PRODUCTION OF GROWTH FACTORS FROM THE HUMAN CARCINOMA CELL LINE, RPMI 2650

by

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A thesis submitted for the degree of Ph.D

The experimental work described in this thesis was carried out under the supervision of Professor Martin Clynes

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I declare that the work described in this thesis is entirely my own work, and has not been previously submitted for a degree at this at any other university.

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ABSTRACT

The human carcinoma cell line RPMI 2650 produces autocrine factors These are detected by the ability of RPMI 2650 conditioned medium (CM) to stimulate growth in soft agar of RPMI 2650 cells plated at low density The autocrine activity in crude CM can be fractionated by ultrafiltration into a lower molecular weight (MW) fraction (R1-30), which concentrates molecules in the 1,000-30,000 kDa range, and a higher MW fraction (R30) with molecules greater than 30,000 kDa in a more concentrated form R1-30 is labile to acid, base and heat treatment, whereas R30 is stable to (and sometimes activated by) these treatments Boiling of R30, however, renders it labile to acid-, base- and trypsin-treatments CM can be separated into a weakly heparin-binding fraction (with stability properties similar to R1-30), and a non-heparin binding fraction (with stability properties similar to R30) RPMI 2650 cells secrete TGF-α- and TGF-ß-like molecules, but the R1-30 can be distinguished from these TGFs, and from most other known growth factors, by the unusual combination of acid lability and weak affinity for heparin Since the R30/non-heparin binding fraction is rendered labile by boiling or acid treatment, it may represent a bound or conformationally stable form of a growth factor Growth factor production from RPMI 2650 cells was successfully scaled up to a 2 litre scale

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1 0 INTRODUCTION

1 1 GROWTH CONTROL

Multicellular organisms have evolved highly coordinated mechanisms to control cellular interactions The initial discovery of epidermal growth factor (Carpenter and Cohen, 1979) has been followed by the identification of more than 20 different polypeptide growth factors, which act *in vivo* by stimulating fetal and placental growth during development (Ohlsson, 1989), regulating growth and differentiation of continuously regenerating tissues e g in haematopoiesis (Metcalf, 1985) and stimulating tissue repair processes (ten Dijke and Iwata, 1989)

In vivo, the arrangement of developing tissues and the control of repair processes require the spatial confinement of growth factor activities Circulating growth factors (PDGF, TGF-ß, and the IGFs) are usually in latent form e g. in complexes with binding proteins Growth factors of the FGF family and certain hemopoietic factors are anchored by interaction with the extracellular matrix These systems allow presentation of the factors in organised microenvironments within which growth control is finely tuned

Growth factors are currently believed to elicit their responses at a cellular level by binding to a specific membrane receptor resulting in a cascade of reactions, the precise mechanisms of which remain to be deciphered Receptors for the major classes of growth factors consist of an external ligand domain which specifically binds to a selected portion of the growth factor This external domain is linked, via a hydrophobic stretch of amino acids, to an internal domain which often incorporates a protein tyrosine kinase catalytic domain (reviewed by Cadena and Gill, 1992) The mechanism of activation is thought to be due to dimerization as has been shown to be the case for platelet-derived growth factor (PDGF) type B receptor (Heldin et al , 1989) and epidermal growth factor (EGF) (Schessinger, 1986) Models of the various receptor tyrosine kinases are shown diagramatically below

The spacing of the cysteine residues in their external domains defines either immunoglobulin-like domains, in the case of PDGF

and EGF receptor families, or cysteine-rich clusters The known ligands for each receptor family also show conservation of cysteine clusters, but are otherwise dissimilar (Aaronson, 1991)

The receptors that constitute each family and growth factors which bind to them are listed in the table below

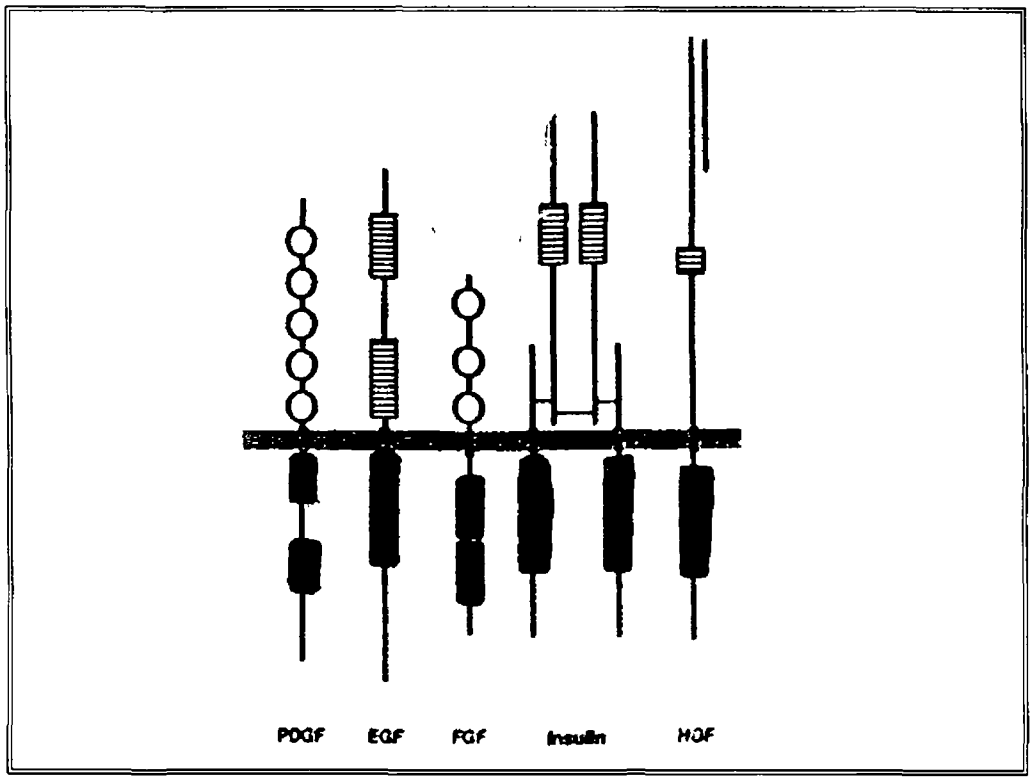


Figure 1 1 **Schematic diagram of transmembrane tryosine kinase receptors The open circles illustrate immunoglobulin-like repeats Dashed boxes indicate cysteine-rich domains Solid boxes indicate conserved tyrosine kinase domains (Aaronson,1991)**

TABLE 1 **Transmembrane Tryosine Kinase Families**

Receptor Prototype	Family members	Ligands
PDGF	PDGF-α, PDGF-ß, M-CSFR (*c-fms*), *c-kit*	PDGFAA, PDGFBB, PDGFAB, *c-sis*, M-CSF
EGF	EGFR, *c-erbB*, ERBB2, ERBB3, *neu*	EGF, TGF-α, Amphiregulin,
FGF	FGFR1 (*flg*), FGFR2 (*bek*), FGFR3, FGFR4	aFGF, bFGF, INT-2, FGF-5, *hst*/K-FGF, FGF-6, KGF
Insulin	IR, IGF-IR, *c-ros*	Insulin, IGF-I
HGF	HGFR, *met*	HGF

Abbreviations: PDGF = Platelet-derived growth factor, M-CSF = Macrophage colony stimulating factor, EGF = epidermal growth factor, FGF = fibroblast growth factor, I = insulin, IGF = insulin-like growth factor, HGF = hepatocyte growth factor R refers to receptor

The complete list of substrates of these transmembrane tyrosine kinases has yet to be deciphered It has been shown that some substrates are protein (serine, threonine or tyrosine) kinases (Hunter et al , 1985, Morrison et al , 1988, Ralston and Bishop, 1985, Gould and Hunter, 1988) Although the receptor, itself, is often the major tyrosine phosphorylated species observed after the receptor is activated by ligand binding (Kock et al , 1991)

A second post-binding event is the phosphorylation of phospholipase C which stimulates phosphatidyl-inositol turnover leading to the degradation of phosphatidyl-inositol biphosphate and release of diacylglycerol and insoitol triphosphate (ITP) Diacylglycerol activates protein kinase C (Nishizuka, 1988) by binding to it and raising its affinity for calcium ITP increases the cytoplasmic calcium ion levels (Berridge and Irvine, 1984).

Formation of the ligand-receptor complex can also result in phosphorylation of Ras guanosine triphosphatase activating protein (GAP) which regulates the function of the Ras protein (McCormick, 1989) Ras, a 21 kDa protein, is a critical component of intracellular signalling It binds guanosine triphosphate (GTP) tightly catalysing the removal of phosphate from GTP to form the inactive guanosine diphosphate Interaction of this complex with GAP, results in increased GTPase activity

A third post-binding event is the rapid induction and transient expression of a number of primary response genes (reviewed by Herschman, 1991) including c-fos, c-jun and c-myc The proteins encoded by these genes include transcriptional modulators, structural proteins, cytokines and proteins of as yet unknown function

Binding of ligands to receptor types distinct from membrane spanning tyrosine kinases is known to stimulate cell proliferation One class includes the receptors for interleukin 2, 3, 4, 5, 6 and 7, granulocyte-macrophage colony stimulating factor (GM-CSF), G-CSF and erythropoietin (Nicola and Metcalf, 1991) These receptors are membrane glycoproteins with a single hydrophobic transmembrane domain Their external domains are similar in size, with conserved cysteine residues at their amino termini Their intracellular domains, however, are not similar They vary in length and possess no tyrosine kinase domain The signal transduction pathways of these receptors have not been elucidated. It is known, however, that ligand binding results in increased levels of GTP-bound ras (Shiraishi et al , 1987), although they are distinct from G-protein linked receptors A number of high affinity cytokine receptors have a two chain structure The interleukin 2 receptor, for example, consists of two subchains α and ß Each bind IL-2 with low affinity, but the αß complex binds IL-2 with high affinity (Waldmann, 1991) Other two chain receptors include the receptors for IL-5 (Takatsu, 1991), IL-6 (Taga et al , 1989), GM-CSF (Cosman et al , 1990) and IL-9 (Hilton, 1992)

Another class of molecules (to which bombesin belongs) capable of causing mitogenic stimulation of certain cell types, are neurotransmitters (Lin et al., 1991) These receptors are different from the ones mentioned above They consist of an extracellular amino terminal domain linked to a looped cytoplasmic carboxyl-terminal by seven transmembrane domains The intracellular domain contains regulatory serine and threonine residues and is coupled to a G-protein

In the course of this introduction, I will give a brief overview of each of the main families of growth factors and cytokines, and show how each are implicated in different systems as autocrine growth regulators

1 1.1 TRANSFORMING GROWTH FACTORS AND EPIDERMAL GROWTH FACTOR

Transforming growth factor (TGFs) are peptides that affect the growth and phenotype of cultured cells and bring about phenotypic properties in non-malignant fibroblast cells that resemble those of malignant cells (Keski-Oja et al , 1987). Two main classes of TGFs have been extensively studied, TGF-α and TGF-ß. More recently epithelial TGF has been isolated and characterised (*Halper* and Moses, 1987) Other members of this structurally and functionally related family include amphiregulin (Shoyab and Plowman, 1991) and vaccinia virus growth factor (Brown et al , 1985).

Epidermal growth factor (urogastrone), a fully sequenced polypeptide of 53 amino acids (Carpenter and Cohen, 1979), contains 6 cysteine residues which form 3 disuphide bonds (Savage et al , 1972, Savage et al , 1973) It is synthesised in a large precursor form (Gray et al , 1983) (133 kd) which consists of the EGF moiety (53 amino acids) flanked by a stretch of 976 amino acids at its amino terminal end that contains 7 peptides, with sequences that are similar but not identical to EGF (Scott et al , 1983), and 188 amino acids at its carboxy terminal.

Transforming growth factor alpha (TGFα), a 50 amino acid polypeptide (5 7 kD), (Derynck et al , 1984) has 40% homology with EGF with conservation of all 6 cysteine residues (Marquardt et al , 1984) and has been shown to possess very similar chain folds (Montelione et al , 1989, Harvey et al , 1991) Larger forms of TGFα, arising from incomplete cleavage and/or heterogenous glycosylation, have been described (Texido et al , 1988)

TGF-α is synthesised as a larger precursor (pro-TGF-α) containing a 23 amino acid hydrophobic signal sequence followed by the mature unglycosylated growth factor sequence, a second hydrophobic domain (which spans the plasma membrane) and finally a cysteine rich domain at the carboxyl terminal (Lee et al , 1985, Texido et al , 1988, Bringman et al , 1987, Gentry et al , 1987) Pro-TGF-α has been cloned and expressed in *E coli* (Derynck et al , 1984) Newly synthesised pro-TGF-α is resistant to cleavage until it reaches the plasma membrane (Texido et al , 1990) Cleavage of the TGF-α precursor in CHO cells that express a transfected pro-TGF-α occurs in two steps (Pandiella and Massagué, 1990). In the first step, pro-TGF-α rapidly ($t_{\frac{1}{2}}$=15 minutes) loses the N-terminal sequence that precedes the TGF-α segment In the second step, pro-TGFα is cleaved at the C terminus releasing soluble TGF-α into the medium and leaving the transmembrane/cytoplasmic fragment associated with the cell This second step is rate limiting and so leads to the accumulation of pro-TGF-α on the cell surface, much of which turns over without ever being converted to the soluble factor

TGF-α and EGF bind to a common cell surface receptor, the TGF-α/EGF receptor (175 kDa) (Ullrich et al , 1984), a 1186-amino acid transmembrane phosphorylated glycoprotein The ligand binding site located in the extracellular 621-amino acid portion of the molecule, is separated by a 23-amino acid hydrophobic membrane spanning section from the 524-amino acid intracelluar region of the molecule which contains protein tyrosine activity (encoded by the *erbB* protooncogene).

