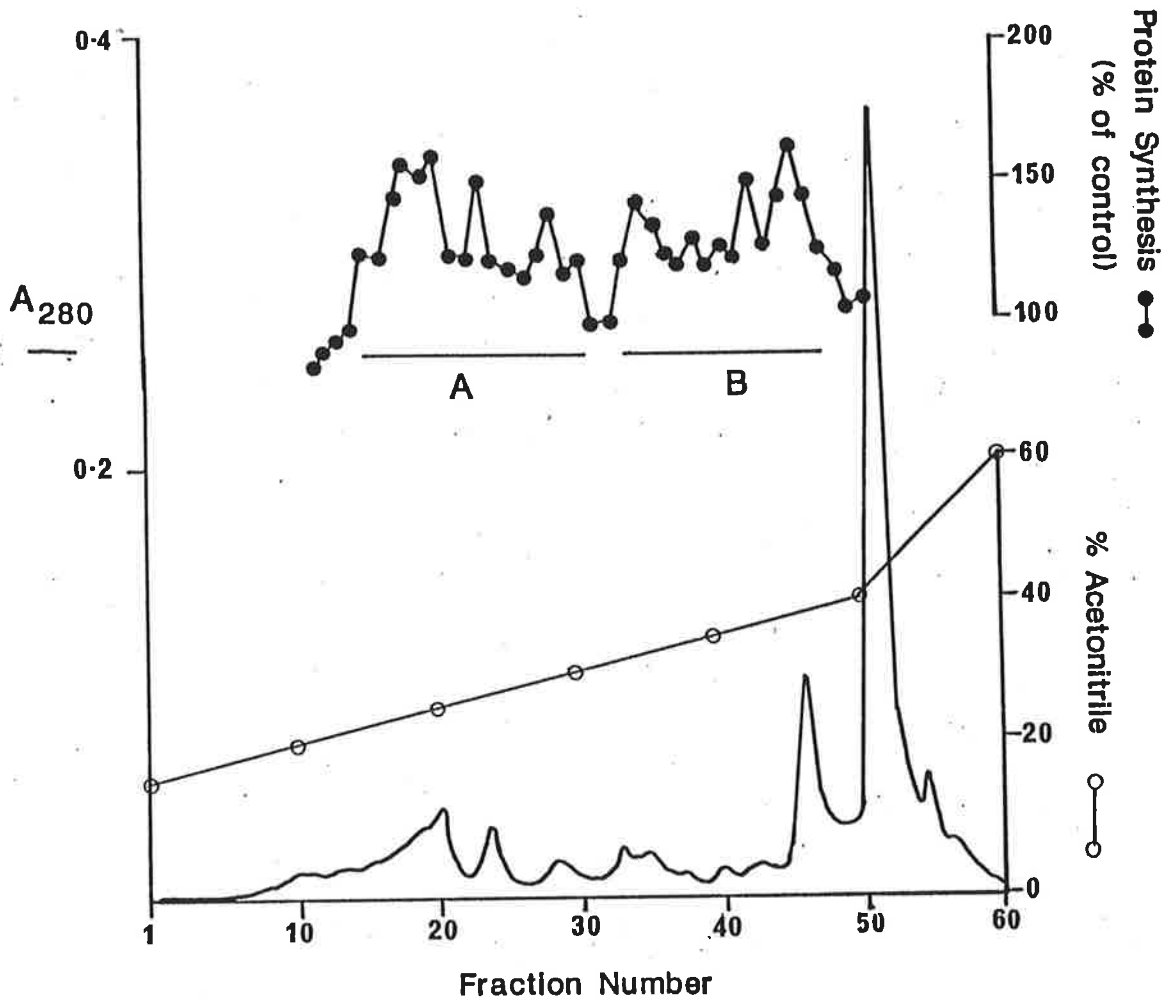


Figure 3.2 Elution profile of the first reverse phase HPLC. The pooled fractions from two cation exchange column runs (equivalent to 25 litres of conditioned medium) were adjusted to 0.1% TFA pH 2.1 and pumped onto an Aquapore Prep 10 RP-300 cartridge equilibrated with 0.1% TFA. The cartridge was washed with 0.1% TFA/15% acetonitrile and growth factor activity eluted with a linear gradient from 15% to 40% acetonitrile over 50 min at a flow rate of 1 ml/min, with fractions of 1 ml being collected. Aliquots of each fraction (2 µl) were lyophilized and assayed for growth factor activity. Fractions pooled for subsequent purification steps are indicated by solid bars (A and B).

Figure 3.3

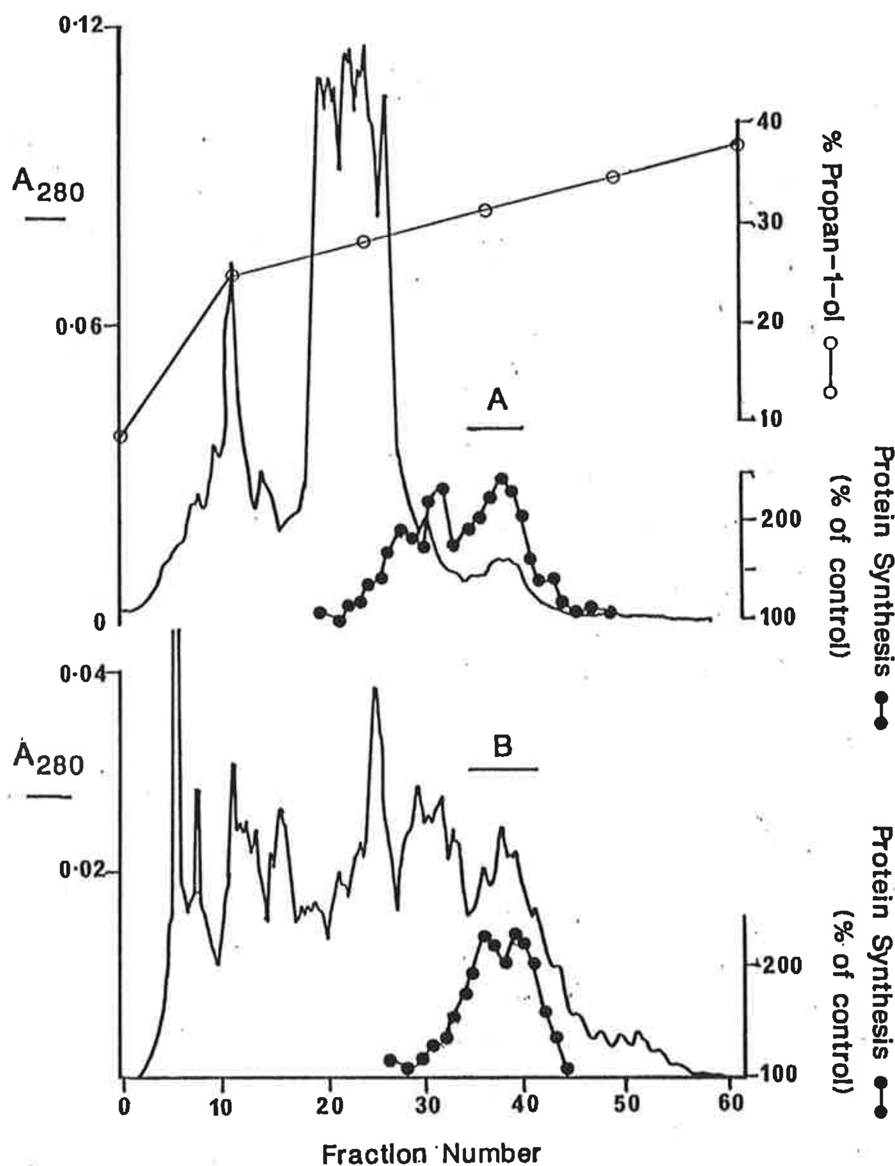


Figure 3.3 Elution profiles of the second reverse phase HPLC. The pooled fractions from two 1st rpHPLC steps (equivalent to 40 litres of conditioned medium in total) were diluted with 0.1% HFBA to adjust the acetonitrile concentration to approximately 10% and pumped onto an Aquapore RP-300 cartridge equilibrated with 0.1% HFBA. The cartridge was washed with 0.1% HFBA/10% propan-1-ol and growth factor activity eluted with a two step linear gradient of 10% to 25% propan-1-ol in 10 min and then to 40% propan-1-ol in 60 min at a flow rate of 1 ml/min. The elution profile for pool A is shown in the *upper panel* and that for pool B in the *lower panel.* Fractions of 1 ml were collected and 5 µl of each fraction was assayed for growth factor activity. Fractions pooled for subsequent purification steps are indicated by solid bars (A and B).

Figure 3.4

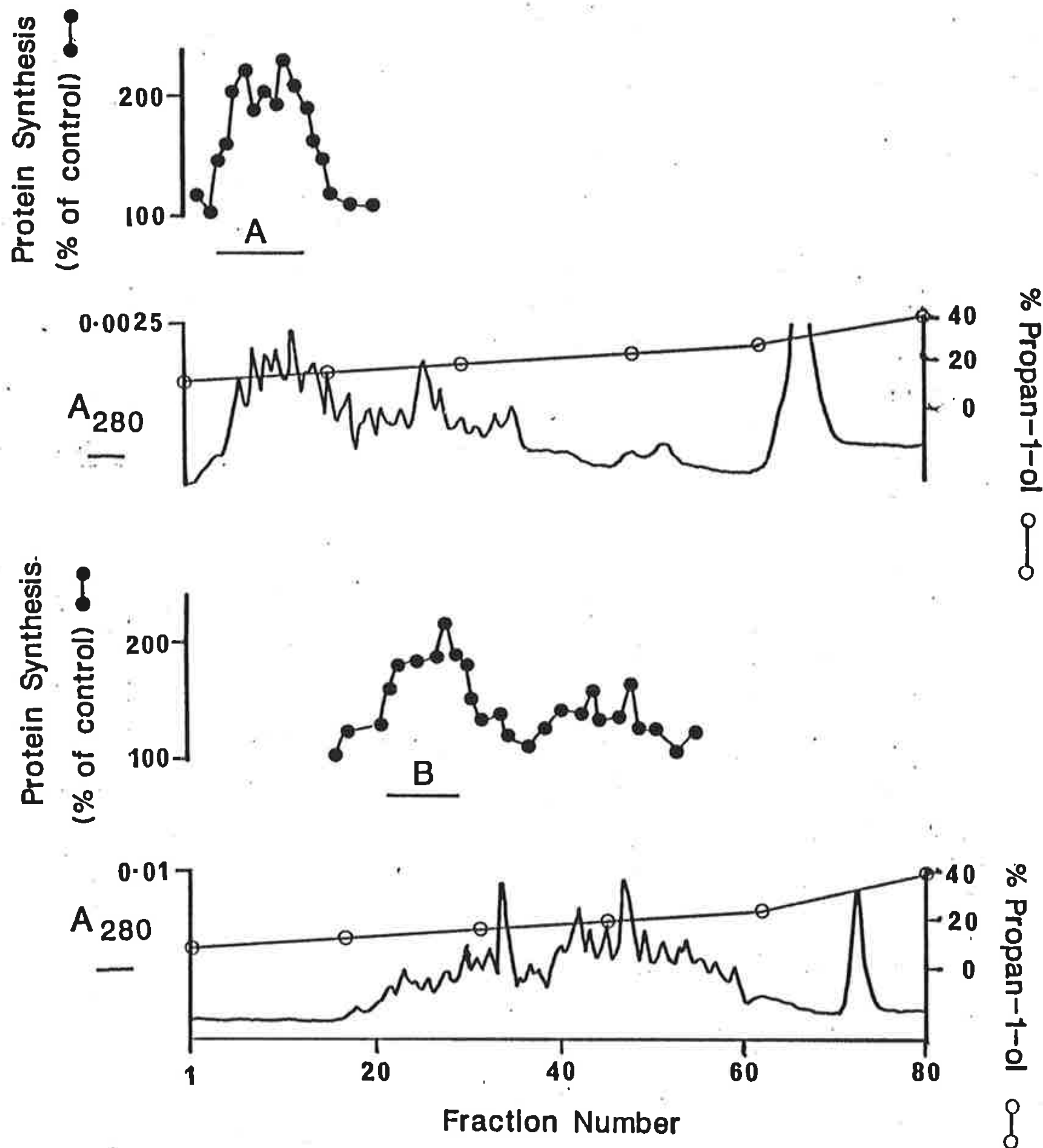


Figure 3.4 Elution profiles of the third reverse phase HPLC. The pooled fractions from the second rpHPLC step were diluted with 0.1% TFA to adjust the propan-1-ol concentration to approximately 5% and pumped onto an Aquapore RP-300 cartridge equilibrated with 0.1% TFA. In the case of pool A (*upper panel*) the cartridge was washed with 0.1% TFA/13% propan-1-ol and growth factor activity eluted with a linear gradient of propan-1-ol from 13% to 28% in 60 min at a flow rate of 1 ml/min. In the case of pool B (*lower panel*) the cartridge was washed with 0.1% TFA/9% propan-1-ol and growth factor activity eluted with a linear gradient of propan-1-ol from 9% to 24% in 60 min at a flow rate of 1 ml/min. Fractions of 1 ml were collected for both pool A and B and 10 μl of each fraction assayed for growth factor activity. Fractions from pool A and pool B chromatography runs which were pooled (consisting of the activity which eluted between 14% and 16% propan-1-ol) for further purification are indicated by solid bars.

Figure 3.5

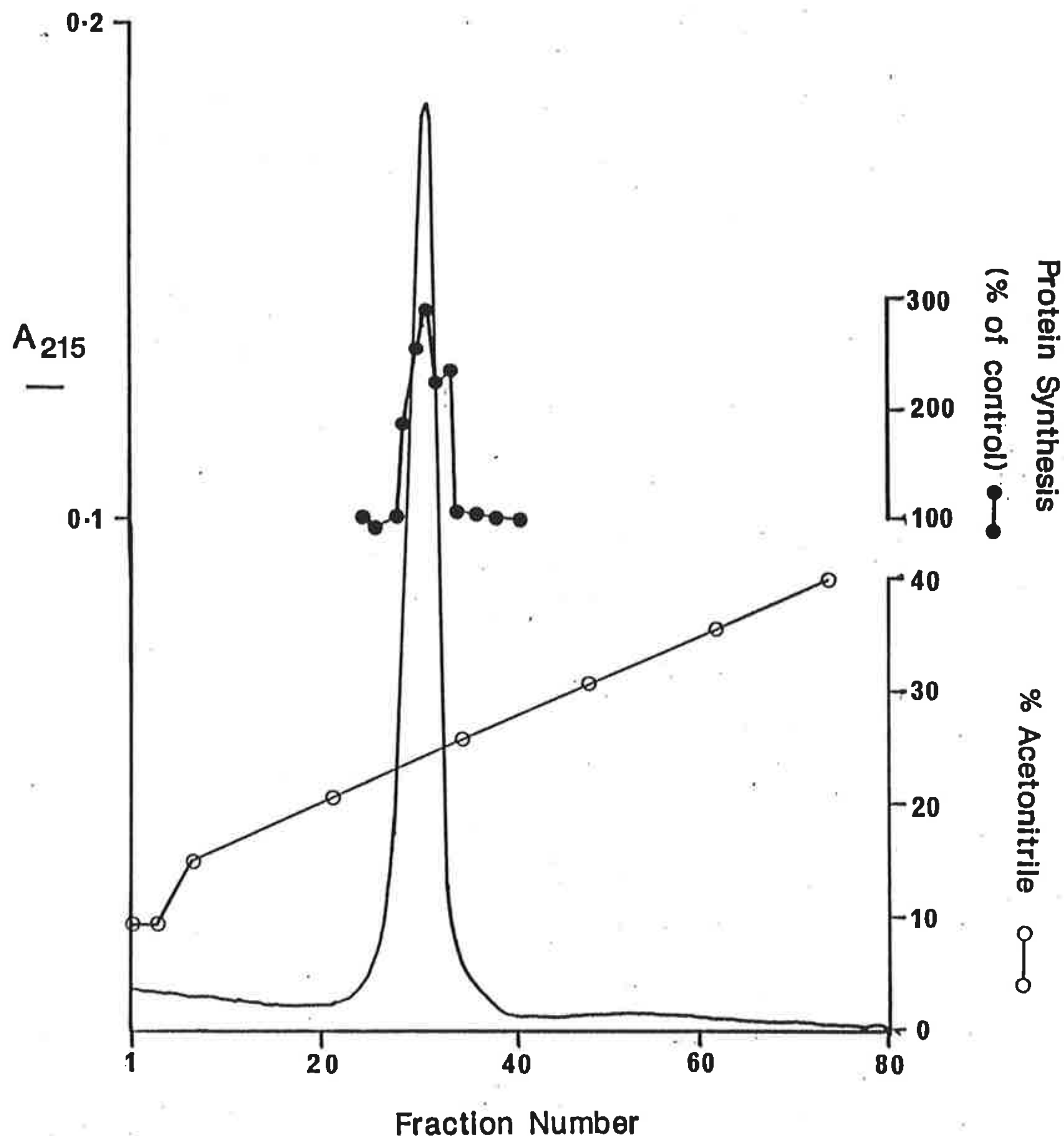


Figure 3.5 Reverse phase HPLC of purified growth factor. The two separate growth factor pools from the third rpHPLC step (i.e. pools A and B) were pooled, diluted with 0.1% TFA to adjust the propan-1-ol concentration to approximately 5% and loaded onto an Aquapore RP-300 microbore cartridge equilibrated with 0.1% TFA. The cartridge was washed with 0.1% TFA/10% acetonitrile and growth factor activity eluted with a two step linear gradient from 10% to 15% acetonitrile in 5 min and then to 40% acetonitrile in 100 min at a flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected and 50 µl of each was assayed for growth factor activity. Fractions with activity were pooled.

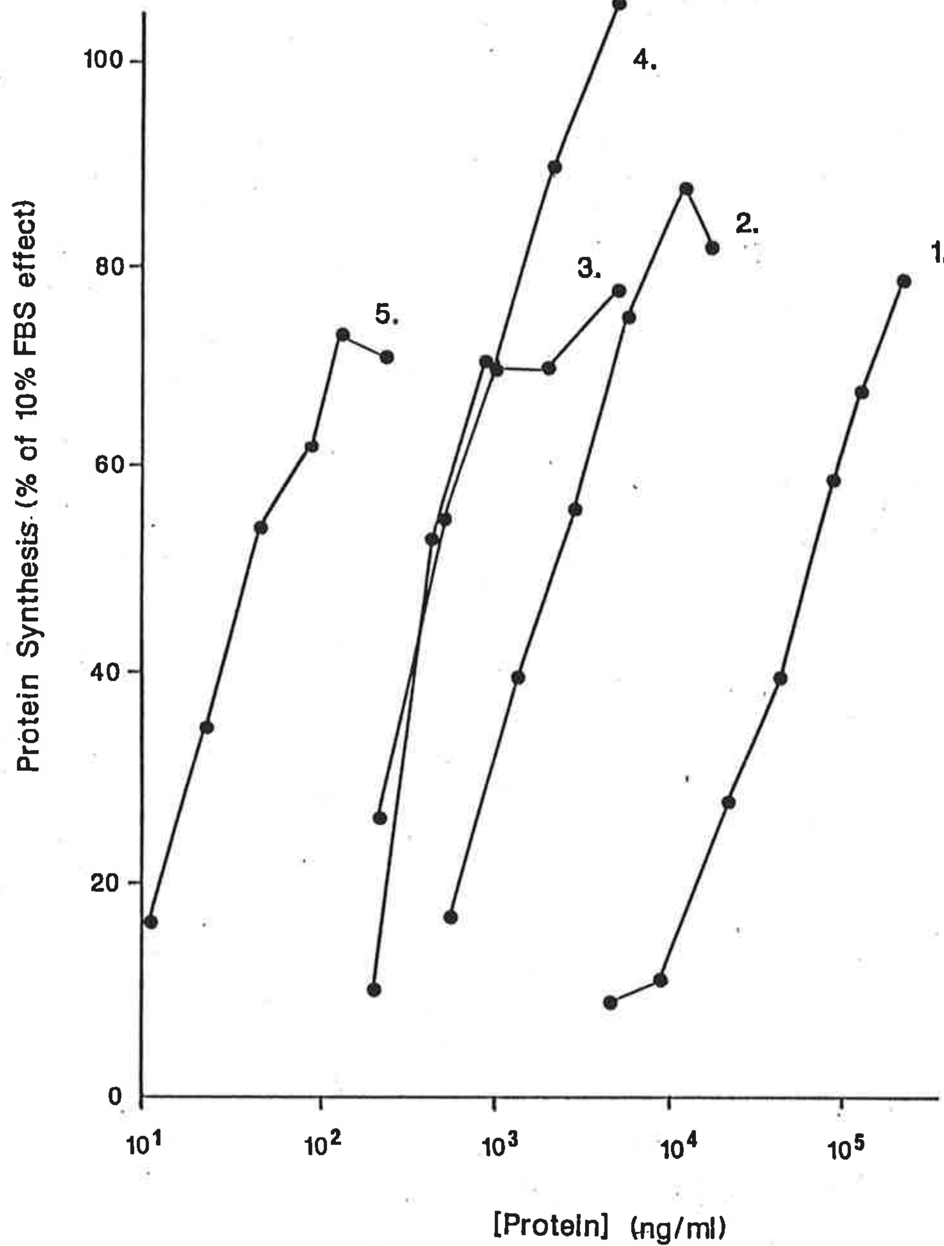
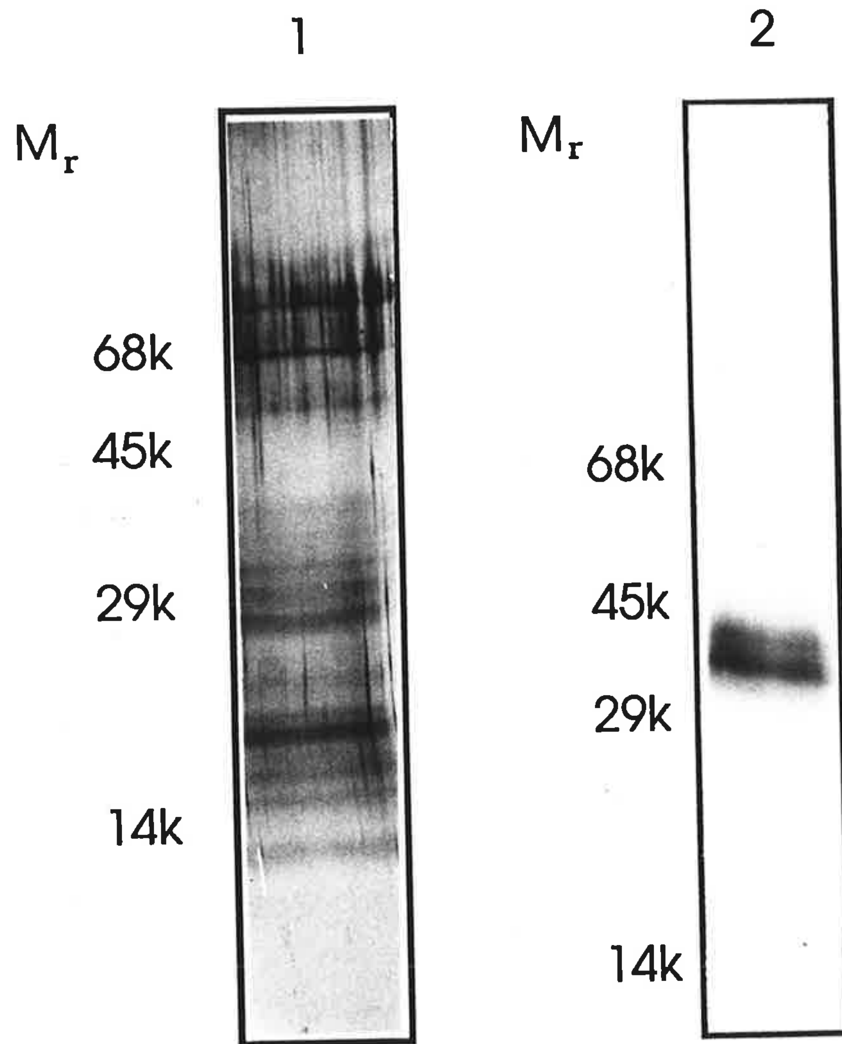


Figure 3.6 Dose response curves at different stages in the purification of growth factor activity from MDBK cell conditioned medium. Values plotted are the mean of triplicate or duplicate determinations and are expressed as a percent of the level of protein synthesis induced by 10% fetal bovine serum in L-6 myoblasts. The curves correspond to; 1, acidified MDBK cell conditioned medium; 2, pooled fractions from S-Sepharose chromatography; 3, pool A from the 1st rpHPLC; 4, pool B from the 1st rpHPLC; 5, purified growth factor.

Figure 3.7 SDS-gel electrophoresis of purified growth factor.
SDS-polyacrylamide gel electrophoresis was performed using a 15% separating gel with (**track 1**) and without (**track 2**) prior reduction of the sample, which consisted of 500 ng (total protein) of purified growth factor. Gels were silver stained as described by Heukeshoven and Derynck, (1985). Molecular weight standards used were, bovine serum albumin (68,000); ovalbumin (45,000); carbonic anhydrase (29,000) and lysozyme (14,000). The staining above 45 k in track 1 is characteristic of the presence of 2-mercaptoethanol in the sample, and not due to any protein components in the sample.

Figure 3.7



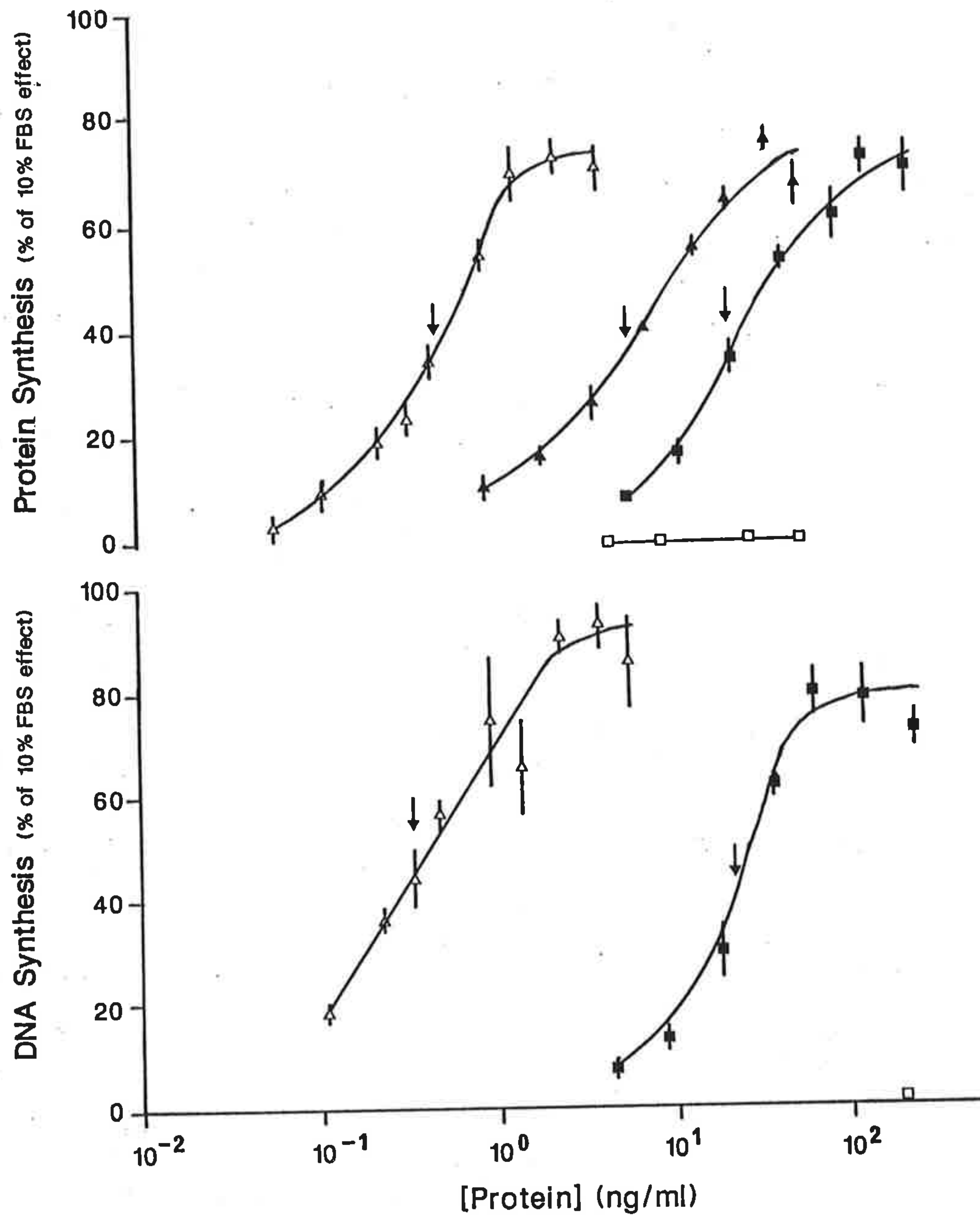


Figure 3.8 Effects of different PDGF isoforms and bPDGF on protein synthesis (*upper panel*) and DNA synthesis (*lower panel*) in L-6 myoblasts. Values plotted are the mean of triplicate determinations ± SEM, and are expressed as a percent of the level of stimulation induced by 10% fetal bovine serum (FBS). The symbols used are: △, hPDGF-BB; ▲, hPDGF-AB; ■, bPDGF; □, hPDGF-AA. Arrows indicate the concentration of protein at which half the maximal stimulation of each growth factor is obtained (i.e. the ED_{50}).