The presence of two distinct EGF/TGF-α receptors (high and low affinity) have been identified using Scatchard analysis (Shoyab et al., 1979, Boonstra et al , 1985) and blocking antibody studies (Defize et al , 1989) Recently, Berkers et al (1991) have shown that, in addition to the high affinity receptor in HeLa cells, there are two low affinity receptors which exhibit different kinetics

In general EGF and TGF-α elict the same effects For example the stimulation of DNA synthesis in several cell lines (Schreiber et al., 1986), induction of anchorage independent growth (Anzano et al., 1983; Salomon et al., 1987), induction of eyelid opening in newborn mice (Tam, 1985) or inhibition of parathyroid hormone-reponsive adenylate cyclase in osteoblasts (Guiterrez et al , 1987)

In other situations, however, TGF-α is more potent TGF-α is 3-10 times more potent than EGF in releasing calcium ions from bone cells in culture and bone resorption (Stern et al , 1985) In vivo, TGF-α has a more potent effect inducing neovascularization (Schreiber et al , 1986) It is more potent than EGF in causing transient ruffling of the cell membrane in normal rat kidney cells (Myrdal et al , 1986) and increasing rate of migration and monolayer growth in keratinocytes (Barrandon and Green, 1987) While TGF-α and EGF bind the receptor with similar affinities, antibody experiments have shown that the binding is not the same (Winkler et al., 1989). The more rapid clearance of intracellular TGF-α than EGF after internalisation (Ebner and Derynck, 1991) has been suggested as a mechanism for their differences in potency

Transforming growth factor beta (TGFß) is produced as a 390 amino acid inactive precursor that is cleaved to a 112 amino acid monomer (12 5 kD) (Derynck et al., 1985) Two monomers linked by disulphide bonds form the biologically active 25 kD form (Assoian et al , 1983) TGFß is found in three homologous forms in humans, each conserving 9 cysteine residues (Ten Dijke et al., 1988) Two

other forms have also been found in chicken cells (Jakowlow et al., 1988) and frogs (Kondaiah et al., 1990) and designated TGFß4 and TGFß5 respectively

TGF-ß in itself represents a large family of factors with diverse activities (reviewed by Massagué, 1990) A number of molecules have been identified that have 25-40% homology with TGFß and conserve at least 7 or the 9 cysteine residues These include Mullerian inhibiting substance (Cate et al , 1986, Blake et al., 1988), inhibins and activins (Mason et al., 1985), Drosophilia decapentaplegic gene (Padgett et al , 1987), BMP-2b and BMP-3 (Worzney et al., 1988), Vg-1 (from Xenopus) (Weeks et al., 1987) and the Vgr-1 gene product (Lyons et al , 1989)

In vivo, all three forms of TGF-ß have been found in a wide variety of normal adult mouse tissues Some forms are expressed at higher levels than others in different tissues TGF-ß1 and ß2 were found to be expressed in spleen, lung and placenta, while TGF-ß3 is only detected in the lung and placenta (Miller et al , 1989) Some cell lines (e g WI-38 human diploid lung fibroblasts), secrete predominately TGF-ß1, some (e g BSC-1 monkey kidney, and MCF-7 human breast carcinoma) secrete TGF-ß2, while others (e g NRK-49F rat kidney cells, HT-1080 human fibrosarcoma, NIH-3T3 rat fibroblasts and MDCK dog kidney cells) secrete both forms in roughly similar amounts (Danielpour et al , 1989) TGF-ß has been detected in human urine (Twardzik et al , 1985), human placenta (Frolik et al , 1983) and more recently in bovine milk (Cox et al , 1991)

TGF-ß is synthesised in an inactive form (Lawerence et al , 1984) in most transformed and non-transformed cells While normal and chemically transformed rat liver epithelial cells typically produce the latent form of TGF-ß, malignant epithelial cells tended to secrete activated TGF-ß de novo (Liu et al , 1988). The latent molecule consists of the mature TGF-ß molecule non-covalently bound to a N-terminal precursor glycopeptide, the latency-associated peptide (LAP) (Gentry et al , 1989, Gentry and Nash, 1990) Sequence analysis of the cDNA clones has suggested the existence of 2 different LAPs for TGF-ß2 (Webb et al , 1988) Latent TGF-ß, isolated from platelets and some other cells, is

associated with a binding protein (TGF-ß-BP) (Wakefield et al , 1989) TGF-ß-BP has been isolated and cloned (Miyazono et al , 1988, Wakefield et al , 1988, Kanzaki et al , 1990) The molecule is made up of multiple EGF-like repeats and hydroxylated asparagine residues that are capable of binding calcium ions Activation of the latent TGF-ß molecule has been well documented (Lyons and Moses, 1990) and can be achieved by transient acid treatment (Pircher et al , 1984), treatment with proteases (Lyons et al , 1988) or brief heat treatment (Brown et al , 1990) Removal of the carbohydrate moieties from the latent molecule also results in activation (Miyazano and Heldin, 1989)

TGF-ß differs from other transforming growth factors by not competing with EGF for its receptors (Chua et al., 1983, Fanger et al , 1986; Like and Massagué, 1986) Three classes of high affinity transmembrane receptors have been described in detail (60-70, 85-95 and 280-330 kDa) (Massague et al , 1987) While type I and II receptors have distinct sets of affinities for each member of the TGF-ß family, Type II receptor shows comparable affinities for all isotypes (Cheifetz et al , 1987) Type III receptor (also called betaglycan) (280-330) is a proteoglycan consisting of 50% heparan sulphate and chondroitin sulphate (Segarini et al , 1989, Cheifetz et al , 1988, Cheifetz et al , 1986) Disulphide linked complexes of type III receptors (560-600 kDa) are the predominant form of the receptor in most mammalian and avian fibroblasts and epithelial cells (Massague, 1985) Some cell lines, however (e g myoblasts) do not express type III receptors (Cheifetz et al , 1986) Betaglycan also exists in soluble forms that are released by cells into the medium and are found in extracellular matrices and serum (Andres et al., 1989) The type III receptor has recently been cloned and characterised (Wang et al , 1991, Lopez-Casillas et al., 1991) It is an 853 amino acid protein with a large N-terminal extracellular domain containing at least one site for glycosaminoglycan addition, a single transmembrane hydophobic domain and a 41 amino acid cytoplasmic tail with no obvious signalling motif. Betaglycan has also been shown to have an affinity for bFGF, although this binding is via its heparan sulphate chains rather than by its core protein (Andres et al , 1992, Rouslahti and Yamaguchi, 1991)

Three other less characterised TGF-ß receptors have been reported (Cheifetz et al , 1988, O'Grady et al , 1991, Mackay and Danielpour, 1991)

While TGF-ß was originally characterised by its ability to induce anchorage independent growth of normal rat kidney cells, its effects on most cell types are antimitogenic (Roberts et a,, 1981; Tucker et al , 1984) It is strongly inhibitory for many cell types, including both normal and transformed epithelial, endothelial, fibroblast, neuronal, lymphoid and hematopoietic cells (Tucker et al , 1984, Moses et al , 1985, Roberts et al , 1985, Shipely et al , 1986, Kehrl et al , 1986, Carr et al , 1986, Cheifetz et al , 1987, 1990, Knabbe et al , 1987, Ohta et al , 1987, Graycar et al , 1989) In addition, TGF-ß plays a central role in regulating the formation of extracellular matrix and cell matrix adhesion processes (Roberts and Sporn, 1990, Massagué, 1990) It also plays a number of roles in embryogenesis (reviewed by Ohlsson, 1989; Bernard, 1990) and differentiation of a number of different cell types (Chakrabarty et al , 1989, Masui et al., 1986, Ignotz and Massagué, 1985, Allen and Boxhorn, 1987, 1989, Florini et al , 1986, Colletta et al , 1989)

Transforming growth factor type-e (TGF-e) is a novel TGF which was first described as a growth factor possibly involved in the autocrine stimulation of anchorage independent growth of carcinoma cells (Halper and Moses, 1983) It is present in normal and neoplastic tissues of mostly epithelial origin and has been detected in both plasma and platelets (Brown and Halper, 1988) It has been reported to act as a mitogen for both fibroblast and epithelial cells of non-neoplastic origin (Brown and Halper, 1990) TGF-e has been purified to homogeneity from bovine kidney (Halper and Moses, 1987) and has been characterised as an acid- and heat-stable polypeptide with an apparent molecular weight of 22,000-25,000, requiring disulphide bonds for maximal activity Human TGF-e differs from bovine TGF-e in a number of respects (Dunnington et al , 1990) Its apparent molecular weight is 59 kDa and, unlike bovine TGF-e, is unstable to SDS Dunnington et al. (1990) reported the detection of a low molecular weight bovine derived TGF-e (13-15 kDa) which appears to be an active

truncated form of the 25 kDa form These two TGF-e forms have a weak affinity for heparin and can be eluted at 0 5M NaCl (Parnell et al , 1990), unlike human TGF-e which has no affinity for heparin (Dunnington et al , 1990)

1 1.2 PLATELET-DERIVED GROWTH FACTOR

PDGF, a potent mitogen for all cells of mesenchymal origin, is produced by a range of cell types including endothelial cells, vascular smooth muscle cells and osteosarcoma cell lines It is a heat stable, 2 chain polypeptide glycoprotein (28-31 kDa) that is sensitive to proteases and treatment with 2-mercaptoethanol (Antoniades, 1981, Heldin et al , 1981; Raines and Ross, 1982) The chains, designated PDGF A and B have been found to be 56% homologous (Josephs et al , 1984). Their genes have been assigned to chromosomes 7 and 22 respectively (Betscholtz et al , 1986, Swan et al., 1982) The two genes can be expressed independently, giving rise to three mature forms of PDGF (AA, BB, AB) (Alitalo et al , 1987). Both chains have eight cysteine residues, located in the same positions, suggesting that the homo and hetrodimeric forms of the molecule have similar tertiary structures The PDGF B chain has been shown to be identical to a carboxy-terminal piece of the transforming gene product (v-sis gene product) of the simian sarcoma virus (SSV) (Waterfield et al , 1983, Doolittle et al , 1983)

Two forms of PDGF receptors have been identified, both of which have tyrosine kinase activity PDGF receptor α is activated by PDGF-AA and PDGF receptor ß by PDGF-BB and the v-sis oncogene product PDGF-AB can interact with both receptors, (Yarden et al , 1986, Matsui et al , 1989, Gronwald et al , 1988, Claesson-Welsh et al , 1989, Hart et al , 1988, Ostman et al , 1991) The two receptors have a similar domain organisation with five immunoglobulin-like domains extracellularly. There is an inserted sequence (approximately 100 amino acid residues) in the tyrosine kinase domain, splitting it in two parts (Claesson-Welsh et al , 1989)

PDGF is a potent mitogen for cells of mesenchymal origin (e.g. fibroblasts and smooth muscle cells) but has little effect on the growth of epithelial or endothelial cells (Ross et al , 1986), which lack PDGF receptors Binding of PDGF with its receptor rapidly induces phosphatidylinositol turnover, release of arachidonic acid and formation of prostaglandins (Habenicht et al., 1985, Berridge et al , 1984) PDGF induces synthesis of collagenase, an important enzyme involved in wound healing, development and differentiation (Bauer et al , 1985, Circolo et al., 1991) Fibroblasts overexpressing the c-sis oncogene have been shown to have enhanced fibronectin levels (Allen-Hoffmann et al , 1990) At low concentrations, PDGF is chemotatic for fibroblasts, monocytes and neutrophils and has been shown to activate monocytes and neutrophils (Deuel et al , 1982, Tzeng et al , 1985) Although PDGF is a mitogen for rat L6 myoblasts, it has been shown to inhibit their differentiation (Jin et al , 1991).

Vascular endothelial growth factor (VEGF) has been assigned to the PDGF gene family (Tischer et al , 1989) cDNA analysis suggests that VEGF is synthesised in two forms (possibly due to alternative RNA splicing), which are 21% and 24% homologous to the A and B chains of PDGF and conserve all eight cysteine residues VEGF, a 40-46 kDa glycoprotein, has been isolated from medium conditioned by bovine pituitary folliculo-stellate cells (Gospodarowicz et al , 1989) rat glioma cells (Conn et al , 1990), U-937 human histocytic lymphoma cells (Keck et al , 1989) and from A431 human epidermoid carcinoma cells (Myoken et al , 1991)

1 1 3 INSULIN-LIKE GROWTH FACTOR FAMILY

The insulin like growth factor family consist of insulin, insulin-like growth factor I (Sm-C) and insulin-like growth factor II (MSA). IGF I and II are 7.5 kDa, highly conserved, single chain peptides consisting of four peptide domains B, C, A and D

The two domains, A and B are homologous to the insulin A and B chains, and domain C is analogous to the connecting C peptide in proinsulin The 70 amino acid and 67 amino acid sequences in mature IGF-I and II have been remarkably conserved between human, cow, pig, rat, chicken and sheep (Rotwein, 1991) The expression of IGF I, and to a lesser extent IGF II, is under the control of growth hormone (GH). IGF I and II are single genes products, localised in man on the long arm of chromosome 12 and the short arm of chromosome 11 respectively (Brissenden et al , 1984, Tricoli et al , 1984) The genes for IGF-I and II have been described (Kajimoto and Rotwein, 1991, Rotwein and Hall, 1990) The gene structures are complicated with alternative promoters acting on multiple initiation sites, differential RNA splicing, and variable RNA polyadenylation sites resulting in the production of multiple IGF-I and II mRNA species

IGF I is secreted in a precursor form consisting of a signal peptide (48, 25 or 22 residues, depending on the start methionine (Rotwein et al , 1987)), followed by the 70 amino acid mature peptide and a propeptide region at the C-terminus (E-peptide) which contains either 35 or 77 residues (depending on the mRNA transcript) (Bell et al , 1985, Ullrich et al , 1984, Rotwein et al , 1986). IGF II precursor consists of a signal peptide of 24 residues, the 67 amino acid residue of the mature IGF II molecule and an E peptide, 89 residues long (Sussenbach, 1989).

Over 95% of the IGFs present in serum are bound to binding proteins (IGFBP) (Zapf et al., 1975) The function of these proteins is not clear Using partial amino acid sequence information to construct oligonucleotide probes, four distinct classes of IGFBPs have been identified by molecular cloning

IGFBP-1 (28-30 kDa) has been isolated from HEP-G2 hepatoma cell line (Lee et al , 1988), human placenta (Brinkman et al , 1988), human decidua (Brewer et al , 1988) and rat decidua (Murphy et al , 1990). Its gene has been cloned and mapped to the p12-13 site on chromosome 7 (Brinkman et al , 1988) The primary structure of IGFBP-2 was determined from cDNA clones isolated from BRL-3A cells (Brown et al., 1989) It has a molecular mass of 33-35 kDa on SDS-PAGE under non-reducing conditions (Hossenlopp et

al , 1990) IGFBP-1 and -2 contain Arg-Gly-Asp (RGD) sequences which suggest interaction with binding to integrin on cell surfaces (Rouslahti and Pierschbacher, 1987) Most of the IGF in serum is found in a 150 kDa complex consisting of either IGF I or II bound to IGFBP-3 (53 kDa) and an acid labile third component (80 kDa) (Baxter and Martin, 1989) IGFBP 1 and 2 have high affinities for IGF I and II respectively, while IGFBP3 binds both forms with equal affinity (Baxter and Martin, 1989) A fourth type of
binding protein (IGFBP 4) (25 kDa) was isolated from human bone cell conditioned medium (Mohan et al., 1989) Two distinct isoforms of IGFBP-4 were detected by N-terminal amino acid sequence analysis in porcine serum (Coleman et al , 1991) Recently a fifth binding protein (IGFBP-5) has been cloned (Shimasaki et al , 1991) Northern analysis of IGFBP-5 in rat tissues demonstrated that transcription of this gene is highly active in kidney, although mRNA was detected in all tissues examined The gene for IGFBP-5 has been assigned to chromosome 5