Figure 3.9

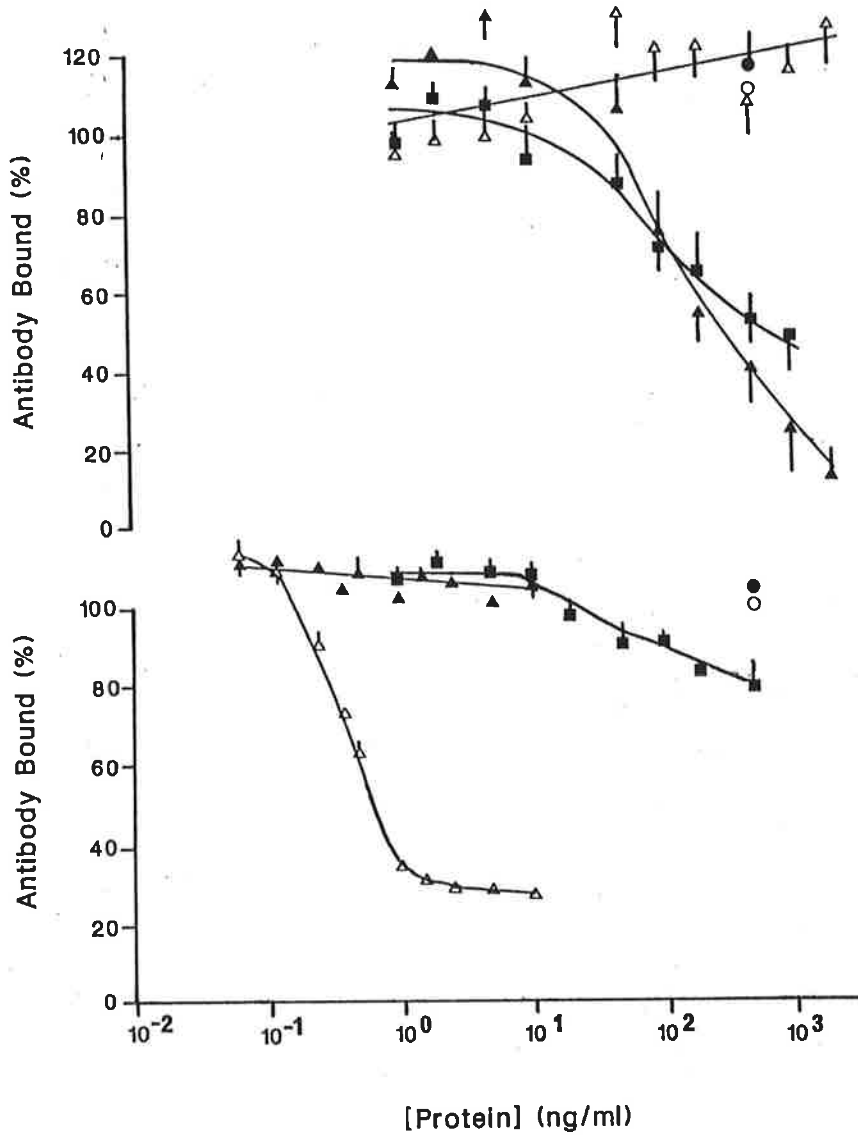


Figure 3.9 Effects of different PDGF isoforms and bPDGF in PDGF ELISA assays. Inhibition of binding of antibody to peptide-coated microtitre wells was determined for hPDGF-AA (▲); hPDGF-BB (△); bPDGF (■); IGF-1 (●); and insulin (○), in a PDGF A chain specific (*upper panel*) and a PDGF B chain specific (*lower panel*) ELISA assay. Increasing concentrations of growth factor were incubated with a 1/800 dilution of primary antibody in PBS/0.1% Tween 20/ 1 mg/ml BSA for 18 hours at 4°C. The mixture (100 µl) was applied to microtitre wells coated with either hPDGF-AA (*upper panel*) or hPDGF-BB (*lower panel*) and the assay developed as described in the **Materials and Methods.** Results are plotted as the mean of four determinations ± SEM, and are expressed as a percent of the amount of antibody bound in the absence of competing ligand.

Table 3.1 : Summary of the Purification of the MDBK cell-derived growth factor

Step	Protein[1] (mg)	Activity[2] (units)	Specific Activity (units/mg)	Yield (% overall)	Fold Purification step	Fold Purification overall
Acidified MDBK CM	1,500	26,000	17	100%	1	1
S-Sepharose	24	15,000	625	58%	37	37
rpHPLC #1	2.7	7,100	2,600	27%	4.2	153
rpHPLC #2	2.0×10^{-1}	ND				
rpHPLC #3	3.1×10^{-2}	ND				
microbore rpHPLC	2.5×10^{-2}	1,000	40,000	4%	15	2,300

1. The amount of protein in the pooled fractions at each chromatography step. For the rpHPLC steps the total amount of protein in the A and B pools was combined. 2. The amount of activity in the pooled fractions at each chromatography step. For rpHPLC #1 the total amount of activity in the A and B pools was combined. One unit of activity is the amount which causes 50% of the maximum level of stimulation. ND, not determined.

Table 3.2 : Effects of PDGF Isoforms and bPDGF on He[39]L Fibroblasts Monitored by the Stimulation of DNA Synthesis[1]

Concentration[2] (ng/ml)	Growth Factor hPDGF-BB	hPDGF-AA	bPDGF
1	374%	178%	117%
10	995%	730%	314%
20	1,118%	898%	515%

1. Results are expressed as a percent of the negative control (i.e. no growth factor added) and are the mean of triplicate or duplicate determinations. 2. The concentration of bPDGF is base on total protein.

Table 3.3 : Antibody Neutralization of bPDGF Mitogenic Activity[1]

Growth Factor[2]	Antibody[3] control	α-AA	α-AB/BB	α-PDGF
hPDGF-AA	200±5%	100±10%	187±10%	100±10%
hPDGF-BB	931±24%	888±55%	469±22%	100±10%
hPDGF-AB	759±39%	855±19%	518±33%	100±10%
bPDGF	878±46%	823±43%	508±5%	NA[4]

1. Results are the mean of triplicate determinations ± SEM, and are expressed as a percent of the negative control (i.e. no growth factor added) 2. Concentrations of the various growth factors used were: hPDGF-AA, 2.0 ng/ml; hPDGF-BB, 0.6 ng/ml; hPDGF-AB, 1.0 ng/ml; bPDGF, 0.5 ng/ml (actual PDGF as determined by RRA). 3. α-AA is a monoclonal antibody specific for the AA homodimer of PDGF; α-AB/BB is a monoclonal antibody specific for the BB and AB forms of PDGF; α-PDGF is a polyclonal antibody raised against the human form of PDGF-AB. 4. NA = not applicable, since this particular anti-PDGF polyclonal antibody does not recognize bovine PDGF.

CHAPTER FOUR : Production of TGF-β-related Growth Inhibitors by MDBK Cells

Chapter 4: Introduction

Growth regulation of cells in culture, and possibly *in vivo*, is a balance between the actions of stimulatory and inhibitory growth factors (Sporn and Roberts, 1988). A number of stimulatory growth factors have been purified and characterized (James and Bradshaw, 1984; Deuel, 1987) whereas growth inhibitory factors are generally less well characterized, with the exception of transforming growth factor β (Wang and Hsu, 1986). The type β transforming growth factors (TGF-β) form a family of structurally related proteins which act on a wide variety of cells to alter their proliferative and phenotypic properties (Sporn *et al.*, 1987; Roberts and Sporn, 1988). Although TGF-β is now better known as a growth inhibitor, it was originally purified using a proliferation assay, *viz*. the stimulation of NRK fibroblast growth in soft agar (Assoian *et al.*, 1983). Since this time it has become apparent that TGF-β has different effects depending on the conditions under which it is acting. For example, TGF-β inhibits the growth of NRK fibroblasts when grown in monolayer culture as opposed to soft agar (Roberts *et al.*, 1985). The growth inhibitory effects of TGF-β are apparent on a number of cell types, including hepatocytes (Nakamura *et al.*, 1985; Carr *et al.*, 1986; McMahon *et al.*, 1986; Strain *et al.*, 1987), endothelial cells (Fràter-Schröder *et al.*, 1986; Heimark *et al.*, 1986; Jennings *et al.*, 1988), hematopoietic progenitor cells (Ohta *et al.*, 1987), prokeratinocytes (Shipley *et al.*, 1987), bronchial epithelial cells (Masui *et al.*, 1986), intestinal epithelial cells (Barnard *et al.*, 1989), and T lymphocytes (Wrann *et al.*, 1987). However, the effects of TGF-β on cells are not limited to cell proliferation. Numerous other effects have been discovered, particularly effects related to differentiation processes, including those on the production of extracellular matrix components (Ignotz and Massagué, 1986; Bassols and Massagué, 1988), myoblast differentiation (Florini *et al.*, 1986b; Massagué *et al.*, 1986; Olson *et al.*, 1986), adipogenic differentiation (Ignotz and Massagué, 1985), the activation of macrophages (Tsunawaki *et al.*, 1988), and bronchial epithelial cell differentiation (Masui *et al.*, 1986). These effects and those on cellular proliferation, suggest that TGF-β is of importance in the regulation of many developmental and tissue repair processes.

During the characterization of the growth factors present in the tissue culture medium conditioned by the bovine kidney cell line, MDBK, I have obtained evidence for the

presence of activities which inhibit DNA synthesis in the rat L-6 myoblast cell line (**Chapter 2**). Here I report the partial purification of these growth inhibitors and show that they are related to TGF-β.

4.1 Materials and Methods

Materials

Fetal-bovine serum was purchased from Flow Laboratories; bovine serum albumin (RIA grade), protein molecular weight standards, amino acids and vitamins for the preparation of media were obtained from Sigma Chemical Co.; L-[4,5-^{3}H]leucine (40-60 Ci/mmol) and [*methyl*-^{3}H]thymidine (20 Ci/mmol) were from New England Nuclear. Sources for the antibiotics used in media are given by Ballard *et al.* (1986). All dilutions of growth factors were made in the presence of 0.1% (w/v) BSA.

Rabbit anti-porcine TGF-β1 IgG fraction (cat. # AB-10-NA) was purchased from R&D Systems, Inc., Minneapolis, MN. This antibody recognizes both TGF-β1 and TGF-β2. Recombinant human TGF-β1 was kindly provided by Genentech, Inc., South San Francisco, CA.

Cell cultures and the collection of conditioned medium

L-6 myoblasts, obtained from Dr. J. M. Gunn, Texas A&M University, College Station TX, U.S.A., were grown by serial passaging of prefused cultures in Dulbecco-modified Eagle's Minimal Essential Medium containing 5% (v/v) fetal-bovine serum, together with 50 mg of gentamycin, 100 mg of streptomycin, 60 mg penicillin and 1 mg of fungizone/litre of growth medium. The cells were grown and used for experiments as monolayers at 37°C under an atmosphere of CO_2/humidified air (1:19). For experimental purposes the cells were subcultured into Linbro 24-place multiwell dishes in growth medium and used during the 4 days after the monolayers first became confluent. At this stage myotubes were not evident. MDBK cells (Flow Laboratories ATCC CCL22) were grown as for L-6 cells except the growth medium contained 10% (v/v) fetal-bovine serum. For the production of conditioned medium, MDBK cells were grown in a Nunc cell factory in growth medium until confluent. Subsequently, cells were transferred to growth medium without serum supplementation. After 24 hours, conditioned medium was replaced with

fresh growth medium without serum. This collection was discarded. Conditioned medium was henceforth collected every third day while the cells remained viable (about 8 weeks) and stored at -15°C until used.

Dialysis

Dialysis, unless otherwise stated, was carried out using Spectrapor #3 dialysis tubing (3,500 molecular weight cut off) against at least a 100-fold excess of 1% (v/v) acetic acid at 4°C.

Protein synthesis determination

The measurement of protein synthesis in L-6 myoblasts has been described previously (Francis *et al.*, 1986). Briefly, the measurement involves the incorporation of [^{3}H]leucine into total cell protein during an 18 hour incubation of confluent cell monolayers in 24-place multiwell dishes. Activity is expressed as the percent stimulation of protein labelling over that occurring in monolayers incubated without growth factors in DMEM.

DNA synthesis determination

Confluent monolayers of L-6 cells in 24-place multiwell dishes were washed with DMEM for 2 hours and exposed to growth factors for 18 hours in 1 ml of DMEM. At the end of this period, 5 nmol of thymidine containing 1 μCi of [^{3}H]thymidine was added for a further 6 hours. Harvesting of the monolayers was carried out as described previously (Ballard *et al.*, 1986). Activity is expressed as the percent stimulation of DNA labelling over that occurring in monolayers incubated without growth factors in DMEM.

Assay of growth inhibitor activity

Inhibition of DNA synthesis in L-6 myoblasts was monitored by the incorporation of [^{3}H]thymidine into the cell monolayer as described in the previous section, except for the presence of 1% fetal-bovine serum in all cultures during the incubation period with the test sample. Percent inhibition (%I) was calculated by the formula,

$\%I = 100 - 100(a\text{-}b/c\text{-}b)$ where:

a = the radioactivity incorporated (c.p.m./well) in the test sample well, a being the mean of three determinations.

b = the radioactivity incorporated (c.p.m./well) in the negative control wells (i.e. wells without any fetal calf serum or growth factor added), b being the mean of at least three determinations.

c = the radioactivity incorporated (c.p.m./well) in the positive control wells (i.e. wells with only 1% fetal calf serum added), c being the mean of at least three determinations.

IGF binding protein assay

IGF binding protein activity was measured using the charcoal binding assay essentially as described by Martin and Baxter (1986), except that the incubation time with the charcoal solution (which contained 0.25% bovine serum albumin) was increased to 30 min to improve assay precision, and the charcoal was removed by centrifugation at 10,000g for 3 min. Assays contained approximately 4000 dpm per tube ^{125}I-IGF-2, iodinated using the Chloramine T method, to a specific activity of between 20 and 80 Ci/g, as previously described (Francis *et al.*, 1988).

Purification of growth inhibitors

Conditioned medium (13 litres) collected from MDBK cells was thawed and filtered through Whatman No. 1 paper. All further steps were carried out at room temperature. The filtrate was acidified to 50 mM acetic acid with glacial acetic acid and the pH adjusted to 3.0 with concentrated HCl. After a second filtration through Whatman No. 1 paper the clear filtrate was pumped onto a S-Sepharose Fast Flow column (3.2 cm diam. x 25 cm, Pharmacia) at a flow rate of 10 ml/min. The column was washed with 1 litre of 50 mM acetic acid, followed by 1 litre of 50 mM ammonium acetate pH 6.5. Protein was eluted with a 1.2 litre linear gradient of 50 mM ammonium acetate pH 6.5 to 2.0 M ammonium acetate pH 8.0 at a flow rate of 3 ml/min. Fractions were collected and assayed in IGF binding protein, growth inhibitor and protein synthesis assays. Samples to be assayed for growth inhibition and protein synthesis activity were dialysed against 1% acetic acid prior to assay. Fractions were pooled as indicated (**figure 4.1**) resulting in two pools of growth inhibitor activity termed GI-1 and GI-2.

Following S-Sepharose chromatography, trifluoroacetic acid (TFA) was added to the pools designated GI-1 and GI-2 to achieve a final concentration of 0.1% (v/v), and the pH adjusted to 2.1 with concentrated HCl. The resulting solutions were both filtered through a 0.45 μm filter. The GI-1 pool was pumped onto an Aquapore Prep 10 RP-300 HPLC

cartridge (1 cm diam. x 10 cm; Brownlee Labs) equilibrated with 0.1% TFA. This concentration of TFA was maintained throughout the rpHPLC of both GI-1 and GI-2. The cartridge was washed with 0.1% TFA/10% acetonitrile until the absorbance at 280 nm returned to baseline. Protein was eluted with a two step linear gradient of 10 to 25% acetonitrile in 10 min and then to 45% acetonitrile in 60 min at a flow rate of 3 ml/min. Fractions were collected and aliquots assayed in IGF binding protein, growth inhibitor and protein synthesis assays. Active fractions were pooled as indicated in **figure 4.2**, to give an IGF binding protein pool (IBP) and a growth inhibitor pool (GI-1).

The GI-2 pool was pumped onto an Aquapore RP-300 HPLC cartridge (4.6 mm diam. x 3 cm; Brownlee Labs) equilibrated with 0.1% TFA. The cartridge was washed with 0.1% TFA/10% propan-1-ol until the absorbance at 280 nm returned to baseline. Protein was eluted with a two step linear gradient of 10 to 16% propan-1-ol in 5 min and then to 40% propan-1-ol in 48 min at a flow rate of 1 ml/min. Fractions were collected during the second step of the gradient and assayed in growth inhibitor and protein synthesis assays. Fractions with growth inhibitor activity were pooled as indicated (**figure 4.3**).

HPLC gel filtration chromatography

The pooled fractions from reverse phase HPLC (IBP) or an aliquot there of (GI-1 and GI-2) were lyophilized and resuspended in 0.1% TFA/40% acetonitrile in a volume of 200 µl and applied to a TSK G 3000 SW column (7.5 mm diam. x 60 cm) equilibrated and eluted with 0.1% TFA/40% acetonitrile (**figures 4.4 a, b** and **c**). The flow rate was 0.4 ml/min, with absorbance measured at either 280 or 215 nm.

Protein concentration

Throughout this study protein content was monitored by absorbance at 280 or 215 nm. Protein concentrations were calculated by assuming that A_{280}=1.0 for a 1 mg/ml solution in a 1 cm light path and an A_{280}/A_{215} ratio of 0.1.

Neutralization of TGF-β activity

TGF-β1, GI-1 and GI-2 were lyophilized and resuspended in phosphate buffered saline. Each growth inhibitor was incubated with either 0, 0.5, 2.5 or 10 µg of anti-TGF-β IgG for one hour at 37°C in a total volume of 50 µl. Following this preincubation period, samples

were transferred to wells containing L-6 cells in a final volume of 0.5 ml of growth medium supplemented with 1% fetal-bovine serum, and inhibition of DNA synthesis monitored as described for the assay of growth inhibitor activity.

4.2 Results

Initial characterization of the growth factors produced by MDBK cells suggested the presence of inhibitors of DNA synthesis in the serum-free conditioned medium of this cell line (**Chapter 2**). I have recently purified and characterized a growth factor from MDBK cell conditioned medium, that I have identified as the bovine equivalent of PDGF-AB (**Chapter 3**). The initial chromatography step in the purification of this form of PDGF (referred to as bPDGF) was cation exchange chromatography of acidified MDBK cell conditioned medium. When DNA synthesis assays on L-6 myoblasts were carried out on the fractions from this chromatography step (protein synthesis in the L-6 myoblast cell line having been used to detect bPDGF activity during its purification), the results indicated the presence of two peaks of inhibitor activity (**figure 4.1**). The first peak of inhibitor activity (referred to as GI-1 or growth inhibitor No.1) co-eluted with the IGF binding protein activity produced by MDBK cells (Szabo *et al.*, 1988). The peak fraction of GI-1 caused virtually 100% inhibition of DNA synthesis induced by 1% fetal-bovine serum on L-6 myoblasts. The second peak of inhibitor activity (referred to as GI-2) appeared to plateau at approximately 75% inhibition of DNA synthesis. This activity eluted at a similar concentration of ammonium acetate to that at which bPDGF eluted during this chromatography step (**Chapter 3**). The presence of bPDGF activity in the fractions with GI-2 activity was suggested by a peak of protein synthesis activity associated with the same fractions (**figure 4.1**). A small amount of protein synthesis stimulating activity was also associated with GI-1.

The growth inhibitors GI-1 and GI-2 were separately pooled as indicated (**figure 4.1**) and each loaded onto a reverse phase HPLC cartridge. GI-1 activity was eluted with a gradient of acetonitrile, which separated the IGF binding protein activity from the growth inhibitor activity (**figure 4.2**). GI-1 was also associated with protein synthesis stimulating activity after elution from the reverse phase HPLC column. Overlapping with the elution position of the MDBK binding protein were coincident peaks of protein and DNA

synthesis stimulating activity (**figure 4.2**). To further characterize the growth inhibitor and IGF binding protein activities, two pools were formed consisting of the IGF binding protein and GI-1, as indicated (**figure 4.2**).

GI-2 activity was eluted from a reverse phase HPLC cartridge with a gradient of propan-1-ol (not acetonitrile as bPDGF had previously shown a tendency to split into two peaks of activity under such elution conditions) resulting in the separation of GI-2 and putative bPDGF activities (**figure 4.3**). The bPDGF-like activity was collected as a pool eluted between 10 and 16% propan-1-ol which exhibited protein synthesis-stimulating activity. GI-2 eluted as a single peak of growth inhibitor activity at approximately 20% propan-1-ol, which coincided with a small peak of protein synthesis-stimulating activity. The most active fractions with growth inhibitor activity were pooled as indicated (**figure 4.3**).

IBP, GI-1 and GI-2 were chromatographed on a HPLC gel filtration column to determine whether the protein synthesis stimulating activity associated with these pools could be resolved from the IGF binding protein activity and growth inhibitor activities. This procedure also enabled approximate molecular weights for each activity to be determined. The IGF binding protein eluted with a molecular weight corresponding to approximately 28,000 and was completely separated from the protein and DNA synthesis stimulating activity associated with this pool (**figure 4.4a**). The protein and DNA synthesis-stimulating activities eluted as coincident peaks with a molecular weight of approximately 9,000. This growth factor activity is likely to be IGF-2, which I have previously shown to be present in MDBK cell conditioned medium (**Chapter 2**). No protein or DNA synthesis activity was associated with the MDBK-derived IGF binding protein (**figure 4.4a**).

Upon gel filtration HPLC, GI-1 and GI-2 both eluted as peaks of growth inhibitor activity corresponding to a molecular weight of approximately 25,000 (**figure 4.4b & c**). A peak of protein synthesis-stimulating activity coincided with both GI-1 and GI-2 activity, suggesting that either GI-1 and GI-2 stimulate protein synthesis in L-6 myoblasts or alternatively one or both of these growth inhibitors may be contaminated by another activity which stimulates protein synthesis in L-6 myoblasts.

Dose response curves of DNA synthesis inhibition were performed using recombinant human TGF-β1, acidified MDBK cell conditioned medium and the pools of GI-1 and GI-2 activity after cation exchange and reverse phase HPLC. The results, shown in **figure 4.5**, indicated that hTGF-β1 inhibits DNA synthesis in L-6 myoblasts and that on a weight basis, hTGF-β1 is approximately 100-fold more potent than GI-1 or GI-2. The sensitivity of DNA synthesis in L-6 myoblasts to the effects of hTGF-β1 and the growth inhibitors GI-1 and GI-2, appears to be variable as assaying the same quantity of these growth inhibitors on different occasions has produced varying degrees of inhibition in the growth inhibitor assay.

The dose response curve of DNA synthesis inhibition associated with acidified MDBK cell conditioned medium or the GI-2 pool after cation exchange chromatography plateaued at approximately 50% (**figure 4.5**). This is likely to be caused by the presence of bPDGF in both of these samples, as the maximum level of DNA synthesis inhibition caused by GI-2 after rpHPLC (i.e. after the removal of the putative bPDGF activity) was the same as that caused by GI-1 and was equivalent to approximately 80%.

To determine whether GI-1 and GI-2 are structurally related to TGF-β, an antibody specific for TGF-β was tested for its ability to neutralize the activity of these growth inhibitors on L-6 myoblasts. The results (**table 4.1**) demonstrate that this antibody does partially inhibit the effect of these growth inhibitors, indicating that at least part of the inhibitory activity of GI-1 and GI-2 is due to TGF-β. The form of TGF-β involved in this growth inhibitory activity can not be determined from these results as the antibody used recognizes both TGF-β1 and TGF-β2.

The interferons are another well characterized family of proteins with anti-proliferative activity on many cells (Clemens and McNurlan, 1985), including MDBK cells (Zoon *et al.*, 1986a). Hence, it was of interest to establish whether GI-1 or GI-2 exhibited any interferon activity. To determine this both growth inhibitors were tested for antiviral activity using MDBK cells challenged with Semliki Forest Virus. The results indicated that GI-1 and GI-2 do not have antiviral activity and therefore are not related to the interferons (B. Cheetham, *pers. comm.*, 1989).

4.3 Discussion

Here I report the partial purification of two growth inhibitors produced by MDBK cells and show that they are related to TGF-β. These MDBK cell-derived growth inhibitors (known as GI-1 and GI-2) were discovered due to their effect on DNA synthesis in L-6 myoblasts. TGF-β is a well characterized inhibitor of myogenesis in skeletal muscle cells, including the rat L-6 cell line (Florini *et al.*, 1986b; Massagué *et al.*, 1986; Olson *et al.*, 1986). Florini *et al.* have also reported that TGF-β caused a slight inhibition of cell growth in L-6 cells, as determined by cell number and DNA content. These effects were reported using TGF-β at a concentration of 1 ng/ml. In the same study, detectable inhibition of myogenesis was observed at 0.06 ng/ml, while maximum inhibition was reached at 0.5 ng/ml. The results I have obtained for the inhibition of DNA synthesis by TGF-β in L-6 cells indicate a detectable inhibition at approximately 0.05 ng/ml, while maximum inhibition is reached at levels greater than 10 ng/ml. There appears to be a difference between my results and those obtained by Florini *et al.* (1986b) and Massagué *et al.* (1986) in the magnitude of the effect of TGF-β on the inhibition of DNA synthesis in L-6 myoblasts. I have obtained up to 80% inhibition of the level of DNA synthesis caused by 1% fetal-bovine serum on L-6 cells, whereas Florini *et al.* and Massagué *et al.* describe the effect of TGF-β on DNA synthesis as "slight". Possibilities to explain this discrepancy include differences in the L-6 cells used in the different laboratories and the culture conditions under which the assays were performed.

TGF-β is a ubiquitous growth regulator which is produced by and acts on many cell types, including kidney cells (Wakefield *et al.*, 1987; Danielpour *et al.*, 1989). Indeed, the first indication of the growth inhibitory activity of TGF-β was obtained from studies on a growth inhibitor produced by the monkey kidney cell line BSC-1 (Holley *et al.*, 1978), an inhibitor that has since been identified as TGF-β (Tucker *et al.*, 1984). Kidney was also one of the first sources for the purification of TGF-β (Roberts *et al.*, 1983), so it is not surprising that the growth inhibitors produced by the kidney cell line, MDBK, are related to TGF-β. Other sources that have been utilized for the purification of TGF-β have been platelets (Assoian *et al.*, 1983), bone (Seyedin *et al.*, 1985, 1986, 1987), placenta (Frolik *et al.*, 1983) and the conditioned medium of a number of cell lines (Massagué, 1984; Fernandez-Pol *et al.*, 1986; Ikeda *et al.*, 1987; Wrann *et al.*, 1987; Smith *et al.*, 1988; Van

den Eijnden-Van Raaij *et al.*, 1989). More recently, methods similar to those used in this study have been incorporated into protocols used for the purification of TGF-β from platelets (Cone *et al.*, 1988; Van den Eijnden-Van Raaij *et al.*, 1988) and conditioned medium (Van den Eijnden-Van Raaij *et al.*, 1989). In particular, these methods have combined the use of cation exchange chromatography with reverse phase HPLC. The impure nature of GI-1 and GI-2 is apparent from the HPLC gel filtration profiles of both growth inhibitors (**figure 4.4b** and **c**), however, my aim in this study has been to separate the growth inhibitors produced by MDBK cells from the other growth factors produced by this cell line, and to determine the identity of these growth inhibitors. The techniques of cation exchange chromatography and reverse phase HPLC have proved useful in achieving this aim.

TGF-β has been isolated in at least three different forms, TGF-β1, TGF-β2 and TGF-β1.2, each consisting of two polypeptide chains, β1 and β2, forming homo- and heterodimers of 25,000 M_r (Cheifetz *et al.*, 1987). Recently, cDNA cloning has provided evidence of further TGF-β chains, known as TGF-β3 (Derynck *et al.*, 1988; Dijke *et al.*, 1988; Jakowlew *et al.*, 1988a) and TGF-β4 (Jakowlew *et al.*, 1988b). The MDBK-derived growth inhibitors, GI-1 and GI-2, have not been characterized in terms of which TGF-β chains they consist. To determine this assays specific for the different chains of TGF-β need to be developed. Using such assays Danielpour *et al.* (1989) have determined the relative amounts of TGF-β1 and TGF-β2 produced by a number of cell lines, including two epithelial cell types of kidney origin, BSC-1 and MDCK. In both of these cell lines, the majority of the TGF-β activity produced is neutralized by an antibody specific for the TGF-β2 chain. Results obtained by northern analysis using BSC-1 cell RNA support these antibody neutralization experiments (Derynck *et al.*, 1988). The type of TGF-β chain(s) of which a particular growth inhibitor consists is an important question, as differences between the biological potency of the different forms of TGF-β have been reported (Cheifetz *et al.*, 1987, 1988; Ohta *et al.*, 1987; Jennings *et al.*, 1988).

IGF binding proteins have previously been reported to have both inhibitory (Knauer and Smith, 1980; De Mellow and Baxter, 1988; Ritvos *et al.*, 1988) and stimulatory (Elgin *et al.*, 1987) effects on cells in culture. The MDBK cell line produces an IGF binding protein (Szabo *et al.*, 1988), which Ross *et al.* (1989) have shown inhibits the biological

activities of IGF-1 and IGF-2 on chicken embryo fibroblasts, and inhibits the binding of IGF-1 and IGF-2 to L-6 myoblasts. Therefore it was important to determine if the growth inhibitor activity which co-eluted with the IGF binding protein activity, after cation exchange chromatography of MDBK cell conditioned medium, was actually a property of the MDBK IGF binding protein. This is not the case as the growth inhibitor activity (GI-1) was separated from the IGF binding protein activity upon reverse phase HPLC. Subsequent to this chromatography step, IGF binding protein activity was resolved from a peak of DNA and protein synthesis-stimulating activity by HPLC gel filtration chromatography. Hence, the MDBK IGF binding protein does not appear to affect DNA synthesis induced by fetal-bovine serum, nor the basal level of protein or DNA synthesis in L-6 myoblasts.

The production of TGF-β by MDBK, MDCK (Danielpour *et al.*, 1989) and BSC-1 (Tucker *et al.*, 1984) kidney epithelial cells, and the isolation of TGF-β from bovine kidney (Roberts *et al.*, 1983), raises the question of the function of TGF-β *in vivo*, particularly in terms of kidney growth and development. Relevant to this question is the finding that TGF-β is active on primary cultures of proximal tubular cells (Fine *et al.*, 1985). In this study, TGF-β inhibited the mitogenic effect of insulin and hydrocortisone without blocking protein synthesis induced by these agents. The net effect of this action is to cause cellular hypertrophy evidenced by an increase in cell volume and protein content with minimal mitogenesis. Therefore, as a consequence of these results, it has been suggested that TGF-β may play a role in compensatory regrowth of the kidney (Roberts and Sporn, 1988) in a similar fashion to its possible involvement in hepatic regrowth (Russell *et al.*, 1988).