IGFs have two types of transmembrane receptors (reviewed by Nissely and Lopaczynski, 1991; Kasuga et al., 1981). The Type I receptor is a glycosylated hetrotetramer composing of two extracellular alpha subunits (115 kDa, the ligand binding site) and 2 beta subunits (90 kDa) which span the membrane and provide an intracellular tyrosine kinase domain The four subunits are linked by disulphide bonds The type II IGF receptor shows no structural similarity to the type I and does not have tyrosine kinase activity The extracellular domain of the type II receptor is large, consisting of 15 contiguous repeats with an average size of 147 amino acids A single transmembrane segment connects the extracellular domain to a relatively short 164 amino acid cytoplasmic domain that lacks sequence similarity to any known protein (Lobel et al , 1988) The cDNA for the human mannose-6-phosphate receptor is 99 8% homologous to the human IGF II receptor (Oshima et al , 1988) A soluble form of the extracelluar portion of the IGF-II/Man-6-P receptor has been described (Causin et al., 1988) The type I receptor binds IGF I with higher affinity than IGF II, while the type II receptor has a higher affinity for IGF II than I (Massagué and Czech, 1982)

IGF has been shown to have mitogenic effects and metabolic effects both in vivo and in vitro (reviewed by Humbel, 1990) The mitogenic effects include increased DNA and RNA synthesis, cell proliferation and increased body weight Both IGF I and II show insulin-like metabolic effects (glucose transport, stimulation of glycogen and lipid synthesis in adipose tissue IGFs have also been implicated in differentiation of cells of mesodermal origin (Kurtz et al., 1982). IGFs are also thought to be important in the development and function of the nervous system (DiCicco-Bloom et al , 1988)

1.1 4 HEPARIN BINDING GROWTH FACTORS

Fibroblast growth factors (FGF) are encoded by a family of genes containing at least seven members, acidic FGF, basic FGF, *int*-2, K-FGF/Hst-1, FGF-5, FGF 6/Hst-2 and KGF (FGF 1 to 7) Their gene structures are similar (3 exons separated by 2 large introns) (Abraham et al , 1986, Jaye et al., 1986) and they share the conservation of two cysteine residues, which do not appear to be involved in disulphide bonds (Seno et al , 1988) The FGF-related oncogenes differ from bFGF and aFGF in that they have signal sequences (implying secretion) and they are rarely found in adult tissues (Klagsburn, 1989, Burgess and Maciag, 1989, Rifkin and Moscatelli, 1989)

Basic FGF is a single chain non-glycosylated cationic polypeptide with a molecular weight of about 18,000 and a pI of 9.6 The mature form has 154 amino acid residues (Ueno et al , 1986) although extraction under acidic conditions resulted in the isolation of a 146 amino form (Klagsburn et al , 1987) Higher molecular weight forms of bFGF have been isolated (Sommer et al , 1987, Prats et al , 1989) These multiple forms are derived from a single gene or mRNA whose translation is initiated at either AUG (18 kDa form) or CUG (21 and 22 5 kDa forms) start codons (Prats et al , 1989; Florkiewicz and Sommer, 1989) The smallest form

(18 kDa) occurs predominantly in the cytosol, while the higher molecular weight forms are associated with the nucleus and ribosomes It has been shown that target signalling sequences within the extended amino terminal direct the processed molecule to its final destination (Tessler and Neufeld, 1990, Quarto et al , 1991, Powell and Klagsburn, 1991)

Acidic FGF is a single chain anionic polypeptide with a molecular weight of about 18 kDa and a pI of 5 6 (Thomas et al , 1984, Burgess and Macaig, 1989) The pure growth factor is mitogenic for a variety of types of normal cells in culture including fibroblasts, glial cells, osteoblasts and vascular endothelial cells. It has been shown to share homology with human interleukin 1 (IL-1) (Thomas, 1987). Like bFGF, amino terminal truncated forms have been described (Burgess et al , 1986)

The third member of the FGF family, the product of the *int*-2 proto-oncogene, is expressed in adult mouse essentially only in the brain and during embyrogenesis (Dickson and Peters, 1989, Robinson, 1991) *Int*-2 has not yet been demonstrated to be a mitogen or to bind heparin and has not been detected in adult tissues. The protein product of *int*-2 consists of 240 amino acids which have 44% and 38% homology to bFGF and aFGF, respectively (Dickson et al , 1987) Unlike aFGF and bFGF, the amino terminus of *int*-2 has a short sequence of 18 or 19 non-charged amino acids, which may play some role in secretion (Dickson et al , 1989)

hst was originally identifed as an oncogene present in DNA isolated form a human stomach tumour (hst) and from a non-cancerous portion of human mucosa that had the ability to transform NIH 3T3 cells after transfection (Sakamoto et al , 1986, Yoshida et al , 1987, Taira et al , 1987) The predicted protein consists of 206 amino acids with approximately 43%, 38% and 40% sequence homology with bFGF, aFGF and *int*-2 respectively. It has a 55 amino acid N-terminal signal sequence and appears to be secreted. K-*fgf*, an oncogene derived from transfection of Kaposi's sarcoma DNA into NIH 3T3 cells is identical to *hst* (Delli Bovi et al , 1987) The protein, K-FGF, has been purified from conditioned medium from the transfected NIH 3T3 cells (Delli Bovi

et al , 1988) It is a mitogen for fibroblasts and endothelial cells. It has an affinity for heparin, and (like aFGF) heparin enhances its activity

FGF-5, an oncogene isolated by transfection of human bladder tumour DNA into NIH 3T3 cells, encodes for a 267 amino acid secreted heparin binding growth factor which exhibits 45% and 42% homologies with bFGF and aFGF, respectively. FGF-5 is expressed in neonatal brain and in some human tumour cell lines (Zhan et al , 1988, Haub et al., 1990) More recently the gene was found to be expressed in exponentially growing normal human fibroblasts In quiescent fibroblasts, expression of FGF-5 is strongly induced by serum and several growth factors (PDGF, EGF and TGF-α) (Werner et al , 1991).

FGF-6 was cloned by sequence similarities with the *hst* product (Marics et al , 1989) Although the proteins are 70% homologous, FGF-6 has been mapped to chromosome 12, while *hst* is located near the gene for *int*-2 on chromosome 11 Translation of the FGF-6 transcript from three alternate start sites yielded three mitogenic peptides with molecular weights of 21, 22 and 24 kDa It is suggested that these peptides could be glycosylated to give a single 25 kDa species (Coulier et al., 1991). The gene for FGF-6 has been recently cloned from a normal human genomic library and RNA blot analysis revealed its expression in human leukemia cell lines (Iida et al , 1992)

Keratinocyte growth factor (KGF/FGF-7) (Rubin et al , 1989), consists of a single polypeptide chain (28 kDa) that shares homology with the other members of the FGF family cDNA analysis suggests the present of a amino-terminal hydrophobic region which indicates secretion (Finch et al , 1989) KGF has been characterised as a epithelial cell specific mitogen which is secreted by normal stromal fibroblasts The mitogenic signal of KGF is mediated by specific receptors with tyrosine kinase activity (Bottaro et al , 1990) which have a weaker affinity for basic and acidic FGF

Two distinct classes of FGF receptors have been described (reviewed by Klagsbrun and Baird, 1991); high affinity receptors with tryosine kinase activity and lower affinity heparan sulphate proteoglycans. Four human genes have deen identified that encode distinct high affinity receptors (Houssain et al , 1990, Johnson et al , 1990; Keegan et al., 1991, Partanen et al , 1991) The receptor/ligand interaction model is complicated further by alternative mRNA splicing giving rise to multiple proteins from each gene. In general, the cell surface receptors are single chain glycosyated polypeptides with molecular weights ranging form 110 to 165 kDa (Neufield et al , 1985, Burgess and Macaig, 1989) On binding aFGF or bFGF, the receptors form dimers This leads to a conformational change in the cytoplasmic domain with either auto- or cross-phosphorylation occuring FGF interaction with heparan sulphate proteoglycans provides a mechanism for regulation of the growth of capillary blood vessels in normal and pathological situations, while providing protection from proteolytic degradation (Vlodavsky et al , 1991; Saksela et al , 1988) Prevention of bFGF binding to heparan sulphate results in reduction of binding to the high affinity surface receptors and subsequent signal transduction (Rapraeger et al , 1991) A 150 kDa heparan sulphate proteoglycan has recently been described as a high affinity receptor for aFGF (Sakaguchi et al , 1991)

1 1.5 MISCELLANEOUS GROWTH FACTORS

1.1.5.1 Non-FGF Heparin binding growth factors A new family of growth factors have recently been described which have a strong affinity for heparin, but are structurally unrelated to the FGF family. The midkine gene (MK), expressed temporarily during the early stages of retinoic acid induced differentiation of embryonal carcinoma cells as well as during the mid-gestation period of mouse embyrogenesis, encodes for a 14 kDa heparin-binding protein (Tomomura et al , 1990, Kadomatsu et al , 1988) It is also expressed in adult kidney (Kadomatsu et al , 1990) It's protein structure is highly conserved between the human and the mouse (Tsutsui et al , 1991)

The protein structure of MK has been found to be 50% homologous with heparin-binding growth associated molecule (HB-GAM)/ pleiotrophin, an 18 kDa protein produced by neonatal brain, osteoblasts and other cell types that promotes neurite extension of embryonic nerve cells and is mitogenic to fibroblasts (Li et al , 1990, Merenmies and Rauvala, 1990, Rauvala, 1989) MK and HB-GAM differ in a number of respects MK is a basic protein, while HB-GAM is neutral Also HB-GAM is mitogenic to both 3T3 and NRK cells (Li et al , 1990, Milner et al , 1989) while MK has no mitogenic effects on NRK cells but stimulates proliferation of 3T3 cells (Muramtsu et al , 1991).

The amino-terminal sequence of HB-GAM and MK was found to share 50% and 66% homology, respectively, with a 19 kDa heparin binding protein isolated from chicken embryos (retinoic acid induced heparin binding protein, RI-HB) (Vingy et al , 1989, Raulais et al , 1991) While RI-HB showed no mitogenic activity on bovine epithelial lens cells (Vigny et al , 1989), fibroblast 3T3 cells, human or bovine umbilical vein endothelial cells, it stimulated thymidine incorporation into PC12 cells and induced rapid neurite outgrowth (Raulais et al , 1991) It has yet to be determined if RI-HB and the MK protein are 2 distinct proteins or whether MK is the mammalian homologue to RI-HB

1 1 5.2 Platelet Derived Endothelial Cell Growth Factor. Platelet derived endothelial cell growth factor (PD-ECGF) (Miyazono et al , 1987), is composed of 2 subforms of 44kD and 46kD (Miyazono and Heldin, 1989) and has been shown to be susceptible to both heat and acid, but stable to reducing agents Unlike other endothelial mitogens, it does not bind heparin and it does not stimulate proliferation of fibroblasts (Miyazono et al , 1987). PD-ECGF stimulates endothelial cell growth and chemotaxis in vitro, and it exhibits angiogenic properties in vivo (Ishikawa et al , 1989)

1 1 5 3 Bombesin Bombesin, an amphibian tetracapeptide, and related peptides (for example gastrin-releasing peptide, GRP) stimulate fibroblasts and epithelial cells in culture Antibodies bind to the carboxyl terminal of bombesin, blocking its binding

to its receptor and inhibiting the clonal growth of small cell lung cancer cells in vitro (Cuttitta et al , 1985) This indicates the presence of an autocrine loop It has been reported, however, that this inhibition is not a property of all small cell lung cancer cells (Layton et al , 1988)

1.1.5.4 Melanoma Growth Stimulating Activity (MGSA): MGSA, originally isolated as an autocrine growth regulator of Hs294T human malignant melanoma cells (Richmond and Thomas, 1986, Lawson et al , 1987), has been found to be produced by about 70% of the primary cell cultures from human melanoma biopsies (Richmond et al , 1986) The MGSA protein is homologous with the *gro* gene product (Anisowicz et al , 1987) and it is related to connective-tissue-activating peptide III, a mitogenic polypeptide that is stored in platelets and released on thrombin-induced platelet aggregation (Castor et al , 1983) CTAP-III belongs to the ß-thromboglobulin super family which also includes platelet factor 4 (PF4), macrophage derived growth factor (MDGF), platelet basic protein (PBP), and interleukin 8 (among others). CTAP-III is derived from PBP, a biologically inactive precursor protein (Deuel et al , 1977, Holt et al , 1986) ß-thromboglobulin is formed following proteolytic cleavage of the amino terminal tetrapeptide of CTAP-III, which results in the loss of its biological activities (synthesis of DNA, hyaluronic acid, sulphated glycosaminoglycan chains) (Castor et al , 1983)

1.1.5 5 Hepatocyte Growth Factor (HGF) HGF was first isolated from rat platelets as a potent mitogen for mature hepatocytes (Nakamura et al , 1985) A 87 kDa form of HGF was subsequently isolated from human lung fibroblasts and found to stimulate proliferation in both melanocytes and endothelial cells, as well as epithelial cells (Rubin et al , 1991) HGF is derived from a single-chain precursor of 728 amino acid residues and proteolytically processed to form a heterodimer molecule composed of a 69 kD *α*-chain and a 34 kDa ß-chain, and although it exhibits no proteolytic activity, HGF shares structural homology with plasminogen (Matsumoto et al., 1991). A 145 kDa protein has been identifed as the receptor for HGF Immunoblot analysis revealed the this protein was the ß subunit of the c-*met* proto-oncogene

product, a membrane spanning tyrosine kinase (Bottaro et al , 1991). It was recently reported that human HGF and human scatter factor (SF) are identical (Weidner et al , 1991)

1 1 6 INTERLEUKIN FAMILY AND HAEMOPOIETIC GROWTH FACTORS

Interleukins mediate the interactions between immune and inflamatory cells, promoting cell growth, differentiation and function (reviewed in Mizel, 1989). At present 11 different interleukins have been described in molecular detail and their genes have been cloned The functions of a number of the members of interleukin family (IL-1, IL-3, IL-4, IL-5, IL-6) overlaps to a large extent with the haemopoietic growth factor family, erythropoitein (Epo), granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF) and macrophage colony stimulating factor (M-CSF) A summary of their alternative names and biochemical characteristics are presented in Appendices A and B Originally interleukins and haemopoietic growth factors were described in terms of their effects on the immune and blood systems growth factors, (reviewed by Nicola, 1989, Morstyn and Burgess, 1988) but it is now apparent that these factors are produced by a wide number of cell types and have a variety of effects on them. Here I will give a brief overview of the non-haematopoietic functions of some of these factors

IL-1, for example, has been isolated from fibroblasts, endothelial cells, keratinocytes and smooth muscle and induces the synthesis of prostaglandins and collagenases by fibroblasts (Oppenheim et al , 1986). Binding of IL-1 to its receptor in human foreskin fibroblasts induces increased levels of cAMP by activation of a pertussis toxin sensitive GTP binding protein (Zhang et al , 1988).

IL-6 has also been found in a variety of cells including fibroblasts, endothelial cells, keratincytes and a number of tumour types Except for tumour cells that produce IL-6 constitutively, normal cells do not produce IL-6 unless they have been stimulated The list of possible stimulants includes IL-1,

PDGF and cAMP (Billiau et al , 1989). IL-6 has also been implicated as a putative growth factor for renal cell carcinoma cells (Miki et al., 1989).