Figure 4.1

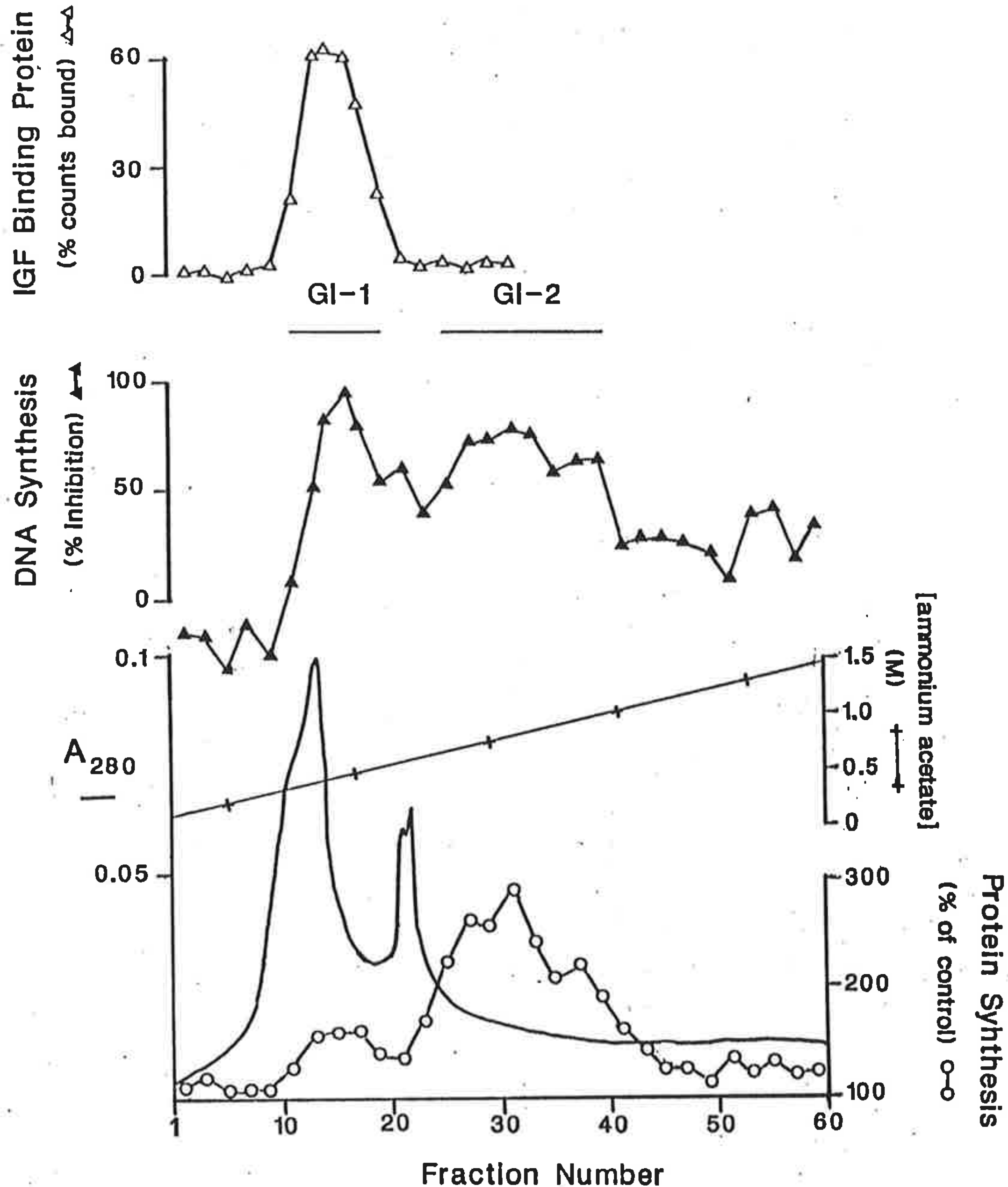


Figure 4.1 Cation exchange chromatography of MDBK cell conditioned medium. Acidified MDBK cell conditioned medium (13 litres) was pumped onto a S-Sepharose Fast Flow column equilibrated with 50 mM acetic acid. The column was washed with 50 mM ammonium acetate pH 6.5 and protein eluted with a 1.2 litre linear gradient of 50 mM ammonium acetate pH 6.5 to 2.0 M ammonium acetate pH 8.0 at a flow rate of 3 ml/min. Fractions (15 ml) were collected and aliquots of every second fraction tested in protein synthesis (150 µl), growth inhibitor (15 µl) and IGF binding protein (20 µl) assays. The horizontal bars represent those fractions pooled for subsequent purification steps.

Figure 4.2

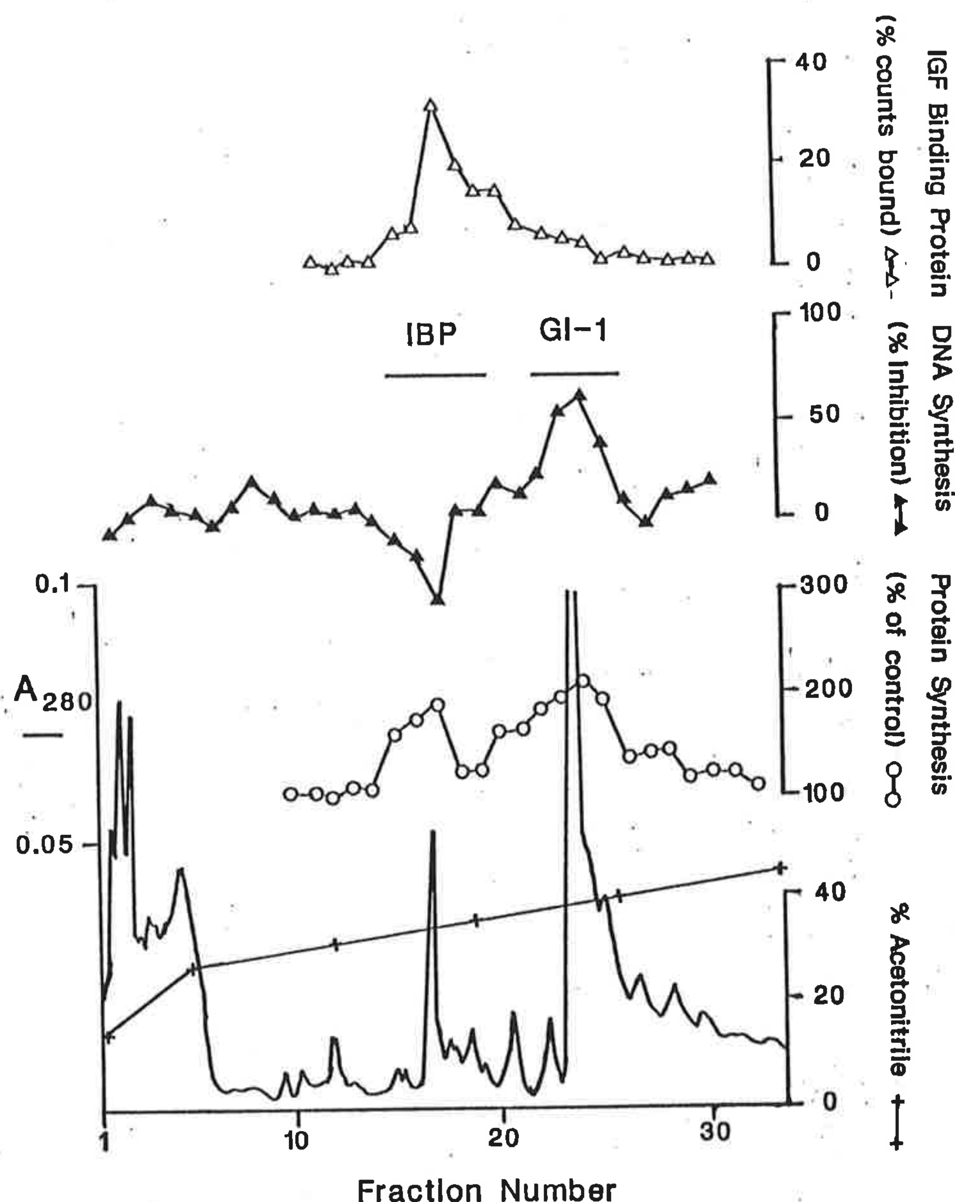


Figure 4.2 Reverse phase HPLC of GI-1. The pooled fractions from cation exchange chromatography were adjusted to 0.1% TFA pH 2.1 and pumped onto an Aquapore Prep 10 RP-300 cartridge equilibrated with 0.1% TFA. The cartridge was washed with 0.1% TFA/10% acetonitrile and protein eluted with a two step linear gradient of 10 to 25% acetonitrile in 10 min and then to 45% acetonitrile in 60 min at a flow rate of 3 ml/min. Fractions were collected (6 ml) and aliquots of each tested in protein synthesis (150 μl), growth inhibitor (30 μl) and IGF binding protein assays (20 μl). The horizontal bars represent those fractions pooled for subsequent characterization steps.

Figure 4.3

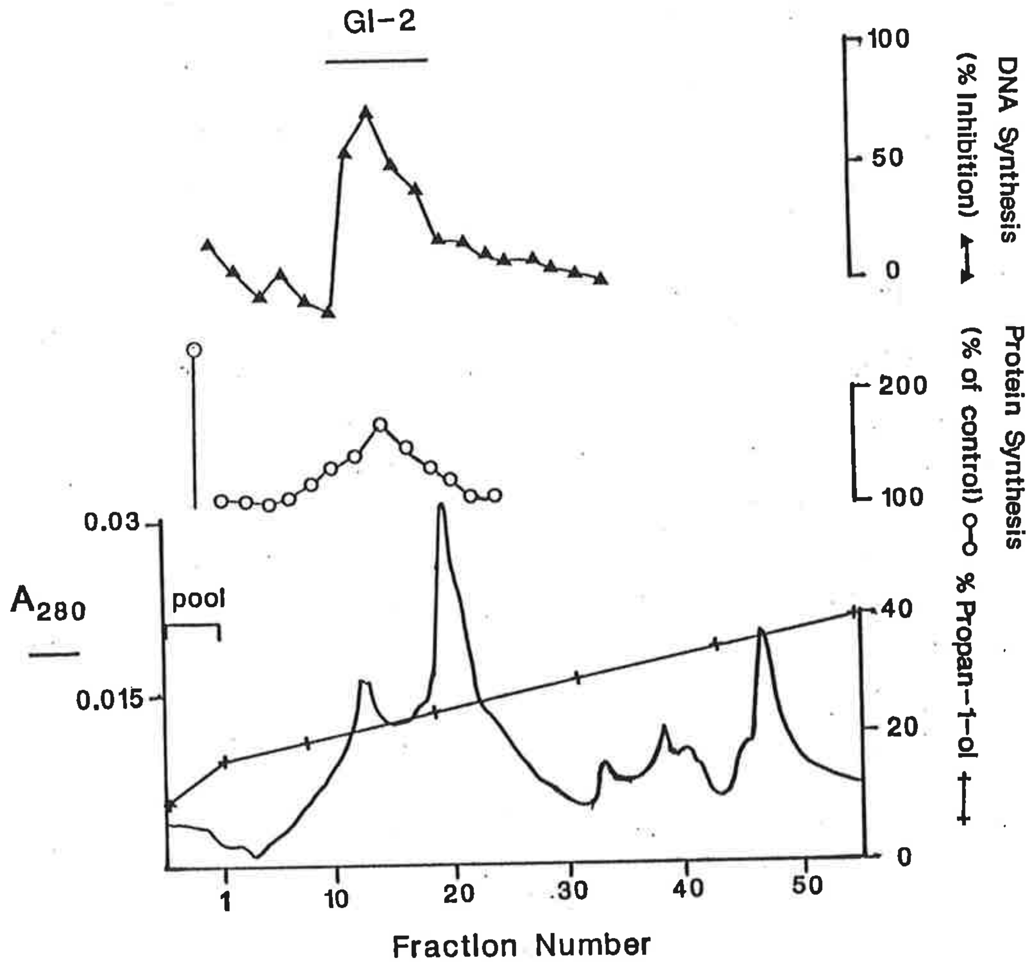


Figure 4.3 Reverse phase HPLC of GI-2. The pooled fractions from cation exchange chromatography were adjusted to 0.1% TFA pH 2.1 and pumped onto an Aquaporé RP-300 cartridge equilibrated with 0.1% TFA. The cartridge was washed with 0.1% TFA/10% propan-1-ol and protein eluted with a two step linear gradient of 10 to 16% propan-1-ol in 5 min and then to 40% propan-1-ol in 48 min at a flow rate of 1 ml/min. The protein eluted in the first step of the propan-1-ol gradient was collected as a pool, whereas in the second step of the gradient, 1 ml fractions were collected. Aliquots of every second fraction were tested in protein synthesis (20 µl) and growth inhibitor (5 µl) assays. The horizontal bar represents those fractions pooled for subsequent characterization steps.

Figure 4.4a

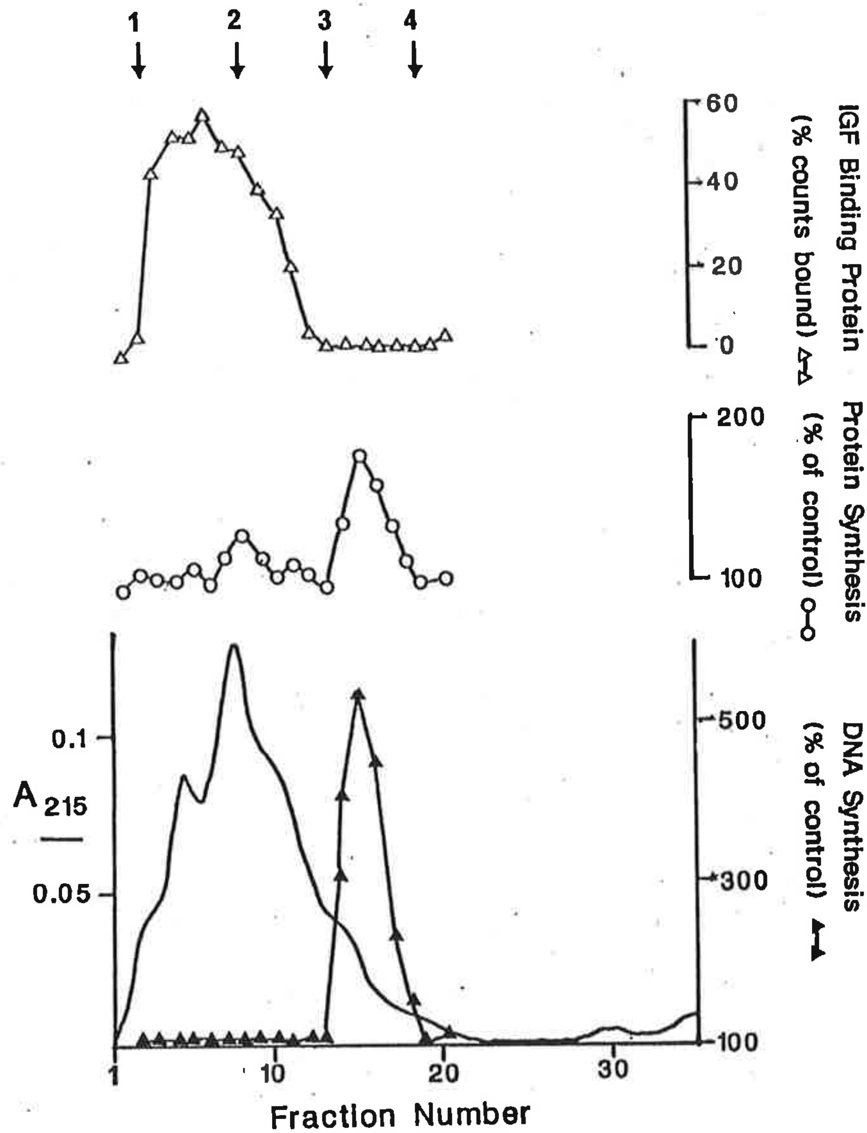


Figure 4.4 HPLC gel filtration chromatography of a) IBP, b) GI-1 and c) GI-2. The pooled fractions from reverse phase HPLC were lyophilized, resuspended in 0.1% TFA/40% acetonitrile (200 μl) and applied to a TSK G 3000 SW gel filtration column equilibrated and eluted with the same solution at a flow rate of 0.4 ml/min. Fractions were collected (400 μl) and in the case of IBP, aliquots tested in IGF binding protein (7.5 μl), protein synthesis (20 μl) and DNA synthesis (20 μl) assays, and in the case of GI-1 and GI-2, aliquots tested in protein synthesis (20 μl) and growth inhibitor (5 μl) assays. Molecular weight standards were chromatographed separately, their elution position indicated by arrows. The molecular weight standards used were (1) bovine serum albumin (68,000), (2) α-chymotrypsinogen A (25,000), (3) insulin dimer (11,000) and (4) insulin (5,500).