IL-2 receptors have been found on endothelial cells (Hicks et al., 1991) IL-4 has been shown to inhibit both colon and breast carcinoma cells in a paracrine manner (Toi et al , 1992)

Likewise GM-CSF has been implicated as having a non-hematopoietic function Recently, GM-CSF has been shown to be produced by human tracheal epithelial cells (Churchill et al , 1992) It has also been shown to enhance colony formation in nonhematopoietic ovarian cancer cells (Cimoli et al , 1991)

1 2 AUTOCRINE GROWTH REGULATION

The growth of malignant cells is generally been considered to be more autonomous than that of their normal counterparts (Holley et al , 1975) This observation was later developed into the autocrine hypothesis for cell growth (Todaro and deLarco, 1978), which postulates that malignant cells have the ability to produce polypeptide growth factors which can act on the producer cells via specific cell surface receptors resulting in a growth response Sporn and Todaro (1985) extended the hypothesis to normal cells and suggested that this mechanism may be implicated in normal processes e g during embyrogenesis and wound healing (Slack et al , 1989, Barbul et al , 1988) Thus, cells that synthesise a growth factor, display its receptor and respond to its activation have the potential for autocrine growth

The classic view of autocrine control suggests that cells secrete biologically active growth factors into the culture medium, they proliferate at relatively high cell densities in the absence of added factors and their growth is arrested by the presence of

antibodies which block the response to that growth factor (Browder et al , 1989) Such a loop, where the growth factor is secreted, is termed a "public" autocrine control loop

More recently this theory has been extended to account for growth factors that are not secreted and remain cell-associated In these "private" autocrine loops, proliferation of the cells is not density dependent, even in the absence of exogenous factors and neutralizing antibodies do not prevent growth This mechanism of growth control is particularly important in cases where the growth factor or receptor is synthesised at low concentrations

1 2 1 GROWTH FACTOR CONTROL OF EMBRYOGENESIS

Growth factors control the process of embryogenesis by their mitogenic, chemotactic and inductive properties

In the very early amphibian embryo there are only two tissue layers (the ectoderm and endoderm) A third layer, the mesoderm, is induced from the ectoderm *in vitro* by the action of bFGF (Slack et al., 1987) and possibly TGF-$ß_2$ (Kimelman et al., 1987) *In vivo*, bFGF has also been implicated in mesoderm formation (Gillespie et al , 1989)

Embyro research in mammalian systems concentrates mainly on in-situ hybridisation and immunocytochemistry on embryo sections, the culture of embryonal carcinoma (EC) cells, derived from germ cell tissues, or the culture of primary cells grown directly from pre-implantation embryos (in the presence of IL-9 to inhibit differentiation, (Smith et al , 1988) These studies again implicate TGFß and FGF in embryo development Normal chicken, mouse and human embryo fibroblasts secrete latent TGFß (Lawerence et al , 1984). High levels of TGF-ß were found in fetal rat skeletal muscle, liver and lung extracts (Hill et al , 1986) mRNA for TGFß has been detected in mouse embryo hematopoietic and liver cells (Wilcox et al , 1988). TGF-α may play a role in lung development (Catterton et al , 1979) In mouse embryos, the

int-2 gene is expressed at high levels in the extraembryonic (perietal) ectoderm when this is formed shortly after implantation (Wilkinson et al., 1987). IGF-II also plays a role in embryogenesis, being described as the fetal growth factor in rats (Van Wyk, 1984)

1 2 2 GROWTH FACTOR CONTROL OF WOUND HEALING

The process of wound healing can be divided into three main overlapping stages inflammation, formation of granulation tissue, matrix formation and remodeling (Ten Dijke and Iwata, 1989)

PDGF is one growth factor whose role in the area of wound healing has been well documentated PDGF is released, along with TGF-ß and FGF, from the α-granules of platelets at the site of tissue injury In addition to acting mitogenically, PDGF acts as a chemoattractant for fibroblasts and smooth muscle cells, neutrophils and monocytes (Senior et al , 1983, Deuel et al , 1982) The conversion of monocytes to macrophages is critical for the initiation of tissue repair They remove pathogenic organisms and tissue debris and release further quantities of PDGF and TGF-ß, augmenting their effect. TGF-ß has been reported to be three times more potent than PDGF as a chemoattractant for both monocytes and fibroblasts (Wahl et al., 1987, Postlethwaite et al , 1987) After injury, the macrophages are suppresssed by TGF-ß to protect healthy tissue being attacked by the secreted products of activated macrophages (Tsunawaki et al , 1988).

The second stage of wound healing is granulation This consists of closely packed fibroblasts, macrophages and neovasculature embedded in a matrix of collagen, fibronectin and hyaluronic acid TGF-ß, FGF and PDGF all play a part in the stimulation of

extracellular matrix (ECM) components (Ignotz and Massagué, 1986, Narayanan and Page, 1983, Gospodarowicz et al , 1986) FGF stimulates endothelial cells to produce u-PA, which is necessary for neovascularisation (Gospodarowicz et al , 1986). The FGF family are potent mitogens for endothelial cells and so promote angiogenesis (Folkman and Klagsbrun, 1987)

Within hours of injury, epithelial cells begin to proliferate and migrate from the free edges of the tissue across the wound. This process is mediated by the members of the EGF family which are chemotatic and mitogenic for epithelial cells (Blay and Brown, 1985) Once re-epithelialization is complete the epithelial cells revert to their non-migrating phenotype.

Finally the granulation tissue is broken down and replaced with connective tissue consisting of collagen and elastin fibers, promoted by TGF-ß Extracellular protease activity is controlled by a balance of TGF-ß and PDGF TGF-ß controls extracellular protease activity by inducing the synthesis of the endothelial protease inhibitor, plasminogen activator inhibitor, inhibiting synthesis and secretion of both u- and t-type plasminogen activators (uPA and tPA) and the thiol protease cathepesin L (Laiho et al , 1986, Chiang et al , 1986) PDGF, on the other hand, stimulates production of collagenases by fibroblasts (Bauer et al , 1985) This protease activity combined with fibroblast proliferation and collagen synthesis results in the formation of scar tissue (Kovacs, 1991) Recently, monclonal antibodies to TGF-ß injected into the margins of skin wounds in adult rats were found to reduce the resulting level of scarring (Shah et al , 1992)

1 2 3 GROWTH FACTOR CONTROL OF CANCER

Carcinogenesis is a multi-stage event Malignant growth may be the result of a combination of mutations in a number of the several gene products involved in the mitogenic pathway These mutations may be expressed in a number of ways (i) excessive production, expression or action of growth stimulators (ii) excessive production or activity of receptor molecules for these factors, or (iii) failure of the cells to synthesise or respond to specific

negative growth factors. The mutated genes have been designated oncogenes while their normal cellular counterparts are called proto-oncogenes Oncogenes have been found to encode for growth factor receptors, signal transducers and transcriptional factors (see table below).

TABLE 1.1 Partial list of Oncogenes (Drucker et al , 1990)

Category	Oncogene
Growth Factor	*sis, int-2*
Transmembrane receptor	*erb*B, *neu, fms, ros, kit*
Membrane Associated tryosine kinase	*abl, src* family, *fes, fps*
Membrane associated guanine nucleotide binding protein	K-, N-, and H-*ras*
Cytoplasmic serine-threonine kinase	*raf/mil, mos*
Cytoplasmic hormone receptor	*erb*A
Nuclear factors	*c-myc*, N-*myc*, L-*myc*, *fos, jun, myb, ets, ski*

A further class of oncogenes has been described that encode for negative growth regulators. These genes have been termed "tumour-suppressor" genes, "recessive oncogenes", "anti-oncogenes" and "growth suppressor" genes (Klein, 1987) Transformation can occur when these genes are mutated in such a way that their protein product is not expressed This results in the removal of normal constraints on the cells' proliferation thereby triggering uncontrolled neoplastic growth (reviewed by Marshall, 1991) This type of mutation has been linked to allele losses in a number of human cancers including retinoblastoma, osteosarcoma, Wilms' tumour and renal carcinomas

The mitogenic response occurs in two steps, each of which is mediated by specific growth factors (Aaronson, 1991). Quiescent cells must first be advanced from the resting or G_0 phase into the G_1 phase This is achieved by the action of "competence" factors such as PDGF (Pledger et al , 1978) Transition through the G_1 phase into the S phase is achieved by "progression" factors (for example, EGF, FGF, IGF-I and insulin) There is a critical period in G_1 where both the competence and progression factors need to be present for the cycle to continue (Leof et al , 1983) Transverse of S, G_2 and M was found to be independent of added growth factors (Wharton, 1983) Negative growth modulators (e g TGFß) can antagonize the proliferative effects of growth factors in G_1 (Moses et al , 1990)

The retinoblastoma protein, pRB, has the properties of a cell cycle regulatory factor. pRB is a 103 kDa nuclear protein that is underphosphorylated in the G_1 phase of the cell cycle and hyperphosphorylated in the S and G_2 phases (DeCaprio et al , 1989) It is hypothesised that the underphosphorylated form suppresses growth by restricting cell cycle progression at a specific point in G_1 (Ludlow et al , 1989, Goodrich et al , 1991) Laiho et al (1990) observed that TGF-ß1 treatment of mink lung epithelial cells is associated with a block of phosphorylation of pRB in the mid to late G_1 This result provides an important link between TGF-ß and the control of the cell cycle.

In the remaining part of this introduction, I will describe the main classes of growth factors and how each has been implicated in autocrine control loops.

1 2 4 AUTOCRINE CONTROL AND TGFs

TGF-α has been linked to the autocrine regulation of breast cancers (Lippman et al , 1987) All estrogen-dependent epithelial breast cancer cell lines secrete high levels of

TGF-α-like activity (Dickson et al , 1983) The EGF receptor has also been identified in breast cancer cells lines (Osborne et al , 1982, Fitzpatrick et al , 1984, Davidson et al., 1987), and primary breast tumours (Sainsbury et al , 1987) Ennis et al (1989) studied the effect of a monoclonal antibody against the EGF receptor in a range of EGF receptor-positive malignant and non-malignant transformed human breast cell lines They found that while some breast cell lines did not respond to exogenous EGF or whose growth was not inhibited by antibodies, MDA-468 human breast cancer cells and two transformed non-malignant breast cell lines appeared to exhibit TGF-α autocrine control of growth.

TGF-α is secreted into the conditioned medium of melanoma cells and TGF-α mRNA has been detected in melanoma cell extracts (Derynck et al , 1987, Marquardt et al., 1983, Imanishi et al., 1989) Ellem et al (1988) reported that TGF-α promoted the growth of both melanoma and melanocytes While TGF-α mRNA transcripts are generally found in melanoma cells, trace levels or no levels were detected in melanocytes (Albino et al , 1991), suggesting that mRNA for TGF-α may be used as a marker for melanomagenesis Some doubt was cast on the actual autocrine role for TGF-α/EGF in the melanoma system Kudlow et al (1984) found that a monoclonal antibody against the EGF receptor did not inhibit the cellular growth of a melanoma cell line, although this would not necessarily rule out the possibility of TGF-α being the factor involved (Winkler et al , 1989)

Recently, evidence was put forward indicating an involvement of TGF-α in the autocrine growth of ovarian cancer cells lines <u>in vitro</u> (Morshige et al., 1991, Stromberg et al , 1992), <u>in vivo</u> (Kurachi et al , 1991) and in primary human ovarian cancers <u>in vitro</u> (Morishige et al., 1991)

TGFα/EGF has been implicated in the normal autocrine growth of keratinocytes It has been shown that human keratinocytes plated at high densities into medium lacking TGF-α, EGF or FGF are capable of maintaining growth in the absence of exogeneously added growth factors (Shipely et al., 1989) Coffey et al (1987) reported that normal human keratinocyte cell lines both require TGF-α for proliferation and and express its mRNA. Also, TGF-α

enhanced TGF-α gene expression Furthermore the high density growth factor-independent growth of human keratinocytes was inhibited by treatment of these cultures with a monoclonal antibody which acts as a competitive antagonist of the human EGF receptor (Cook et al , 1991)

TGF-α autocrine control is apparently involved in the growth of a range of other normal and cancer cells Tracheobronchial epithelial cells (Jetten, 1991) secrete and respond to TGF-α Aberrant growth control in certain lung carcinoma or transformed cells has been linked at least in part to increased or constituitive expression of TGF-α (Damstrup et al , 1989) The growth of two human lung adenocarcinoma cell lines A549 and PC-9, which produce TGF-α, was blocked by neutralising TGF-α antibody (Imanishi et al , 1989) Mydlo et al (1989) detected mRNA for TGF-α in malignant kidney tissue specimens but not in their normal counterparts, which suggests a role for TGF-α in promoting transformation and/or proliferation of kidney neoplasms by an autocrine mechanism

LIke TGF-α, TGF-ß has been implicated in the autocrine growth of keratinocytes (Bascom et al , 1989) It reversibly arrested keratinocytes in the G_1 phase of the cell cycle, but did not effect entry into a differentiation pathway (Coffey et al , 1988, Shipley et al , 1986). These keratinocytes have been found to possess specific receptor sites for TGF-ß and are known to produce TGF-ß in their conditioned media, indicating the presence of a growth inhibitory autocrine loop for these cells

Immunohistochemical studies using anti-TGF-ß antibodies have detected antigen expression among malignant but not benign glioma cells (Samuels et al , 1989) While TGF-ß mRNA has not been detected in normal brain tissues, mRNA for TGF-ß1 and -ß2 have been found in glioblastoma cultures (Bodmer et al., 1989) TGF-ß receptor types I, II and III have been detected in glioma cells (Jennings et al , 1991) The response to TGF-ß varied depending on the cell type Near-diploid gliomas were growth inhibited by TGF-ß, while hyperdiploid glioblastomas showed a positive mitogenic response Under serum free conditions, anti-TGF-ß

antibody neutralised each of these effects, demonstrating the basic elements in support of an autocrine hypothesis (Jennings et al., 1991)

1.2.5 AUTOCRINE GROWTH AND PDGF

Autocrine control by PDGF has recently been reviewed (Browder et al , 1989, Westermark and Heldin, 1991) The basis of most of the work on PDGF autocrine action is a result of research into v-*sis*, an oncogene derived from the transduction of the PDGF-B gene by the simian sarcoma virus (SSV) Apart from the lack of the enogenous signal peptide, the predicted sequence of the v-*sis* product is virtually identical to PDGF B chain (Johnsson et al., 1984) PDGF-BB exerts the same transforming activity as SSV (Westermark and Heldin, 1991), SSV-transformed cells produce a growth factor that binds PDGF receptors and is recognised by antibodies to PDGF (Owen et al , 1984) and only PDGF receptor positive cells can be transformed by SSV (Leal et al , 1985) Thus the autocrine role played by the v-*sis* gene in transformation has been established The mechanism of action of PDGF is however not as clear cut.