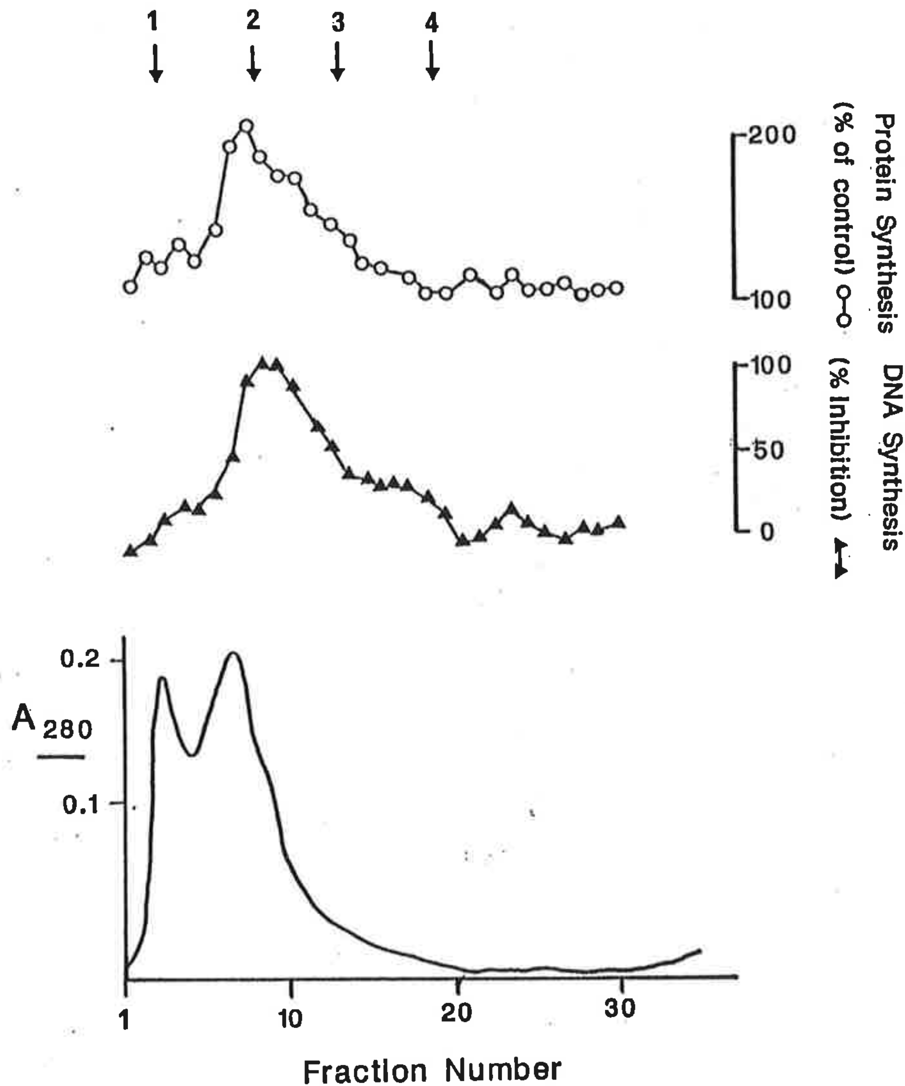


Figure 4.4 HPLC gel filtration chromatography of a) IBP, b) GI-1 and c) GI-2. The pooled fractions from reverse phase HPLC were lyophilized, resuspended in 0.1% TFA/40% acetonitrile (200 μl) and applied to a TSK G 3000 SW gel filtration column equilibrated and eluted with the same solution at a flow rate of 0.4 ml/min. Fractions were collected (400 μl) and in the case of IBP, aliquots tested in IGF binding protein (7.5 μl), protein synthesis (20 μl) and DNA synthesis (20 μl) assays, and in the case of GI-1 and GI-2, aliquots tested in protein synthesis (20 μl) and growth inhibitor (5 μl) assays. Molecular weight standards were chromatographed separately, their elution position indicated by arrows. The molecular weight standards used were (1) bovine serum albumin (68,000), (2) α-chymotrypsinogen A (25,000), (3) insulin dimer (11,000) and (4) insulin (5,500).

Figure 4.4c

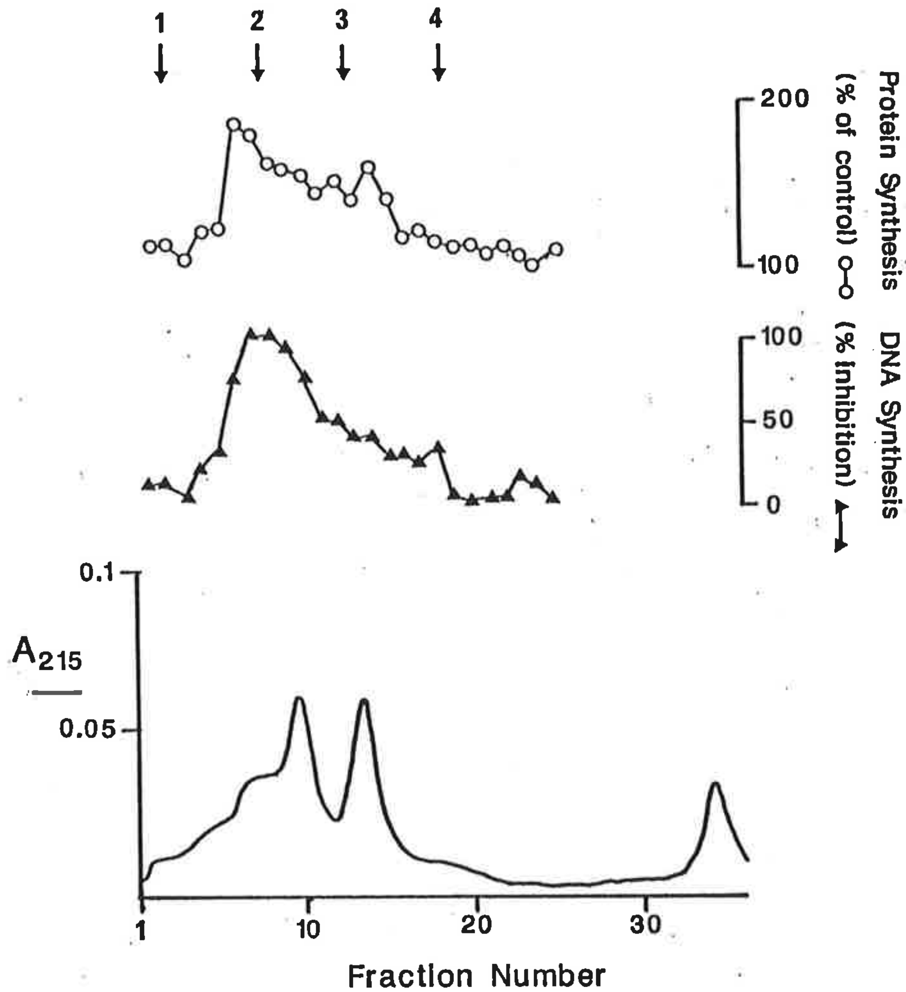


Figure 4.4 HPLC gel filtration chromatography of a) IBP, b) GI-1 and c) GI-2. The pooled fractions from reverse phase HPLC were lyophilized, resuspended in 0.1% TFA/40% acetonitrile (200 µl) and applied to a TSK G 3000 SW gel filtration column equilibrated and eluted with the same solution at a flow rate of 0.4 ml/min. Fractions were collected (400 µl) and in the case of IBP, aliquots tested in IGF binding protein (7.5 µl), protein synthesis (20 µl) and DNA synthesis (20 µl) assays, and in the case of GI-1 and GI-2, aliquots tested in protein synthesis (20 µl) and growth inhibitor (5 µl) assays. Molecular weight standards were chromatographed separately, their elution position indicated by arrows. The molecular weight standards used were (1) bovine serum albumin (68,000), (2) α-chymotrypsinogen A (25,000), (3) insulin dimer (11,000) and (4) insulin (5,500).

Figure 4.5

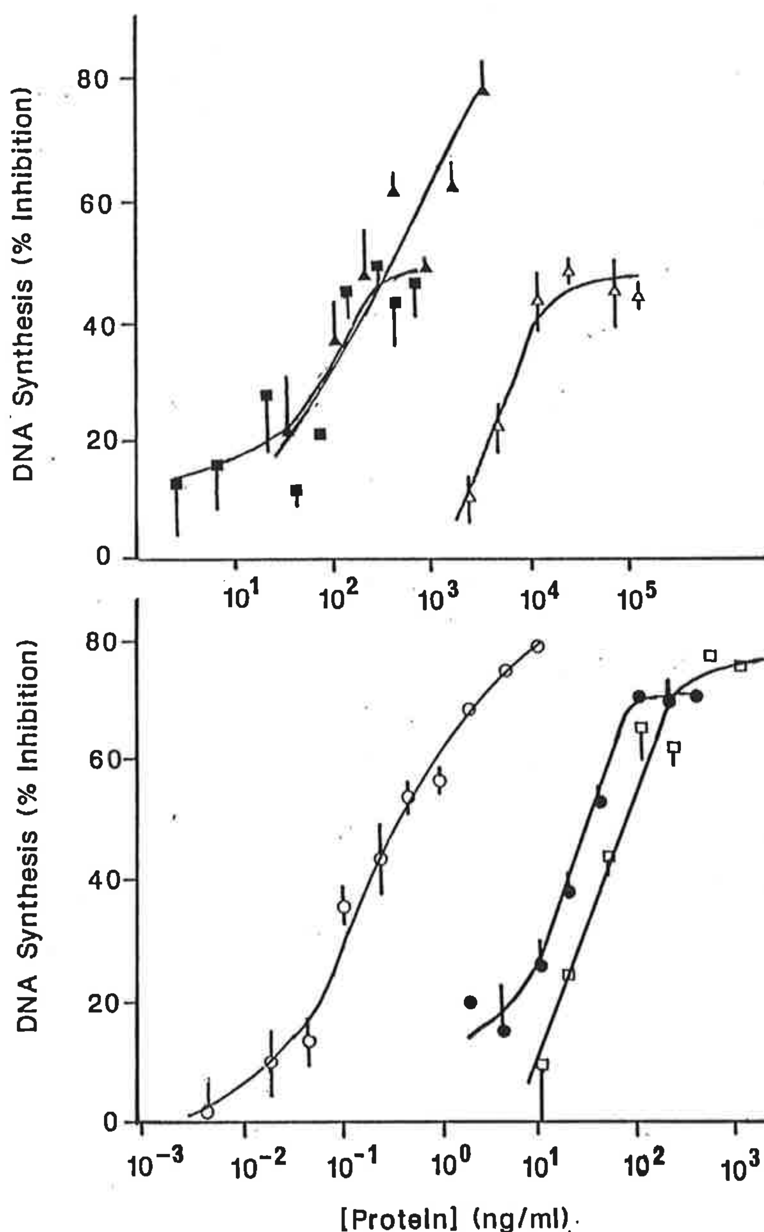


Figure 4.5 Dose response curves of growth inhibition for GI-1, GI-2 (at different stages of purification) and TGF-β1. Values plotted are the mean of triplicate determinations ± SEM, and are expressed as the percent inhibition of DNA synthesis on L-6 myoblasts, calculated as described in the **Materials and Methods**. The curves correspond to (*upper panel*), (△) acidified MDBK cell conditioned medium; (▲) the GI-1 pool from cation exchange chromatography; (■) the GI-2 pool from cation exchange chromatography; (*lower panel*), (□) the GI-1 pool from reverse phase HPLC; (●) the GI-2 pool from reverse phase HPLC; and (○) recombinant human TGF-β1.

Table 4.1: Neutralization of Growth Inhibitor Activity by Anti-TGF-β Antibody[1]

Growth Inhibitor	Antibody Concentration			
	0 μg/ml	1 μg/ml	5 μg/ml	20 μg/ml
GI-1 (10u/ml)[2]	88±1%	86±1%	79±3%	26±3%
GI-2 (5u/ml)[2]	84±2%	80±3%	61±2%	20±2%
TGF-β (1ng/ml)	70±4%	62±3%	18±3%	4±3%
FBS (1%)[3]	0±4%	ND[4]	ND[4]	-10±0.1%[5]

1. Results are expressed as percent inhibition of DNA synthesis and are the mean of triplicate determinations ± SEM. GI-1, GI-2 and recombinant human TGF-β1 were incubated with either 0, 0.5, 2.5 or 10 μg of anti-TGF-β IgG for one hour at 37°C in a total volume of 50 μl. Following this preincubation period, samples were transferred to wells containing L-6 cells in a final volume of 0.5 ml of growth medium supplemented with 1% fetal-bovine serum, and inhibition of DNA synthesis monitored as described in the **Materials and Methods**; 2. u/ml=units/ml, where one unit is defined as the amount of growth inhibitor required to cause a half maximal effect in the DNA synthesis inhibition assay on L-6 myoblasts (as in **figure 4.5**, *lower panel*). These units are only approximate since the sensitivity of DNA synthesis in L-6 myoblasts to the effects of GI-1 and GI-2 appears to be variable, as assaying the same quantity of these growth inhibitors on different occasions has produced varying degrees of inhibition in the growth inhibitor assay. ; 3. Fetal-bovine serum (FBS 1%) is the negative control i.e. no growth inhibitor added; 4. ND=not determined; 5. ± the range not the SEM as n=2.

CHAPTER FIVE : The Response of MDBK Cells to Growth Factors

Chapter 5: Introduction

The survival of cells in culture without serum supplementation has been associated with the endogenous production of growth factors (Dulak and Temin, 1973; De Larco and Todaro, 1978a,b; Kaplan *et al.*, 1982; Messing *et al.*, 1984). In a number of examples, including the growth factors TGF-α, PDGF, bombesin and TGF-β, an autocrine loop operates where the externalized growth factor acts on the cell which produces it, enabling it to be independent of exogenous growth factors (Sporn and Roberts, 1985). In the case of PDGF, the involvement of the growth factor in autocrine stimulation was first established in the process of cellular transformation by the simian sarcoma virus (Huang *et al.*, 1984; Johnsson *et al.*, 1985b). An autocrine role for PDGF in normal growth and development processes has also been suggested following the finding that smooth muscle (Seifert *et al.*, 1984; Sejersen *et al.*, 1986; Majesky *et al.*, 1988; Sjölund *et al.*, 1988) and mesangial cells (Shultz *et al.*, 1988) produce and respond to PDGF. More recently, evidence has been obtained implicating other growth factors in autocrine pathways of growth, including IGF-1 (Minuto *et al.*, 1988; Williams *et al.*, 1989), an insulin-related factor (Yamada and Serrero, 1988) and bFGF (Schweigerer *et al.*, 1987; Sato and Rifkin, 1988).

I have found that the bovine kidney cell line, MDBK, survives in culture without serum supplementation and produces the growth factors IGF-2, PDGF-AB and TGF-β (**Chapters 2, 3** and 4). To answer the question of whether the production of these growth factors is responsible for the low serum requirements of MDBK cells, I have investigated the effects of these and other growth factors on protein metabolism and DNA synthesis in the MDBK cell line.

5.1 Materials and Methods

Materials

Fetal-bovine serum was purchased from Flow Laboratories; bovine serum albumin (RIA grade) and amino acids and vitamins for the preparation of media were obtained from Sigma Chemical Co.; L-[4,5-^{3}H]leucine (40-60 Ci/mmol) and [*methyl*-^{3}H]thymidine (20 Ci/mmol) were from New England Nuclear. Sources for the antibiotics used in media are given by Ballard *et al.* (1986). All dilutions of growth factors were made in the presence of 0.1% (w/v) BSA.

Growth factors

Synthetic IGF-1 and synthetic des-(1-3)-IGF-1 were prepared as previously described (Ballard *et al.*, 1987). Bovine IGF-2 was purified from colostrum (Francis *et al.*, 1988). Insulin was Actrapid from Novo Industri A/S, Copenhagen, Denmark. Recombinant human PDGF-AA, PDGF-BB, EGF and bFGF were kindly provided by Chiron Corporation, Emeryville, CA., and recombinant human TGF-β1 by Genentech, Inc., South San Francisco, CA. Human interferon α was a generous gift from Dr. Bruce Korant, E.I. du Pont de Nemours & Company, Wilmington, DE. Bovine PDGF, GI-1 and GI-2 were purified from MDBK cell conditioned medium as described (**Chapters 3** and **4**).

Cell cultures and the collection of conditioned medium

L-6 myoblasts, obtained from Dr. J. M. Gunn, Texas A&M University, College Station TX, U.S.A., were grown by serial passaging of prefused cultures in Dulbecco-modified Eagle's Minimal Essential Medium (DMEM) containing 5% (v/v) fetal-bovine serum, together with 50 mg of gentamycin, 100 mg of streptomycin, 60 mg penicillin and 1 mg of fungizone/litre of growth medium. The cells were grown and used for experiments as monolayers at 37°C under an atmosphere of CO_2/humidified air (1:19). For experimental purposes the cells were subcultured into Linbro 24-place multiwell dishes in growth medium and used during the 4 days after the monolayers first became confluent. At this stage myotubes were not evident. MDBK cells (Flow Laboratories ATCC CCL22) were grown as for L-6 cells except the growth medium contained 10% (v/v) fetal-bovine serum.

Protein synthesis determination

Measurement of protein synthesis in confluent monolayers of cultured cells was performed as previously described (Francis *et al.*, 1986). Briefly, the measurement involves the incorporation of [^{3}H]leucine into total cell protein during an 18 hour incubation of confluent cell monolayers in 24-place multiwell dishes. Activity is expressed as the percent stimulation of protein labelling over that occurring in monolayers incubated without growth factors in DMEM.

DNA synthesis determination

Confluent monolayers of cultured cells in 24-place multiwell dishes were washed with DMEM for 2 hours and exposed to growth factors for 18 hours in 1 ml of DMEM. At the end of this period, 5 nmol of thymidine containing 1 μCi of [^{3}H]thymidine was added for a

further 6 hours. Harvesting of the monolayers was carried out as described previously (Ballard *et al.*, 1986). Activity is expressed as the percent stimulation of DNA labelling over that occurring in monolayers incubated without growth factors in DMEM.

Effects of growth factors on intracellular protein degradation

Confluent monolayers of cultured cells in 24-place multiwell dishes were prelabelled with [^{3}H]leucine after which rates of protein degradation were measured over a 4 hour period (Ballard *et al.*, 1986) with growth factors present. The percent protein degraded during the measurement period was calculated as 100 times the trichloroacetic acid-soluble radioactivity divided by the total radioactivity in the monolayer (Ballard *et al.*, 1980; 1986). The reduction in degraded protein when growth factor was present in the measurement period is expressed as the percent change from that observed when growth factors were not included.

5.2 Results

In this study I have investigated the effects of a number of growth factors, both growth stimulators and growth inhibitors, on the MDBK cell line. I have previously shown that this cell line is producing growth factors, including IGF-2 (**Chapter 2**), PDGF-AB (**Chapter 3**) and TGF-β (**Chapter 4**). The effects of these and other growth factors on MDBK cells has been monitored by DNA synthesis, protein synthesis and protein degradation assays. These experiments were set up in the form of a side by side comparison between the L-6 rat myoblast and MDBK cell lines. The L-6 myoblast cell line was used for comparison as it has been utilized in assays of growth promotion and growth inhibition during the purification and characterization of the growth factors produced by MDBK cells, hence in this study it functions as a positive control. Representatives of each of the major growth factor "families" active on tissues of mesodermal origin (with the exception of hemopoietic tissue) have been included in this study, including EGF and interferon α, both of which are active on MDBK cells (Zoon *et al.*, 1986a).

The results from the assays of the stimulatory growth factors on DNA synthesis are shown in **tables 5.1** and **5.2**. All of the growth factors assayed stimulated DNA synthesis in MDBK cells with the exception of the PDGFs. Of the insulin-like growth factors (IGFs) the most potent was the amino-terminal truncated form of IGF-1 (des-(1-3)-IGF-1), and the

least potent was IGF-2. Insulin also caused a substantial increase in DNA synthesis, but the concentration assayed was 100-fold greater than that used for the IGFs. It is generally only at these concentrations that insulin exhibits growth promoting effects, suggesting that these effects are mediated by the IGF receptors. Similar results were obtained for the IGFs and insulin on L-6 cells (**table 5.1**), although the magnitude of the growth factor effects on L-6 cells were generally much greater than those on MDBK cells. Of the various growth factors tested, EGF and PDGF-AA had no significant effect on L-6 cells, consistent with previous observations (Ballard, F.J., unpublished observations, & **Chapter 3**), whereas PDGF-BB, bPDGF (bovine PDGF purified from MDBK cell conditioned medium) and bFGF stimulated DNA synthesis. Fetal-bovine serum (FBS) stimulated DNA synthesis in both cell lines, though a marked difference in the magnitude of this response was observed. On MDBK cells fetal-bovine serum consistently produced an increase of only 20% above the control, whereas on L-6 cells this response was more variable, but was often 10-20 fold above the control value (**tables 5.1** and **5.2**).

When the effects of the various growth stimulators on protein synthesis were determined, similar results were found to those obtained in the DNA synthesis assays, i.e. the growth factors which stimulated DNA synthesis also stimulated protein synthesis, although the magnitude of the protein synthesis response was generally less (**tables 5.3** and **5.4**). In both MDBK and L-6 cells a difference between the protein and DNA synthesis results was found in the relative effect of IGF-2 compared to the effects of IGF-1 and des-(1-3)-IGF-1. In both assays IGF-2 was the least potent peptide, however the magnitude of the difference was less in the protein synthesis assays. This is consistent with the production of an IGF binding protein by MDBK cells that preferentially binds IGF-2 (Szabo *et al.*, 1988), but which inhibits the biological activities of both IGF-1 and IGF-2 (Ross *et al.*, 1989). The presence of such a binding protein is likely to cause a greater effect in the DNA synthesis assay as the incubation period with growth factor is longer in this assay compared to the protein synthesis assay.

Inhibition of protein degradation is a consistent response of cells in culture to growth factors (Ballard *et al.*, 1980). In L-6 cells in particular, inhibition of protein degradation has been shown to be a sensitive assay for the insulin-like growth factors (Ballard *et al.*, 1986). This assay has a much shorter growth factor incubation period (4 hours) compared

with the protein (18 hours) and DNA (24 hours) synthesis assays used in this study, so that the protein degradation assay is less prone to interference by growth factor binding proteins produced by the MDBK or L-6 cells. The increased sensitivity of the protein degradation assay was evidenced by the results obtained (**table 5.5** and **5.6**). These results show no significant difference between the effects of the various IGFs and insulin on L-6 cells at the concentrations used in this assay. Since there was a significant difference between these growth factors in the protein synthesis assay at the same concentrations, it is likely that in the protein degradation assay a plateau of the dose response for these growth factors has been reached. In contrast with the results obtained on L-6 cells, a difference was observed between IGF-2 and the other IGFs in protein degradation assays on MDBK cells (**table 5.5**), where again IGF-2 was the least potent peptide. This is likely to be due to the presence of the MDBK IGF binding protein, with the difference in the results obtained for L-6 cells compared to MDBK cells possibly due to the particular selectivity and level of production of the MDBK IGF binding protein.

The PDGFs also inhibited protein degradation in L-6 cells, with the BB homodimer causing an inhibition equivalent to that caused by the IGFs (**table 5.6**). An inhibition of protein degradation by PDGF-AA was observed, possibly reflecting the increased sensitivity of this assay compared to the protein and DNA synthesis assays. The effect of the AA homodimer of PDGF may reflect the presence of a small number of A type PDGF receptors on L-6 cells, or alternatively may be due to a low affinity interaction between the AA isoform and the B type PDGF receptor.

Growth inhibitors are referred to as such because they inhibit DNA synthesis, usually in a number of cell lines. The MDBK cell line produces two growth inhibitors that inhibit DNA synthesis in L-6 cells and are related to TGF-β (**Chapter 4**). Hence it was of interest to determine whether MDBK cells were responsive to these growth inhibitors (referred to as GI-1 and GI-2). Assaying of the various growth inhibitors (**table 5.7, 5.8** and **5.9**) showed that GI-1, GI-2, TGF-β1 and interferon α all inhibited DNA synthesis in MDBK cells (**table 5.7**). TGF-β and interferon α also inhibited the effect of IGF-1 and EGF on DNA synthesis. In L-6 cells the growth inhibitors of the TGF-β type were potent inhibitors of DNA synthesis, as observed previously (**Chapter 4**), whereas interferon α had no observable effect in any of the assays on this cell line.

Effects of the various growth inhibitors on protein synthesis in MDBK cells were only slight (**table 5.8**), interferon α having the greatest effect at 118% of the control. The combination of TGF-β1 or interferon α with either IGF-1 or EGF, also caused a stimulation of protein synthesis in MDBK cells, with a possible synergistic interaction being involved in the case of TGF-β1. In L-6 cells the TGF-β type growth inhibitors appeared to cause a slight stimulation of protein synthesis.

In protein degradation assays the growth inhibitors showed only a slight effect, or none at all, as in the case of interferon α (**table 5.9**). The only exceptions were the effects of GI-1 and GI-2 on L-6 cells, where both growth inhibitors caused a 15% inhibition of protein degradation. These effects do not seem to be only due to TGF-β activity and therefore may reflect the impure nature of both of these growth inhibitors.

5.3 Discussion

The purpose of this study was to determine whether MDBK cells are responsive to the growth factors which they produce, namely, IGF-2, PDGF and TGF-β. I have found that these cells are responsive to IGF-2 and TGF-β, but not to PDGF. The potency of the IGFs decreased in the order des-(1-3)-IGF-1, IGF-1 and IGF-2. This order of potency is consistent with the MDBK cells producing an IGF binding protein which inhibits the biological activity of IGF-1 and IGF-2 (Ross *et al.*, 1989), and which preferentially binds IGF-2 (Szabo *et al.*, 1988). Similar results have been reported for the order of potency of IGFs on L-6 cells (Ballard *et al.*, 1987; Francis *et al.*, 1986, 1988), a cell line that also appears to produce an IGF binding protein with a low affinity for des-(1-3)-IGF-1 (C.J. Bagley, *pers. comm.*, 1989). My results indicating that MDBK cells are responsive to IGFs is not surprising as receptors for the IGFs have been detected on a similar kidney cell line, MDCK (Krett *et al.*, 1986). Furthermore, effects of IGFs on isolated kidney tissue (Mellas *et al.*, 1986; Blazer-Yost and Cox, 1988) and basolateral membranes (Hammerman and Gavin, 1984; Rogers and Hammerman, 1988) have been reported.

Whether the IGF-2 produced by MDBK cells is involved in autocrine stimulation of this cell line can not be definitively answered based upon the results from this study. However, as previously stated, MDBK cells produce a potent inhibitor of the action of IGF-2. Furthermore, this inhibitor, an IGF binding protein, contains the sequence

-Arg-Gly-Asp- (Szabo *et al.*, 1988) which is recognized by many of the integrin superfamily of cell surface adhesion receptors (Pierschbacher and Ruoslahti, 1984). Therefore, as IGF-2 is produced by MDBK cells, it is likely to be bound by IGF binding proteins located on the cell surface and in the culture medium, leaving little opportunity for autocrine stimulation.

PDGF is produced by many cell types, particularly transformed cells (see Heldin *et al.*, 1986a and **table 1.1**), although only a small number of these have been reported as being responsive to PDGF (Huang *et al.*, 1984; Johnsson *et al.*, 1985b; Fraizer *et al.*, 1987; Grotendorst *et al.*, 1988; Shultz *et al.*, 1988; Sjölund *et al.*, 1988). The MDBK cell line is not responsive to PDGF and hence is similar to BSC-1 monkey kidney cells which produce but do not respond to PDGF (Kartha *et al.*, 1988). A number of transformed epithelial cell lines that produce PDGF have also been reported to be unresponsive to PDGF (Sariban *et al.*, 1988; Sitaras *et al.*, 1988). All this data is consistent with the original findings of Heldin *et al.* (1981) that epithelial-derived cells do not express a PDGF receptor. An exception to the above appears to be the rat lens, which recently was shown to respond to PDGF (Brewitt and Clark, 1988).

Both growth inhibitors investigated in this study, namely interferon α and TGF-β (the MDBK cell derived growth inhibitors GI-1 and GI-2 being related to TGF-β) inhibited DNA synthesis in MDBK cells. Interferon α has previously been shown to inhibit DNA synthesis in MDBK cells (Zoon *et al.*, 1986a), the results obtained being similar to those presented in this study. Conflicting results have previously been reported on the effects of interferon on skeletal myoblasts (Lough *et al.*, 1982; Fisher *et al.*, 1983). The report by Lough *et al.* concluded that partially purified chicken interferon inhibited the differentiation of chicken myoblast cultures. In contrast, the report by Fisher *et al.* concluded that recombinant human interferon α induced an acceleration of myotubule formation and creatine kinase isozyme transition in human myoblast cultures. The apparent opposite results obtained by these two studies may well reflect the impure nature of the chicken interferon used by Lough *et al.* My results indicate that interferon α is not active on L-6 myoblasts, a situation that may either reflect a difference between the L-6 cell line

and the myoblast cultures used by Fisher *et al.*, or indicate that although interferon α is active on L-6 myoblasts, this action is not observable using the assays involved in the present study.

The focus of the present study was to determine whether the low serum requirement of MDBK cells is due to the autocrine production of growth factors. The evidence presented here suggests that this is not likely, though the MDBK cell line may be producing growth factors which I have been unable to detect. Alternatively, activation of a growth factor receptor or intracellular pathway may explain the growth properties of the MDBK cell line.

Table 5.1: Effects of Growth Factors on DNA Synthesis in MDBK and L-6 cells

Growth Factor	DNA Synthesis[1] (% of control) MDBK	L-6
IGF-1 (10 nM)	200±8	696±40[2]
des-(1-3)-IGF-1 (10 nM)	311±7	1,076±26[2,3]
IGF-2 (10 nM)	141±10	164±7[2]
Insulin (1 μM)	309±15	810±22
EGF (1 nM)	165±3	104±5
bFGF (1 nM)	162±7	725±49
FBS (10%)	121±2	2,407±32

1. DNA synthesis was monitored by the incorporation of ^{3}H-thymidine into the cell monolayer as per the **Materials and Methods.** Results are expressed as a percent of the negative control (i.e. no growth factor added) and are the mean of four determinations ± SEM; 2. Assayed at 5 nM; 3. ± the range not the SEM as n=2.