Unlike cultured vascular endothelial cells, which do not not express specific PDGF receptors, smooth muscle cells (SMC) bind and respond to PDGF Production of PDGF by smooth muscle cells appears to be developmentally regulated (Seifert et al , 1984, Ross et al , 1986, Hahn et al., 1991) The hypothesis that developmentally regulated production of PDGF by SMC contributes toward autocrine stimulation of SMC proliferation has been suggested but not clearly established PDGF autocrine control loops have been suggested for a number of other systems, although no definite proof has been shown These systems include the adrenal carcinoma cell line, SW-13 (O'Donnell et al , 1989) and endothelial cell hyperplasia in human glioblastoma (Hermansson et al , 1988)

Although human glioma cells have been shown to express mRNA for PDGF, to synthesise all three PDGF combinations, and to express cell surface receptors (Hammacher et al , 1988), neutralising antibodies do not to block its response Neither has protamine, a

polyanionic compound that competitively inhibits extracellular binding of PDGF to its receptor, been shown to have an effect on autocrine growth or transformation (Huang and Huang, 1988). This contradicts earlier findings that suramin, another binding competitor, could inhibit v-*sis* mediated transformation (Hannick and Donoghue, 1988). It was subsequently shown that suramin can penetrate the cells secretory compartments and so would not necessarily block a cell surface interaction (Huang and Huang, 1988)

This inability to block the autocrine response at a cell surface level indicates the presence of a "private" autocrine loop This theory has been corroberated by Munro and Pelham (1987) who discovered a carboxyl sequence common to several proteins which are retained in the endoplasmic reticulum. When this sequence (Lys-Asp-Glu-Leu, or KDEL) was added to the carboxyl terminus of the v-sis protein, the modified protein remains fully transforming, although it is not secreted and cannot be detected on the cell surface (Browder et al , 1989) Keating and Williams (1988) showed that autocrine activation of PDGF receptors in v-*sis*-transformed cells occured in intracellular compartments

Other data, however conflicts with the intracellular activation hypothesis. Binding of PDGF to its cell surface receptor results in tyrosine kinase activation and an increase in c-*fos* expression The intracellular activation of tyrosine kinase, however, is not coupled to c-*fos* expression, and so may be considered futile (Hannick and Donoghue, 1988) A intermediate model of transformation by PDGF is put forward by Westermark and Heldin (1991) They suggest that following formation of the intracellular receptor-ligand complex, translocation to the cell surface is a prerequiste for interaction with the proper substrates for the receptor kinase

1 2 6 AUTOCRINE CONTROL AND IGF

Insulin, IGF-I and IGF-II have all been implicated in autocrine mechanisms A teratoma-derived cell line, 1246-3A, produces a

protein very similar to insulin (Yamada et al , 1988) The growth of this cell line could be inhibited by antibodies to insulin, indicating an autocrine contol mechanism.

IGF-I has been shown to be involved in the autocrine growth of a range of human cancer cell lines including lung carcinoma, osteosarcoma, breast carcinoma and ovarian cancer cell lines

CALU-6 (human lung carcinoma) produces IGF I and its growth is inhibited by antibodies to IGF-I (Minuto et al , 1988) In a similar way, human pancreatic cells and an ovarian cancer cell line produce IGF I, are stimulated by exogenous IGF-I and their growth can be blocked by antibodies to the IGF-I receptor (Ohumura et al , 1990, Yee et al , 1991) Recently, IGF-I has been reported as an autocrine regulator of human colon cancer cell differentiation and growth (Baghdiguian et al , 1992)

Differentiation of muscle cells to form postmitotic myotubes has been shown to be stimulated in an autocrine way by IGF-II (Florini et al , 1986) The rate of spontaneous differentiation of sublines of myogenic cells correlated with their level of expression of IGF-II Cells that endogenously produced high levels of IGF-II were not affected by exogenously added factors An antisense oligodeoxyribonucleotide complementary to the first five codons of IGF-II inhibits myogenic differentiation in the absence, but not in the presence, of exogenous IGF-II

An autocrine role for both IGF-I and II has been described in breast cancers CM from the cell line MCF-7 was found to contain high levels of immunoreactive IGF-I, the cells expressed mRNA for IGF I and exogenous IGF I could stimulate MCF-7 proliferation (Huff et al , 1986). Also addition of a monoclonal antibody against IGF-I in serum free medium caused an inhibition suggesting that endogenous IGF-I does play an autocrine role in MCF-7 growth (Freed and Herington, 1989) A number of breast cell lines have also been demonstrated to be stimulated by IGF-II (Karey et al , 1988, Myal et al., 1984) Yee et al , (1988) reported that out of 9 human breast cancer cell lines, one expressed mRNA for IGF-II αIR_3, a monoclonal antibody to the IGF-I receptor inhibited the mitogenic effects in two human breast cancer cell

lines, MCF-7L and MDA-231, suggesting that this receptor mediates the growth effects of IGF-II in these cell lines (Kent Osborne et al , 1989)

The interpretation of the results from the use of the IGF-I receptor blocking antibody experiments with the α-IR_3 monoclonal antibody have been questioned. Although this antibody can block IGF-I binding to the type I IGF receptor, Steele-Perkins et al (1988) showed that the αIR3 is not physiologically inert and binding to the receptor results in receptor phosphorylation

1 2 7 AUTOCRINE CONTROL AND HEPARIN-BINDING GROWTH FACTORS

Acidic and basic FGF were originally purified from brain and pituitary tissue, and have subsequently been found in a variety of normal (Ferrara et al , 1987, Moscatelli et al , 1986, Winkles et al , 1987) and transformed (Moscatelli et al., 1986, Klagsburn et al , 1986) cells Several of the malignant producer cell types also respond to FGF e g glioma (Morrison et al , 1990) and melanoma (Rodeck et al , 1991)

More recently, bFGF has been associated with the growth control of epithelial cells New and Yeoman (1992) reported the stimulatory effects of bFGF on human colon tumour cell lines which express receptors for bFGF. Immunoprecipitation studies revealed levels of bFGF in both conditioned media and cell lysates in each of the cell lines examined These results imply the existence of an autocrine mechanism

The expression of bFGF may be particularly important for the growth and proliferation of human gliomas Gliomas develop by invasion and local spread rather than by metastasis and so, may be influenced by factors which stimulate the growth invasion and vascularization of the primary tumour in the cranium (Morrison et al , 1990) Human glioma cell lines have been shown to express the gene encoding aFGF (Libermann et al , 1987) and express and respond to bFGF (Morrison et al , 1990) Morrision et al suggest that bFGF may be involved in the autocrine control of glioma cells by its mitogenic effects following release from lysing necrotic cells as the tumour progresses.

Studies by Moscatelli et al. (1986) demonstrated that bFGF protein could be detected in a human melanoma cell line extract bFGF mRNA is expressed in metastatic melanoma cells but not in normal melanocytes (Halaban et al , 1988). Halaban et al also showed that the mitogenic effects for normal melanocytes caused by bFGF-like activity in melanoma cell extracts could be blocked by 60% by intracellular injection of anti-FGF antibodies Antisense oligodeoxynucleotides directed to a region around the AUG codon and to different sites on the bFGF mRNA added to culture medium of primary and metastatic melanoma cells resulted in inhibition of 70-90% of proliferation (Becker et al , 1989) These results provide strong evidence that bFGF is an autocrine growth factor for melanoma cells

Conventional public autocrine loops require that the growth factor is secreted from the cell to interact with the cell surface receptor No evidence of secretion of either bFGF or aFGF has been reported Neither aFGF nor bFGF has a classic signal peptide (Abraham et al. 1986) Also addition of blocking antibodies to the culture medium has no effect on growth, but injection of the antibodies into the cells blocked growth (Halaban et al , 1988). This would suggest that bFGF acts intracellularly in the melanoma system and thus bFGF, like PDGF, is a candidate for the private autocrine loop theory.

In contrast, addition of neutralising antibodies to the culture medium of BALB/c3T3 fibroblasts inhibited proliferation (Saseda et al , 1988) suggesting a public autocrine loop for this system This extracellular autocrine loop is also supported by the fact that transfection of bFGF expression vectors does not result in transformation of NIH-3T3 cells unless the gene for bFGF was linked to an immunoglobulin signal sequence (Yayon et al., 1990) Also significant amounts of bFGF are found extracellularly associated with the heparan sulfate proteoglycans of the extracellular matrix in vitro (Moscatelli et al., 1987; Vlodavski et al., 1987) and are present in basement membranes in vivo (Folkman et al , 1988, DiMario et al , 1989)

Since no defined mechanism for bFGF release has been described, cell death or leakage following cell injury have been proposed as the mechanism for bFGF externalization (Schweigerer et al , 1987, Gajdusek and Carbon, 1989). Although it has also been reported that limited diffusion of bFGF across basement membrane and reconstituted endothelium occurs in the presence of heparin and high concentrations of bFGF (Dabin and Courtois, 1991)

The migration effects of bFGF on isolated single viable cells were examined by Mignatti et al (1992) From their studies they concluded that bFGF may be released from cells by a mechanism of exocytosis that does not involve the endoplasmic reticulum-Golgi pathway

The other members of the FGF family (*int*-2, *hst*/K-*fgf* and FGF-5) are rarely found in normal adult tissues. Instead they appear to be mostly expressed during embryogenesis and in tumours, i e during periods of intense growth Expression of *int*-2 has been reported to be amplifed in melanoma, breast cancer and squamous cell carcinoma of the head and neck (Adelaide et al , 1988, Zhou et al , 1988), which indicates that autocrine stimulation is possible

1 2 8 AUTOCRINE CONTROL AND BOMBESIN/GRP

Gastrin releasing peptide (GRP) is the mammalian homologue of the amphibian neuropeptide, bombesin Nearly all small cell lung carcinomas (SCLC) require GRP to maintain cell growth (Moody et al , 1981) They secrete GRP and express its specific tyrosine kinase receptors (Cuttitta et al , 1985, Gaudino et al , 1988) Neutralising antibody blocks colony formation in semisolid culture and delays tumour growth in nude mice injected with these cells (Cuttitta et al , 1985) These results indicate autocrine growth control for most SCLC Layton et al (1988) found that the inhibitory effect of bombesin antagonists on SCLC cells was not

mediated via the bomesin receptor and that not all SCLC cells were dependent on bombesin *in vitro* So, the autocrine theory does not appear to be the complete story for the SCLC system

1 2 9 AUTOCRINE CONTROL AND THE INTERLEUKINS AND HAEMATOPOIETIC GROWTH FACTORS

Interleukin 6 is a cytokine that can act on a variety of cell types including lymphocytes, hepatocytes, haematopoietic stem cells and nerve cells by either acting as a growth inhibitor or an inducer of differentiation (Kishimoto and Hirano, 1988) IL-6 has also been linked to a number of different malignancies for example plasmacytoma and myeloma (Van Damme et al., 1987, Kawano et al , 1988)

IL-6 has been implicated in the autocrine growth of renal carcinomas (Miki et al , 1989) IL-6 is a growth factor for human renal carcinoma cells *in vitro* Freshly isolated renal cell carcinomas expressed IL-6 mRNA and secreted biologically active IL-6 in the presence of FCS The IL-6 activity was neutralised by anti-IL-6 antibodies The fact that no IL-6 was detected when the cells were grown in serum-free conditions, indicates that the IL-6 autocrine mechanism is induced by serum factors

IL-6 is a major myeloma cell growth factor (Kawano et al , 1988), but whether the action is autocrine or paracrine is unclear (Klien et al , 1989) mRNA transcripts have been widely detected in the bone marrow (Portier et al , 1991) but only recently the transcripts have been detected in myeloma cells (Schwab et al , 1991) Interferon-α (INF-α) has been shown to stimulate the proliferation of myeloma cells in vitro (Ludwig et al , 1983), by inducing an autocrine production of IL-6 which could be blocked by a monoclonal antibody to IL-6 (Jourdan et al , 1991), supporting the autocrine theory.

Acute myeloid leukemia (AML) cells, in general, secrete large amounts of IL-1 in vitro Proliferation of cells from AML patients was enhanced with human recombinant IL-1α and IL-1ß and the spontaneous proliferation of these cells could be modulated by anti-IL-1 antibodies. (Cozzolino et al., 1989) These results suggest that IL-1 may act as an autocrine growth factor in AMLs

Autocrine growth factor production has been suggested as one of the mechanisms responsible for the unregulated growth of haematopoietic cells (Browder et al., 1989) When M-CSF cDNA and *c-fms* (M-CSF receptor) were co-transfected into NIH 3T3 cells, an external autocrine loop was established which was transforming and could be blocked by neutralising antibodies (Retenmier et al , 1987) Macrophage-colony stimulating factor (M-CSF or CSF-1) has been implicated in the autocrine proliferation of murine monocytes by the inhibition of growth due to the presence of either anti-sense oligonucleotides or neutralising antibodies (Birchenall-Roberts et al , 1990)

Autocrine synthesis of growth factors also seems to occur among immortalised cells of haemopoietic origin. Thus some T-lymphoma lines both secrete and respond to IL-2 and the introduction of the v-*src* oncogene stimulates autocrine production of a myeloid growth factor in chicken myeloid cell lines (Heldin et al , 1987)

1 3 BACKGROUND AND AIMS OF THE WORK DESCRIBED IN THIS THESIS

In the course of research into improving conditions for the growth of cells in vitro, McManus and Clynes (1984) observed that the growth of the human carcinoma cell line, RPMI 2650, in soft agar was directly proportional to its plating concentration down to a minimum critical cell density, below which no colony formation occured This suggested that the cells were producing an autostimulatory factor Using feeder layers of RPMI 2650 cells, they showed that this autostimulatory factor was diffusible and did not require cell-cell contact

The RPMI 2650 cell line is particularly interesting since it is an established cell line, derived from malignant cells, with near normal karyotype (Moorehead, 1965) Prolonged culturing of cells often results in chromosomal abberations, so that the cells may not be representative of the original tumour population After two years of continuous culture, RPMI 2650 cells exhibited a quasi-diploid karyotype, which is quite unique RPMI 2650 cells are also available at early passage, so that the cell stocks are closer to the original tumour sample, than is the case with many of the more widely studied human tumour cell lines (e g. HeLa)

Dooley (1987) subsequently developed a soft agar assay for the autocrine activity and determined by bioassay and radioreceptor work that the cells produced TGF-α and TGF-ß into their conditioned medium, along with an uncharacterised factor

The purpose of the work described in this thesis was to further characterise this autostimulatory effect, and to establish if it was an effect caused by a previously described autocrine growth factor or whether it exhibited properties unlike any of those assigned to other growth factors

2 0 MATERIALS AND METHODS

2.1 MEDIA PREPARATION

2 1 1 Water

Highly purified water is critical for the successful culture of animal cells. Water for media and reagent preparation was extensively pretreated using either a Millipore Milli-Q ultrapure water system or a ELGA UMP system Water firstly passes through a reverse osmosis system (Milli-Q) or was double distilled (ELGA) and passed through two pre-filters to remove ionic and non-ionic solutes It was then passed through two ion-exchange filters, a carbon filter and a 0 22μm cellulose acetate filter. The quality of the resulting water was monitored by an on-line conductivity meter and was regarded to be 'ultrapure' when its resistance was between 10-18 megaOhms/cm.

2 1 2 Glassware

All items of glassware (media bottles, roller bottles, spinner flasks, glass universals, etc) that came in contact with cells or media were stringently prewashed and rinsed Glassware was first steeped for about 2 hours in RBS (R Borghraef), a non-toxic detergent, before they were scrubbed manually with bottle brushes. Each item was then rinsed well in tap water followed by three separate rinses in distilled water Finally, they were rinsed in ultrapure water Metal bottle caps were washed and rinsed separately.

2 1.3 Siliconisation

Spinner flasks and bioreactor vessels require siliconisation to prevent cell attachment and growth on the glass sufaces. This involves rinsing clean dry vessels in a fume cupboard with a 2% solution of trimethyldichlorsilane in 1,1,1-trichloroethane (BDH, Cat No. 33164) using a glass pippette The entire inner surface of the vessels must be coated Excess reagent was then poured off and the vessels were left at 37°C for 2 hours or until dry Each item was then rinsed extensively as in Section 2 1 2 (3x distilled, 1x ultrapure water)

2 1 4 Sterilisation

All glassware, water for media preparation, some reagents, and non-sterile plastics were autoclaved before use (121°C/15 psi/20 minutes). Unstable reagents were filter sterilised through sterile disposible 0 22 μm filters (Millex-GV, SLGV025BS). Large batches of culture medium (5L) were peristaltically pumped through a Bell filter (Gelman, G.14238) into sterile 500ml glass bottles.

2 1 5 Growth Media

Liquid media was prepared from 10x stock solutions and diluted with ultrapure water. Either individual bottles (500 mls) or large batches (5 litres) were prepared MEM and DME media were buffered with sodium bicarbonate (Riedel-de-Haen, Cat No 31437) and HEPES (4-(2-HydroxyEthyl)-PiperazineEthaneSulfonic acid, Sigma, Cat No. H9136) at the volumes indicated in Table 2.1 MEMS for use in pH controlled bioreactor cultures was not supplemented with HEPES The pH was adjusted with 1 M NaOH (BDH, Cat No 30167) to pH 7 4-7.5 MEM and MEMS were further supplemented with non-essential amino acids (NEAA, Gibco, Cat No 043-114OH)

Powdered HAMS F12 media (Gibco 072-1700) was reconsituted in 95% total volume with ultrapure water sodium bicarbonate (1 176g/L) was added, and the pH adjusted to 7 2-7 3. The total volume was then brought up to 5 litres with ultrapure water

TABLE 2 1 Preparation of Growth media

Supplier Cat. No	MEM Gibco 042-01430	DME Gibco 042-02501	MEMS Gibco 042-01650
Stock	500 mls	500 mls	500 mls
Ultrapure H_2O	4500 mls	4500 mls	4500 mls
HEPES (1M)	100 mls	100 mls	-
$NaHCO_3$ (7 5%)	45 mls	45 mls	45 mls
NEAA (100x)	100 mls	-	100 mls
	adjust to pH 7.4-7 5 with 1 M NaOH/1 M HCl		

Media were stored at 4°C for up to three weeks, after sterility check (Section 2 1.7).

L-Glutamine (2 mM) (Gibco, Cat No 043-05030) was added to each media type before use (5 mls/100 mls)

Antibiotics were not used during routine cell culture MEMS was supplemented with 1% penicillin/streptomycin (Gibco 043-05140) for bioreactor cultures

2.1 6 Serum

All media were supplemented with 5% of either foetal calf serum (FCS) for monolayer cultures or donor horse serum (DHS) for suspension cultures The sera tested and their sources used are listed in Table 2 2

TABLE 2 2 Serum batches used in the course of this thesis

Batch No	Source		Use
FCS 22	Sera Labs	701051	General culture
FCS 48	Gibco	10F67915	General culture
DHS 32	Flow	29-211-54	Suspension culture
DHS 87	Flow	91-209-52	Suspension culture
DHS 62	Gibco	012-0762	Suspension culture
FCS 8	Flow	028020	Soft agar assays
FCS 15	Sera Labs	801017	Soft agar assays
FCS 46	M&V	4303R	Soft agar assays
Table 3 1 3			
FCS 46 1	M&V	4303R	Soft agar assay
FCS 67 2	Sera Labs.	901022	Soft agar assay
FCS 68 3	Sera Labs	901027	Soft agar assay
FCS 69 4	Sera Labs	901094	Soft agar assay
FCS 70 5	Gibco	20G77835	Soft agar assay
NC 71 6	Gibco	10F5380	Soft agar assay
DHS 72 7	Gibco	10G3074	Soft agar assay
FCS 73 8	Northumbria	33924	Soft agar assay
FCS 74 9	Northumbria	30925	Soft agar assay

2.1.7 Sterility

Each bottle of medium was sterility checked prior to addition of L-glutamine and serum, by incubating a sample (5-10 mls) at 37°C for at least three days Blood agar (Medlabs) plates, thioglycollate broths (Oxoid, CM173) and Sabourand broth (Oxoid, CM143) were inoculated with medium and incubated for three days to one week to detect microbial contaminants

TABLE 2.3 Cell lines used in this assay

Cell line	Source	Details
RPMI 2650	ATCC CRL 1629	Human squamous epithelial cell carcinoma of the nasal septum
NRK	Dr Ian Pragnell, Beatson Institute, Glasgow	Normal Rat Kidney fibroblasts
NRK-49F	ATCC CRL 1570	Clone of NRK
HEP-2	ATCC CCL 23	Human squamous cell carcinoma of the larynx
MSV-3T3	ATCC CRL 1568	Moloney MSV transformed mouse
SCC-9	ATCC CRL 1629	Human squamous cell carcinoma of the tongue
BSC-1	ATCC CCL 26	African Green monkey kidney

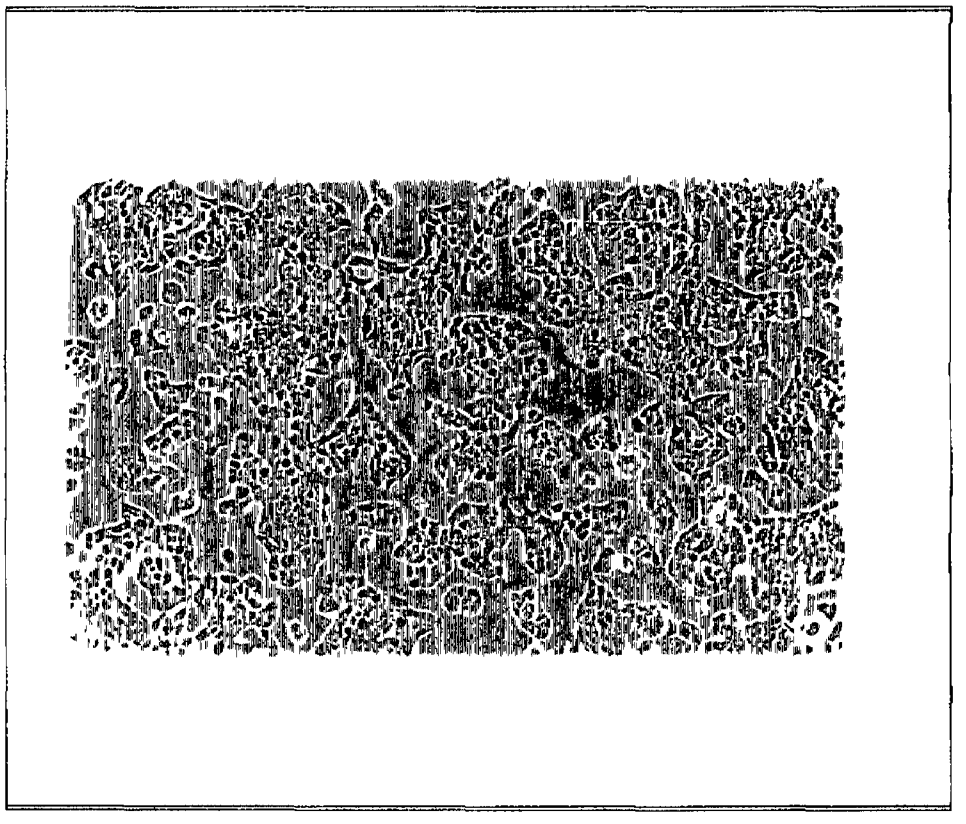


PLATE 1 RPMI 2650 sub-confluent culture grown in monolayer (Phase 1 (150X) magnification

2.3 Maintanence of cell lines in culture

2.3.1 Cell culturing

All the cell lines listed in Table 2 3 are anchorage dependent cell lines which were rountinely grown in 25cm² and 75 cm² tissue culture flasks (Cell Cult, Cat No 32025, Costar, Cat No. 307S) in the appropriate media (indicated in Table 2 4).

TABLE 2.4 Media used to culture cell lines (ATCC*=DME Ham's F12 (50 50))

Cell line	Medium
RPMI 2650	MEM
NRK	DME
NRK-49F	DME
HEP-2	ATCC*
MSV-3T3	DME
SCC-9	ATCC
BSC-1	MEM

2.3.2 Cell feeding

As the cells grow, the culture medium becomes more acidic The phenol red in medium changes from red to orange, indicating that the pH has dropped to below pH 7 2, which can be detrimental to the growth of the cells At this stage the medium is replaced with prewarmed fresh medium supplemented with L-glutamine and serum This also ensures an adequate nutrient supply to the cells

2.3.3 Trypsinisation

When the monolayer culture becomes confluent, usually no further growth can occur as the cells become contact inhibited Other cell lines (e.g Hep 2) form multilayer cultures Before the cells reach confluency, they are subcultured (or passaged)

This was achieved by removing spent medium to a waste bottle The cells were then rinsed in a small quantity of phosphate buffered saline (PBS-A, Oxoid BR12a) to remove trace serum The cells were enzymatically removed from the flask surface by incubating with prewarmed 0 25% trypsin (Gibco, Cat No 043-05090) in PBS-A at 37°C for 5-10 minutes or until the cells detach As soon as a single cell suspension is obtained (probably will require shaking), serum supplemented medium was added Serum contains trypsin inhibitor The cell suspension was then transferred to a sterile universal (Sterlin 128A) and centrifuged at 1,000 rpm for 4 minutes The supernatant was poured into a waste bottle and the cell pellet resuspended in a small volume of medium (about 5 mls) The cells were counted (see Section 2 3 3) and flasks reseeded at the required concentration The volume of the flasks was then brought up to 10

mls (25 cm^2 flask) or 30 mls (75 cm^2 flask) with medium supplemented with serum The cells were incubated at 37°C until they required feeding

2.3 4 Cell Counting

A sample of single cell suspension (1 ml) was mixed well with 200μl of trypan blue (Gibco 043-05250) for 5 minutes. The cells were counted by loading a sample on a Waber haemocytometer (Improved Neubawer) slide by Pasteur pipette. Each of the four large squares at each corner of the grid was counted and an average count obtained By multiplying the count by 1 2 x 10^4, the average cell count per ml of cell suspension was calculated Cells that take up the blue dye were counted as non-viable, while non-stained cells were viable.

2 4 LONG TERM STORAGE OF CELLS

2.4 1 Cell Freezing

Cell stocks were stored, frozen in liquid nitrogen Cells to be frozen must be free from contaminants and be in good condition (growing exponentially) Ideally cells should be frozen at high concentrations (greater than 4 x 10^6 cells/ml)

A single cell suspension was obtained and counted as described previously A solution of 10% DMSO was prepared in 25% FCS and added dropwise to an equal volume of cell suspension. The suspension was then transfered to a cryovial (Greiner, Cat No 122278 or Sterlin, Cat No 35001). After labelling, the vials were left in the vapour phase of the liquid nitrogen container for three hours and then immersed in the liquid phase for long term storage Adequate care should be taken while working with liquid nitrogen.

One vial of frozen stock was thawed shortly after freezing, to ensure the cells remained viable and contamination-free, during the freezing process

2.4.2 Cell Thawing

Vials removed from liquid nitrogen should be thawed quickly at 37°C, once the nitrogen has evaporated off, and added to 5 mls of cold growth medium containing 10% FCS. The cell suspension was then centrifuged at 1,000 rpm/4 minutes and the pellet resuspended in 5 mls of medium plus 10% FCS The cell suspension was added to a

fresh 25cm² flask, and incubated at 37°C The following day, this medium was removed and replaced with 10 mls of medium supplemented with 5% FCS.

2 5 CONTAMINATION

2 5.1 Microbial

Microbial contamination is easily detected in cell cultures. Gross contamination is often associated with cell detachment and acid production. Lower levels of contamination may be detected by careful microscopic examination

Most cases of microbial contamination may be prevented by proper asceptic technique in a class II vertical laminar air flow cabinet All reagents and media should be sterility checked before use (Section 2 1 7)

2 5 2 Mycoplasma

Mycoplasma contamination is not always obvious. Infected cells continue to grow but they appear granular and 'unhealthy'. During the course of this project the cells were rountinely checked for the presence of mycoplasma while they were cultured and before freezing If a culture was found to be contaminated, it was discarded immediately and fresh stocks thawed. The laminar flow cabinet was thoroughly cleaned and media, trypsin, etc that was used in the laminar flow with the infected cells was not re-used

The procedure used to detect mycoplasma was a fluorescent DNA stain Mycoplasma-free stocks of NRK cells were grown overnight at 5% CO_2 on sterile coverslips in 1 ml of medium at a concentration of 5 x 10^2 cells/ml The following day, 1 ml of medium from a flask of cells that were known to be grown in antibiotic-free medium was added (in duplicate). Control plates with added fresh medium were included The cultures were incubated for 3-4 days at 37°C/5% CO_2

The slides were then rinsed twice in PBS followed by a single rinse in a 1 1 dilution of Carnoy's reagent (Acetic acid Methanol, 1·3) in PBS The cells were fixed in Carnoy's reagent for 10 minutes The coverslips were removed and rinsed in ultrapure water Hoeschst 33258 (Sigma Cat No. B2883) at 0 5 mg/ml in PBS was added to the cells (2 mls) and left for 10 minutes at room temperature Because Hoescht is light sensitive, these manipulations were carried out in petri dishes covered in tinfoil.