Table 5.2: Effects of PDGFs on DNA Synthesis in MDBK and L-6 cells

Growth Factor	DNA Synthesis[1] (% of control) MDBK	L-6
hPDGF-AA (10 nM)	88±3	115±14
hPDGF-BB (10 nM)	92±3	737±17
bPDGF (1 nM)[2]	81±4	426±18
FBS (10%)	124±4	1,022±19

1. DNA synthesis was monitored by the incorporation of ^{3}H-thymidine into the cell monolayer as per the **Materials and Methods.** Results are expressed as a percent of the negative control (i.e. no growth factor added) and are the mean of four determinations ± SEM; 2. Based on total protein.

Table 5.3: Effects of Growth Factors on Protein Synthesis in MDBK and L-6 cells

Growth Factor	Protein Synthesis[1] (% of control) MDBK	L-6
IGF-1 (10 nM)	141±3	361±6
des-(1-3)-IGF-1 (10 nM)	169±5	408±10
IGF-2 (10 nM)	122±3	225±5
Insulin (1 μM)	162±2	340±8
EGF (1 nM)	113±1[2]	103±3
bFGF (1 nM)	110±2	196±5
FBS (10%)	105±1	476±12

1. Protein synthesis was monitored by the incorporation of ^{3}H-leucine into the cell monolayer as per the **Materials and Methods**. Results are expressed as a percent of the negative control (i.e. no growth factor added) and are the mean of four determinations ± SEM; 2. ± the range not the SEM as n=2.

Table 5.4: Effects of PDGFs on Protein Synthesis in MDBK and L-6 cells

Growth Factor	Protein Synthesis[1] (% of control) MDBK	L-6
hPDGF-AA (10 nM)	92±2	112±1
hPDGF-BB (10 nM)	92±1	213±4
bPDGF (1 nM)	93±1	167±1
FBS (10%)	93±1	306±2

1. Protein synthesis was monitored by the incorporation of ^{3}H-leucine into the cell monolayer as per the **Materials and Methods**. Results are expressed as a percent of the negative control (i.e. no growth factor added) and are the mean of four determinations ± SEM.

Table 5.5: Effects of Growth Factors on Protein Degradation in MDBK and L-6 cells

Growth Factor	Protein Degradation[1] (% effect) MDBK	L-6
IGF-1 (10 nM)	↓20±1.3	↓28±2.8
des-(1-3)-IGF-1 (10 nM)	↓21±3.0	↓28±4.7
IGF-2 (10 nM)	↓11±2.6	↓28±4.6
Insulin (1 μM)	↓24±1.7	↓31±3.4
EGF (1 nM)	↓2.4±2.0	↓2.8±2.9
bFGF (1 nM)	↓2.9±1.0	↓5.2±1.1
FBS (10%)	↓9.5±0.6	↓21±2.3

1. Protein degradation was measured as per the **Materials and Methods.** Results are the mean of four determinations ± SEM and represent the percent inhibition (↓) or stimulation (↑) of degradation expressed as a percent effect compared to that observed without any growth factor addition.

Table 5.6: Effects of PDGFs on Protein Degradation in MDBK and L-6 cells

Growth Factor	Protein Degradation[1] (% effect) MDBK	L-6
hPDGF-AA (10 nM)	↓5.4±2.1	↓9.9±1.1
hPDGF-BB (10 nM)	↓2.4±1.3	↓25±1.6
bPDGF (1 nM)	↓2.6±2.3	↓19±0.7
FBS (10%)	↓11±1.4	↓28±1.0

1. Protein degradation was measured as per the **Materials and Methods.** Results are the mean of four determinations ± SEM and represent the percent inhibition (↓) or stimulation (↑) of degradation expressed as a percent effect compared to that observed without any growth factor addition.

Table 5.7: Effects of Growth Inhibitors on DNA Synthesis in MDBK and L-6 Cells

Growth Factor	DNA Synthesis[1] (% of control) MDBK	L-6
TGF-β1 (1 nM)	65±6	40±4
TGF-β1(1nM)/IGF-1(10nM)	176±11	184±13
TGF-β1(1nM)/EGF(1nM)	129±7	26±8
IFN-α (1 nM)	72±2	101±3
IFN-α(1nM)/IGF-1(10nM)	166±7	523±30
IFN-α(1nM)/EGF(1nM)	103±2	98±3
GI-1 (20 units/ml)[2]	68±3	9±1
GI-2 (20 units/ml)[2]	72±4	19±2
IGF-1 (10 nM)	197±6	518±15
EGF (1 nM)	157±7	104±5
FBS (10%)	124±4	1,022±19

1. DNA synthesis was monitored by the incorporation of ^{3}H-thymidine into the cell monolayer as per the **Materials and Methods.** Results are expressed as a percent of the negative control (i.e. no growth factor added) and are the mean of four determinations ± SEM; 2. One unit is defined as the amount of growth inhibitor required to cause a half maximal effect in the DNA synthesis inhibition assay on L-6 myoblasts.

Table 5.8: Effects of Growth Inhibitors on Protein Synthesis in MDBK and L-6 Cells

Growth Factor	Protein Synthesis[1] (% of control) MDBK	L-6
TGF-β1 (1 nM)	103±1	122±2
TGF-β1(1nM)/IGF-1(10nM)	134±2	262±4
TGF-β1(1nM)/EGF(1nM)	120±2	128±2
IFN-α (1 nM)	118±2	96±5
IFN-α(1nM)/IGF-1(10nM)	139±2	253±4
IFN-α(1nM)/EGF(1nM)	115±1	104±3
GI-1 (20 units/ml)[2]	93±2	139±3
GI-2 (20 units/ml)[2]	98±1	145±4
IGF-1 (10 nM)	120±1	258±9
EGF (1 nM)	102±2	100±1
FBS (10%)	93±1	306±2

1. Protein synthesis was monitored by the incorporation of ^{3}H-leucine into the cell monolayer as per the **Materials and Methods.** Results are expressed as a percent of the negative control (i.e. no growth factor added) and are the mean of four determinations ± SEM; 2. One unit is defined as the amount of growth inhibitor required to cause a half maximal effect in the DNA synthesis inhibition assay on L-6 myoblasts.

Table 5.9: Effects of Growth Inhibitors on Protein Degradation in MDBK and L-6 Cells

Growth Factor	Protein Degradation[1] (% effect)	
	MDBK	L-6
TGF-β1 (1 nM)	↓3.2±1.4	↓11±8.0
TGF-β1(1nM)/IGF-1(10nM)	↓17±2.2	↓27±1.7
TGF-β1(1nM)/EGF(1nM)	↓4.5±1.2	↓4.2±2.9
IFN-α (1 nM)	↑0.8±2.0	↓0.5±1.7
IFN-α(1nM)/IGF-1(10nM)	↓18±1.8	↓28±2.9
IFN-α(1nM)/EGF(1nM)	↓2.1±6.2	↓4.2±1.6
GI-1 (20 units/ml)[2]	↓3.3±2.3	↓15±0.6
GI-2 (20 units/ml)[2]	↓3.5±0.8	↓15±1.1
IGF-1 (10 nM)	↓20±1.3	↓28±2.8
EGF (1 nM)	↓2.4±2.0	↓2.8±2.9
FBS (10%)	↓9.5±0.6	↓21±2.3

1. Protein degradation was measured as per the **Materials and Methods.** Results are the mean of four determinations ± SEM and represent the percent inhibition (↓) or stimulation (↑) of degradation expressed as a percent effect compared to that observed without any growth factor addition. 2. One unit is defined as the amount of growth inhibitor required to cause a half maximal effect in the DNA synthesis inhibition assay on L-6 myoblasts.

CHAPTER SIX : General Discussion

Chapter 6: General Discussion

The results presented in my thesis have been discussed within each chapter. In the current chapter the possible significance of the results is discussed.

The production of growth factors by the kidney cell line, MDBK, prompts the question, do the growth factors have a role in the growth and function of the kidney? The first step in answering this question is to determine whether the kidney produces the growth factors *in vivo*. Contrasting results have been obtained when the *in vivo* expression of the different growth factors has been examined. For example, TGF-β has been purified from bovine kidney (Roberts *et al.*, 1983), but techniques of *in situ* hybridization suggest that production of TGF-β by the kidney in mouse embryos is slight (Lehnert and Akhurst, 1988). Utilizing northern hybridization techniques, a number of studies have provided evidence of both IGF-1 and IGF-2 production by the kidney (Brown *et al.*, 1986; Murphy *et al.*, 1987b, c). Immunofluorescent antibody techniques have also detected nonsuppressible insulin-like activity (now known to be IGF) in the kidney (Liske and Reber, 1976) and somatomedin C (IGF-1) has been extracted from rat kidney (D'Ercole *et al.*, 1984). PDGF, however, does not appear to be produced by the kidney epithelium as evidenced by tissue immunohistochemistry (Frampton *et al.*, 1988).

Even if the growth factors produced by MDBK cells are not produced constitutively by the kidney they may still be produced at particular periods during the "life" of the kidney, for example in the early morphogenetic development of the kidney and in compensatory re-growth after kidney damage or removal. The nature of the factor(s) involved in compensatory renal growth after kidney damage has been an area of controversy for some time, although there appears to be general agreement for the involvement of a renotropic growth factor (called renotropin) in this process (Preuss, 1983). Evidence for the presence of this growth factor in the serum of nephrectomized rabbits (Yamamoto *et al.*, 1983) and in the urine of rats that have undergone a selective reduction in renal excretory function (Harris *et al.*, 1983) has been reported.

It is not my intention to suggest that any of the growth factors produced by the kidney cell line, MDBK, is renotropin. Indeed, the growth factors PDGF-AB, IGF-2 and TGF-β have a very wide spectrum of target cells and tissues. However, the possibility

remains that these growth factors may be involved in the co-ordinate response of kidney tissue growth after injury with the IGFs (which, in the case of IGF-1, have been reported to stimulate kidney growth *in vivo*, Guler *et al.* (1988)) and TGF-β acting on both epithelial and connective tissue elements and PDGF presumably confined to stimulating kidney associated smooth muscle, mesangial and fibroblastic cells.

An investigation of the production of these growth factors by the kidney during compensatory renal growth versus the normal state or during morphogenetic development may well indicate whether they are involved in kidney growth. It may also be necessary to monitor at the same time the production of growth factor inhibitors (such as IGF binding proteins), the extent of activation of latent growth factor (e.g. TGF-β), the level of expression of the various growth factor receptors and the sensitivity of the post receptor pathways, in order to obtain a complete picture of the involvement of these growth factors in kidney growth.

APPENDICES

Appendix A1: Development of PDGF ELISA Assays

The PDGF A and B chain specific ELISA assays, as described in **Chapter 3**, were developed from a procedure provided by Dr. Elaine Raines, Department of Pathology, University of Washington, Seattle. The procedure was initially performed using PDGF-BB as antigen and Dynatech polyvinyl 96 well microtitre plates. These particular microtitre plates gave very high background absorbance, and therefore better quality microtitre plates specifically designed for ELISA assays were tested. A comparison was made between Nunc-Immuno Plate 1 and Dynatech Immulon II ELISA plates. In this comparison the effect of blocking the plate with BSA after coating with antigen was also examined. Plates were coated with PDGF-BB at 10 ng/ml as described in **Chapter 3** (except for those wells used to determine the nonspecific background binding which were left uncoated), either blocked with BSA (1 mg/ml in PBS for 1 hour at 37°C) or not, and an antiserum dilution curve performed using a rabbit anti-human PDGF-AB antiserum (obtained from Prof. Carl-Henrik Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden). The results indicated that either type of microtitre plate provides an acceptable signal to noise ratio and that it is best to include a BSA blocking step. Since the Nunc-Immuno Plate 1 plates appeared to have a higher binding capacity, reflected in the shorter development time for the assays performed in this type of plate, they were chosen for all further work. Using these plates a competitive ELISA assay was set up with the following parameters: PDGF-BB concentration of 10 ng/ml for plate coating, BSA blocking after plate coating, and an antiserum dilution of 1/1000, a dilution which resulted in a A_{450} absorbance of approximately 0.7 without the addition of competing ligand. This assay was sensitive in the range 0.1 to 1 ng/ml PDGF-BB.

Following the development of this assay the same parameters were applied using PDGF-AA as the antigen to produce a PDGF A chain specific ELISA assay. This resulted in an assay in which the signal to noise ratio was very low. In order to increase this ratio the following parameters were investigated: the concentration of PDGF-AA used to coat the plate, the antiserum dilution and the conjugate dilution (a dilution of 1/5000 of the peroxidase conjugated goat anti-rabbit IgG was originally used in the PDGF-BB ELISA assay). From the results shown in **table A1**, and allowing for the availability of the reagents, a PDGF-AA concentration of 500 ng/ml for plate coating, an antiserum dilution

of 1/800 and a conjugate dilution of 1/2000 (both values interpolated from the data in **table A1**) were chosen as suitable. The PDGF-AA ELISA developed using these concentrations of reactants was not as sensitive as the PDGF-BB assay, the sensitivity of the AA assay being 0.1 to 2μg/ml. This difference in sensitivity presumably reflects a difference in the number and avidity of anti-PDGF B chain antibodies versus anti-PDGF A chain antibodies in the antiserum used. Nevertheless, both assays were specific for the particular PDGF chain used to coat the microtitre plate and have proved very useful in the characterization of bPDGF (see **figures 3.10** and **3.11**).

Table A1 : Absorbance values obtained in the PDGF A chain ELISA assay varying the parameters listed below[1]

PDGF-AA[2] concentration	Antibody Dilutions 1° Ab. 1/500 2° Ab. 1/1000	1° Ab. 1/1000 2° Ab. 1/1000	1° Ab. 1/500 2° Ab. 1/5000	1° Ab. 1/1000 2° Ab. 1/5000
1 µg/ml	1.43±0.06	0.92±0.03	1.19±0.14	0.67±0.02
0.5 µg/ml	1.32±0.01	0.95±0.02	0.98±0.01	0.66±0.02
0.1 µg/ml	0.89±0.03	0.73±0.02	0.72±0.03	0.49±0.02
50 ng/ml	0.81±0.03	0.73±0.02	0.64±0.02	0.46±0.01
10 ng/ml	0.65±0.03	0.64±0.05	0.54±0.02	0.41±0.02
NSB[3]	0.37±0.02	0.33±0.04	0.31±0.04	0.22±0.03

1. The following parameters were investigated, concentration of PDGF-AA used to coat the microtitre plate, antiserum dilution (primary antibody, 1°, dilution) and conjugate dilution (secondary antibody, 2°, dilution). Absorbance values are the mean of triplicate determinations ± SEM. 2. The PDGF-AA concentration used for coating the microtitre plate. 3. NSB=nonspecific binding.

Appendix A2: Publications

Szabo, L., Mottershead, D.G., Ballard, F.J. and Wallace, J.C. (1988) The bovine insulin-like growth factor (IGF) binding protein purified from conditioned medium requires the N-terminal tripeptide in IGF-1 for binding *Biochem. Biophys. Res. Commun.* **151**, 207-214

Szabo, L., Mottershead, D.G., Ballard, F.J., and Wallace, J.C., (1988) The bovine insulin-like growth factor (IGF) binding protein purified from conditioned medium requires the N-terminal tripeptide in IGF-1 for binding.
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