Excess stain was removed by rinsing in ultrapure water and the slides were then mounted in glycerol The cells were observed under oil immersion using 405 nm light Extranuclear fluorescence indicated the presence of mycoplasma contamination

2.5.3 Cell line

If proper procedures are adhered to, cell line cross contamination should not be a problem The precautions taken against this type of contamination included

* Working with only one cell line in the laminar flow cabinet at any one time Allowing at least 15 minutes between cell lines Swabbing all surfaces well with 70% IMS (Lennox, Cat No 1170) before and after use

* Using separate media bottles, sera, trypsin, L-glutamine and waste bottles for each cell line, labelling them clearly

* Ensuring all flasks and cryovials are clearly labelled and documented

Cross contamination of cell lines was monitored occasionally by DNA fingerprinting (ICI, UK)

2 6 COLLECTION OF CONDITIONED MEDIA (CM)

2.6.1 Roller bottle culture

Bellco glass roller bottles (670 cm^2 internal surface area) were washed well and autoclaved damp at 120°C/20 minutes with the lids slightly open and covered in tin foil Before inoculation, the bottles were equilibrated at 37°C to prevent clumping 100 mls of medium plus 5% FCS and 1% penicillin/streptomycin (p/s) was added and the bottle was seeded with a single cell suspension of cells. The seeding concentration depends on the cell line RPMI 2650 roller bottle cultures were inoculated from one confluent 75cm^2 flask. The bottle was then transferred to a Bellco roller bottle apparatus at an initial speed of 0 25 rpm for 24 hours and then increased to 0.75 rpm

The cells were grown for one week with feeding on day 3 at 37°C On day 7 medium was removed and the cells were rinsed twice with approximately 10 mls of PBS or medium without serum and then 50 mls of serum-free medium was added to the cells and incubated overnight This medium was discarded and replaced with 100 mls of fresh medium without serum, which was allowed to be conditioned by the cells for twenty four hours This conditioned medium was designated CM2. This medium was collected and replaced with another 100 mls of serum-free medium. The CM is stored at 4°C. This procedure was continued for 6 days resulting in the collection of 6 batches of CM (CM2-CM7) which were normally pooled, before processing

2 6 2 Microcarrier culture

The growth of RPMI 2650 on a number of different types of microcarrier beads were studied in 250 ml and 500 ml spinner flasks (Techne, Cambridge, U.K)

Whatman	Plastic Biospheres	8302-2001
	Collagen coated Biospheres	8402-2001
	Glass coated Biospheres	8502-2001
Pharmacia	Cytodex 1	II-32125
	Cytodex 2	KF-35315
	Cytodex 3	HL-24905

The Pharmacia beads are composed of dextran and require hydration before use. For each 100 mls culture, 0 3 g of microcarrier bead was hydrated in 30 mls of PBS for 3 hours in a siliconised glass univeral (Section 2.1.3) This PBS was removed and replaced with fresh PBS before autoclaving. Whatman beads (0 2g/100 mls) were also autoclaved in siliconised glass universals. When sterile the PBS was removed and 30 mls of pre-warmed growth medium with 5% serum and 1% p/s was added The bead slurry was added to a sterile siliconised spinner flask The universal was rinsed out with 20 mls of growth medium, which was also added to the spinner flask (total volume, 50 mls) The flask was inoculated with an appropriate cell suspension (in approximately 10 mls) The spinner flask was then placed on a stirrer base with intermittent stirring at 37°C (30 seconds at 30 rpm every 2 minutes) for 3 hours This procedure allows even cell attachment The total volume of the culture was brought up to 100 mls with growth medium and incubated with continous stirring (30 rpm) for 6 days

Conditioned medium was collected from microcarrier cultures by allowing the beads to settle and removing bead-free spent medium Because of the density of the beads they settle quite well and almost 90 mls of spent medium may be removed. The beads were then rinsed three time with 20 mls of PBS, allowing the beads to settle before removal The culture was then set up with 100 mls of medium without serum The cells conditioned this medium for three days after which time it was removed and stored at 4°C until further processing

2.6.2 1 Sampling and Counting

Samples (3 mls) were taken from the spinner flasks after agitation 2 mls were placed on a petri dish for microscopic examination, while the remaining ml was placed in an eppendorf As soon as the beads settled (2-3 minutes) a bead-free sample was removed (200 μl) and counted This count represented the number of free cells, not attached to the beads

The number of attached cells was estimated by the method of Sanford et al (1951) All the bead-free medium was removed carefully and the bead pellet was resuspended in 500 μl of of 0 1M citric acid containing 0.1% (w/v) crystal violet. The eppendorfs are mixed well (by Whirlimixer) and incubated for 1 hour at 37°C. This caused the cells to burst due the hypertonic citric acid and the nuclei were released and stained by crystal violet The freed nuclei could then be counted by haemocytometer The microcarriers did not interfere with the counting since they were too large to pass under the coverslip

2 6.3 Suspension Culture

RPMI 2650 cells were grown in suspension in 250 mls and 500 mls siliconised and autoclaved Techne flasks Medium supplemented with serum (DHS) and 1% p/s was added to the flask and incubated at 37°C while the cell suspension was being prepared A single cell suspension was prepared and added to the spinner flask so that a

final concentration of about 1 x 10^5 RPMI 2650 cells per ml was obtained The spinner flask was incubated at 37°C with continous stirring (20-30 rpm for a 100 ml culture)

Conditioned medium was collected from spinner flasks after 4 days by the following method The contents of the spinner flask were removed to sterile universals and centrifuged at 1,000 rpm for three minutes The supernantant was carefully poured off so as not to disturb the cell pellet. The pellet was resuspended in culture medium without serum and replaced in the spinner flask The volume was brought up to 100 mls The flasks were incubated at 37°C/20-30rpm for the length of time required for conditioning (normally 3 days), at which time the cells were removed from the conditioned medium by centrifugation and were either re-set up with fresh serum-free medium or were discarded The CM was stored at 4°C until further processing.

2.6.3.1 Sampling and counting

A 2 ml homogeneous sample of cells was carefully removed from the suspension culture and centrifuged at 1000 rpm for 4 minutes Waste medium was removed and 500μl of 0 25% trypsin (Gibco, 043-05090) added to the cell pellet This was incubated at 37°C until a single cell suspension was obtained (up to 15 minutes, depending on the size of the cell aggregates) 500 μl of serum supplemented medium was added For viability counts, 200 μl trypan blue was added The cells were then counted on a haemocytometer (Section 2 3 3)

2 7 LARGE SCALE CULTURE

2 7 1 Bioreactor System and control package

The Biostat MC (Braun AG) mammalian cell bioreactor (working volume 1 8 litres) was used to scale up the collection of CM from suspension cultures The system provides

* temperature control by circulating heated water through the jacket of the double walled glass culture vessel
* agitation by a top driven impeller with large adjustable pitch blades
* oxygen control by diffusion of nitrogen or oxygen through a internal basket of silicone tubing
* pH control by addition of CO_2 gas and sodium bicarbonate (1M).

The bioreactor was monitored using the Micro-MFCS control package which was set up on an IBM AT PC with 640 KB memory, with a mathematical coprocessor (80287), EGA graphic card and a EGA color monitor, connected to a dot matrix printer. The control system is operated through menus, where the operations are selected by function keys

The main function of the package used in this project was its on-line monitoring The computer collected analog signals from the oxygen, pH and temperature probes and from tacometer readings on the impeller and converted them into digital signals which were displayed both graphically and numerically in a number of different graphic screens The readings were averaged every hour and stored in batch reports which could be retrieved after a batch run was completed

An alarm function was incorporated into the system whereby each parameter could be assigned limits If the measured value fell outside these limits, its numerical value and graphical bar representation would turn from green to red An alarm message was also printed out.

The computer package also had facility to control pH and dissolved oxygen (DO) levels, overriding the controllers on the Biostat MC unit This function was not employed for the bioreactor work described in this thesis because the 'dead-band' of the controller could not be varied This had dramatic effects on the DO levels which varied by 20-30% The dead-band on the Biostat MC unit could be reduced to ± 5%.

2.7.2 Vessel preparation

Careful vessel preparation is essential for successful bioreactor cultures Each step outlined below must be adhered to.

1. The glass vessel was washed and rinsed as described before (2.2 1) The blades were removed from the impeller shaft and the aeration basket from the head plate and were washed and rinsed The metal parts of the oxygen and temperature probes were also cleaned

2 The glass vessel, the impeller blades, the aeration basket and the temperature probe were all siliconised (in theory cells should not adhere to the metal surfaces, but when these were not treated, some cells did grow on them)

3. Each blank plug and head-plate insert was removed and cleaned individually, paying careful attention to the thread area. Their o-rings were examined for nicks or distortions and replaced if required. All o-rings were greased with silicone grease before each plug was fastened in place

4. The pH probe was inspected If the level of electrolyte was low (more than 1 cm below the blue hole), it was topped up. The procedure for pH probe calibration was followed as described in the Braun Operating Manual with pH 7 and pH 10 standard buffers (Lennox) If slope of the electrode was not between 56 5 to 59 mV/pH or the zero point displacement not within the range -0.5 to

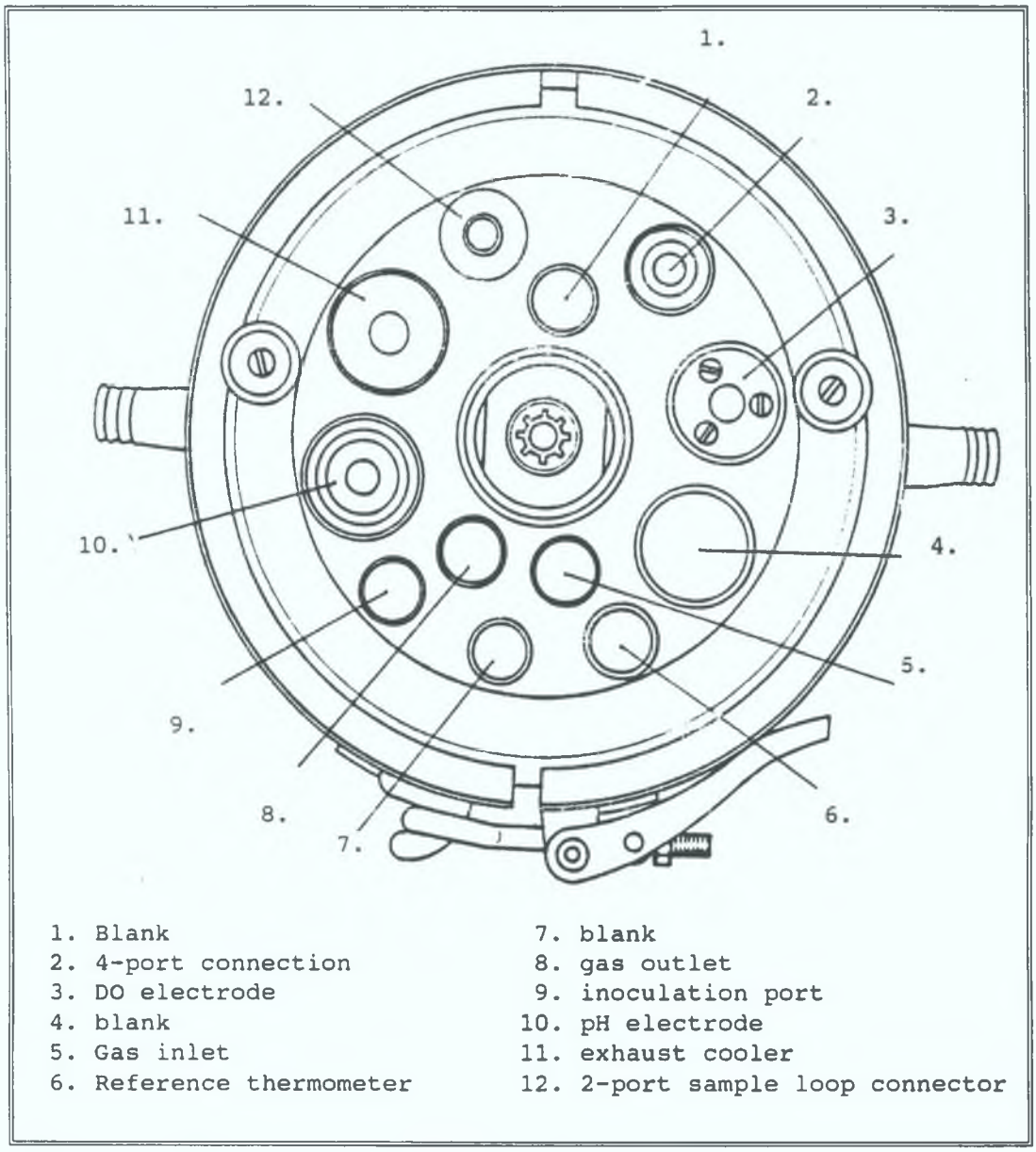
+0 5 pH, then maintenance action was taken (Section 2 7 3) or the electrolyte was removed and replaced with fresh 3M KCl electrolyte (Ingold AG), and the calibration procedure repeated

5. The response of the oxygen probe was checked The probe was polarised for at least 6 hours before the inspection started The inspection is made in three steps.

Step 1. Current Measurement With the 'RANGE' switch in TEST position, the value displayed was recorded At 20-25°C, the reading should be between 40-80
Step 2. Zero Current Measurement The electrode was placed carefully into a 50 mls syringe barrel connected to a nitrogen source The nitogen supply was turned on (less than 1 bar was sufficient) With the 'RANGE' switch in TEST position, the displayed value was recorded after 5 minutes This value must be less than 1.5% of the current that was measured in air.

Step 3 Response Time The electrode was taken carefully out of the syringe barrel. 98% of the original current measurement must be displayed within 40-80 seconds If a malfunction in one of the parameters was discovered during inspection, then maintenance action was taken. In all doubtful cases it is suggested that the membrane body be replaced (Section 2 7 4)

6 The head plate was assembled according to the diagram below. Electrodes, inoculation tubes, sampling tubes, etc which extend to the bottom of the vessel must be accomodated in the outer ports, otherwise they will interfere with impeller rotation. The pH probe and the DO probe were inserted last.



1. Blank
2. 4-port connection
3. DO electrode
4. blank
5. Gas inlet
6. Reference thermometer
7. blank
8. gas outlet
9. inoculation port
10. pH electrode
11. exhaust cooler
12. 2-port sample loop connector

FIGURE 2.1 Assembly of head plate (Biostat MC)

One outlet of four-port connection piece was connected to a solution bottle containing 1 M sodium bicarbonate. The solution bottle had a specially adapted lid with two outlets, one of which served as a vent and was attached to a 0.22 μm autoclavable filter (Gelman, 4464).

A second port on the 4-port connector was used to aerate the head space. It was connected via a forked connection to the gas inlet into the aeration basket and a 0.22 μm filter which was attached to the main gas supply.

The third and fourth ports were not used and were connected by a short loop of tubing to each other.

The sampling loop consisted of a loop of tubing connecting one outlet of the 2-port connection to the other. A sampling device was inserted in the loop. This was made up of four parts: the stainless steel bottom housing with outlets for the loop tubing; a

rubber (self sealable septum) screwed in place via a metal o-ring with a stainless steel upper housing A sampling tube was attached to the inside of one of the outlets, while the second was left free

The septum was replaced before each run It was important to clean the device carefully before assembling it. This involved running water through it and through the tubing after soaking in RBS It was important that the treads were free from grease or dirt

The long piece of tubing (about 1 meter) was connected to the inoculation port A blue tip-fin was inserted in the free end with its narrow tip pointing out. The top 4-5 mm of the tip was cut off

7. The blades were attached onto the impeller shaft The blades were angled so that they are 90° to each other and 45° to the shaft

8. The air filters (Millex FG 50 Cat No SLFG05010) attached to the gas inlet, the gas outlet and the exhaust cooler were examined by forcing air through them with a syringe. If water bubbles could be observed inside the filter housing, then they may be water-logged They were replaced if necessary. Normally these filters were changed every 3-4 runs (about 8 autoclave cycles).

9. Silicone tubing was attached onto the aeration basket. It was observed that cells grew in between the rows of tubing when they were very close together Also cells attached and grew around the area of the sampling tube This possibly was due to insufficient medium agitation near the bottom of the aeration basket. These problems appeared to be solved by wrapping the tubing around every second rung on the metal basket This did not appear to be detrimental to the oxygen control

The tubing was then checked for holes or nicks This was done by forcing air through the tubing by syringe, while it was immersed in water, and looking for air bubbles Water must not enter the silicone tubing or it will wet the exhaust air filters If holes were present, the tubing was replaced.

The aeration basket was connected to the head plate (checking orientation of the gas inlet and outlets against the head plate gas inlet and outlets) and the gas tubes were attached to the head plate

10 The head-plate was assembled to the vessel and clamped shut with the quick locking strap The headplate was positioned on the vessel so that the bottom hose connection is on the left The fastening fittings should lie behind the hose connections as viewed from the top, and the buckle of the locking strap should be to the front

11 The completed culture vessel was mounted to the holding, fastened down with screws and the impeller coupled to the driving shaft The agitation was turned at a slow rate (about 10 rpm) to check that the blades are not obstructed All electrical leads, tubings and hoses should be attached to their appropriate connections, to check everything is in place

12 Before autoclaving about 1 litre of water was added to the vessel (enough to cover probes) and all inlet and outlet tubing (except the exhaust cooler) and the tubing to the sampling bottle were clamped to prevent the air filters becoming wet The open ends

of the air filters were covered with tin foil The top of the impeller shaft, the top of the pH and DO probes and the electrical lead of the temperature probe were also covered well with tin foil. The vessel was now ready for sterilising The vessel was autoclaved for 20 minutes at 121°C/15 psi with the autoclave vent closed It is very important that the autoclave cools slowly (preferably overnight) since sudden pressure drops could damage the oxygen electrode

13 When cool, the autoclaved vessel was placed in the laminar flow cabinet and the water was pumped out via the inoculation tubing using a peristaltic pump (Braun FE411) in to a sterile roller bottle.

The tubing was connected (via the blue tip-fin) to a 1 litre Schott bottle containing 1 litre of medium without serum, L-glutamine or antibiotics, which was pumped into the culture vessel The inoculation tube was removed from the bottle tubing and was covered with IMS soaked cotton wool and wrapped in tin foil

The vessel was then assembled into the fermenter unit, and the impeller coupling connected and the connector hoses inserted in place The temperature is turned on and controlled at 37°C, and the agitation rate is set at 40 rpm. The vessel is covered in tinfoil to protect against light-inactivation of vitamins Incubation of the vessel in this manner for 2-3 days served as an added sterility check

14. The oxygen probe was connected to its meter and switched on The probe must be on for a minimum of 6 hours, to polarise, before it can be calibrated. The oxygen probe was calibrated by first pumping nitrogen gas into the culture vessel and waiting for a steady reading The display was then set to zero with the ZERO potentiometer (RANGE switch set to 20%) The nitrogen was turned off Air was pumped into the vessel until a steady reading was displayed. The RANGE switch was set to 100% and with the SLOPE potentiometer, and the display adjusted to 100%

2 7 3 Inoculation

Inoculation of the bioreactor was carried out in the laminar flow cabinet For RPMI 2650 cultures, three 75 cm^2 flasks (almost confluent) were sufficient to seed 1.8 litres of medium ($1\ 8 \times 10^8$ cells total) A single cell suspension of these cells was prepared in 90 mls of DHS and added to a sterile, siliconised Schott bottle. This was pumped very slowly into the vessel containing 1 litre of medium. L-glutamine (18 mls), Non-essential amino acids (NEAA) (18 mls) and p/s (18 mls) were added to the Schott bottle, swirled around, and pumped in also Finally the remaining medium (650 mls approximately) was pumped in The vessel was re-assembled into the bioreactor unit and all the meters and control modules switched on

2 7.4 Collection of CM

CM was collected in serum free medium, so to remove the medium containing serum from the cells, the entire culture contents was centrifuged, while maintaining sterility This was achieved by pumping the cell suspension directly into 50 ml sterile universals (Sterilin Cat No 125 AP), centrifuging at 1000 rpm for 4 minutes, combining the pellets and resuspending them in a small volume in medium without serum The cells could then be re-inoculated by injection into the vessel containing 1.8 litres of fresh medium supplemented with L-glutamine, NEAA and p/s via the sample port, or as before through the inoculation port

2.7.5. Sampling

Samples were withdrawn by needle via the sample port. The sample loop tubing was connected to the 'ACID' pump on the main bioreactor and with the CO_2 gas turned off, the acid pump was activated Culture medium was circulated around the loop, drawn up from the sample tube inside the vessel, through the sampling port and back to the vessel via the head plate The lid of the sampling port was removed and filled with IMS Samples were withdrawn by inserting a sterile needle straight into the septum The lid was then snapped back in place and the direction of flow through the tubing changed, so that the tubing contents were pumped back into the vessel

2 7 6 pH electrode maintenance

The electrode was cleaned by soaking the membrane and diaphram areas alternatively in pepsin-hydrochloric solution and thiocarbamide solution for a few hours This removed protein and sulphur deposits. After soaking, the probes were rinsed well in distilled water and stored in 3M KCl

2 7.7 DO electrode maintenance

The maintenance of the DO electrode involved changing the electrolyte, replacing the membrane body and cleaning the anode.

The electrolyte was replaced after 2-3 runs (about six autoclaving cycles) carefully following the procedure detailed in the Braun manual. The membrane body was changed whenever there was a doubt as to its operational capability or when it was mechanically damaged.

2.8 PROCESSING OF CM

2.8 1 Ultrafiltration

Ultrafiltration was used in as a means of concentrating and fractionating the CM collected. The stirred cell method was used (Amicon No 8499 - 400 ml capacity chamber), with a variety of molecular-weight cut-off membranes (Amicon)

YM 30	30,000	MW cut-off	R30 + F30
YM 10	10,000	MW cut-off	R10 + F10
YM 5	5,000	MW cut-off	R5 + F5
YM 2	1,000	MW cut-off	R1 + F1

In general CM was concentrated in the range 5-20x by ultrafiltration

CM could be fractionated by concentrating filtrate from one ultrafiltration membrane on a smaller one For example the R1-30 fraction was obtained by concentrating F30 on the YM 2 membrane

2 8 2 Diafiltration

Diafiltration was used as a means to desalt fractions following elution at high salt concentrations from heparin sepharose columns A 10 ml sample to be diafiltered was diluted with 100 mls of medium and concentrated by ultrafiltration to its original volume (10 mls) This was repeated Thus the salt in the sample had been effectively diluted 1/121 with medium

2.8.3 Dialysis

Concentrated CM samples and column fractions were dialysed against 50 volumes of PBS and 25 volumes of medium using a low molecular weight, benzoylated, 1,200 cut-off tubing (Sigma D7884)

Before use the required length of tubing was boiled for 5 minutes in 10 mM EDTA This was then washed well in distilled water and finally rinsed in ultrapure water. Dialysis was carried out at 4°C, with the buffer changed twice daily for 2 days

2.8 4 Lyophilisation

Lyophilisation of CM was carried out using a 4 5 L Consol Freeze Dryer (Virtis) Samples were frozen to -30°C overnight The freeze drying cycle was started and usually lasted for 36 hours or until the samples were dry

2 9 SOFT AGAR ASSAYS

Conditioned media samples were assayed for growth factor activity using three different soft agar assay systems, the basic protocols of which are similar A 2 ml test sample is required for assay in triplicate. The assays vary with regards to basal medium, indicator cell line and indicator cell concentration as indicated below.

TABLE 2 5 Soft Agar Assays

Assay Type	Autocrine	TGF-α-like	TGF-ß-like
Cell line Cells/35 mm dish Basal Medium	RPMI 2650 3×10^4 MEM	NRK 6×10^3 DME	NRK-49F $1\ 95 \times 10^4$ DME

2.9.1 Cell pretreatment

Cells were trypsinised three days prior to assay and fed at least three hours before trypsinisation.

It is advisable that the passage number of NRK cells be kept between passage 19-21.

2.9.2 Assay Protocol

The following protocol was followed for each assay.

1. Weigh out 1.548 g agar (Bacto Difco) and dissove in 100 mls of ultrapure water. Autoclave. Incubate at 44°C immediately.

2. Calculate volume of agar medium (AgM) required by multiplying the number of samples by 7. Divide this figure by 2.58 to find the volume of MEM2x or DME2x required. Prepare the AgM in the following ratio:

MEM2x/DME2x	50 mls
HEPES (1mM)	2 mls
$NaHCO_3$ (7.5%)	1 ml
P/S	1 ml
Growth Medium (-FCS)	14 mls

Equilibrate to 44°C.

MEM2x/DME2x was prepared as follows:

MEM/DME 10x	20 mls
Ultrapure water	76 mls
HEPES (1mM)	4 mls
$NaHCO_3$ (7.5%)	2.2 mls
adjust to pH 7.4 with 1 M NaOH	

3. Place 35 mm plates (Greiner, 627160) on trays and label appropriately. Each sample is assayed in triplicate.

4. Add the thermo-labile components to the AgM at the following concentrations:

L-glutamine	1 ml
FCS	10 mls

5. Add agar to the AgM in the same volume as MEM2x or DME2x (i.e. in this case 50 mls). Mix well and quickly dispense 1.5 mls onto each 35 mm dish. The remaining AgM is returned to the water bath and the temperature reduced to 41°C. The plates are allowed to set at room temperature.

5. Trypsinise cells and prepare a single cell suspension in medium without serum. The required concentration per ml to give the final concentration per dish is as follows:

RPMI 2650	2×10^5 cells/ml
NRK	4×10^4 cells/ml
NRK-49F	1.3×10^5 cells/ml

6. Add 0.5 mls of each cell suspension to each 2 ml test sample and unconditioned medium control samples.

7 Add 2 5 mls AgM (41°C) to each sample (containing cells) and immediately dispense out 1 5 mls per plate (x3) After every five samples (maximum), re-equilibrate the AgM to 41°C

8 Cover trays in tin foil and incubate at 37°C/ 5% CO_2 for 10 days

2 9 3 Colony counting

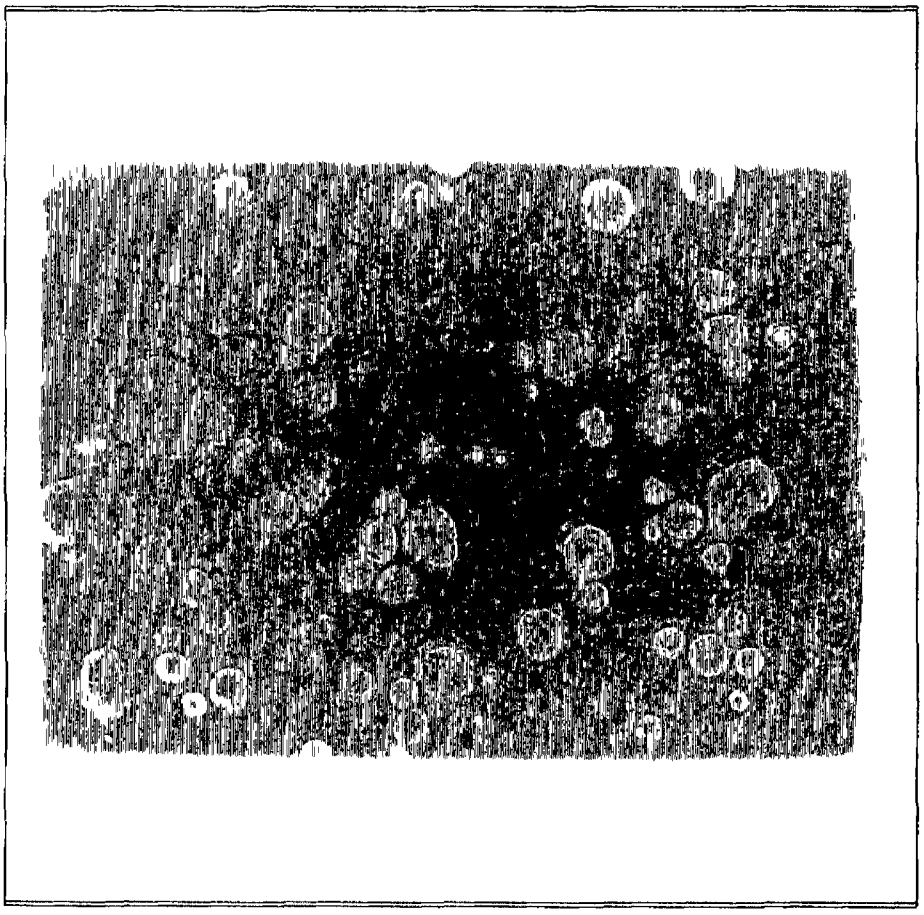


PLATE 2 **Anchorage-independent growth of RPMI 2650 cells in soft agar Phase 1 (150X) magnification**

Colonies were counted on an inverted microscope (CK Olumpus, Tokyo) at 40x All colonies greater than 50μm in diameter after 10 days in culture were counted An eye-piece graticule was graduated using a stage micrometer and was used to size colonies

All agar plates were viewed, superimposed on a transparent gridded disc with 4 mm^2 grids 25 grids were viewed for each 35 mm dish The average total number of colonies per plate was then estimated and the percentage colony forming efficiency (% CFE) determined

$$\% \text{ CFE} = \frac{\text{Number of colonies per plate}}{\text{Number of cells plated}} \times 100$$

Each sample was set up in triplicate and a mean % CFE was determined for each sample and the standard error of the mean calculated by the following formula

$$SEM = \frac{\sqrt{\frac{\Sigma x^2 - (\Sigma x)^2/n}{n-1}}}{\sqrt{n}}$$

Where n = number of replicates per sample
x = CFE of each replicate
Σ = sum of the samples from 1 to n

2.10 PURIFICATION OF CM

2.10 1 Ion exchange chromatography

A preliminary experiment was set up to test the affinity of the autocrine activity for cation and anion exchangers. The gels used were Cellex-CM, a weak cation exchanger, (Bio-Rad, Cat No 7481530), and Cellex-D, standard capacity (Bio-Rad, Cat No 7480530) The gels (0 25 g/test tube) were pre-equilibrated in 10x buffers (25 mls) and adjusted to the pH values required with the acidic or basic component of the buffer The buffers used for each pH are listed in Table 2 6. The buffers were made up to 0 5M (10x) and 0 05 M (1x), for the equilibration and separation steps respectively

TABLE 2 6 Ion-exchange chromatography buffers

Cellex-CM		Cellex-D	
pH	Buffer	pH	Buffer
4 0	Sodium Acetate	5 0	Sodium Acetate
4 8	Sodium Acetate	5 6	Sodium Acetate
5 2	Sodium Acetate	6 0	Potassium phosphate
5 6	Sodium Acetate	6 4	Potassium phosphate
6 0	Potassium phosphate	6 8	Potassium phosphate
6 4	Potassium phosphate	7 1	Tris HCl
6.8	Potassium phosphate	7.4	Tris HCl
7 2	Potassium phosphate	7 8	Tris HCl
7 9	Potassium phosphate	8.2	Tris HCl
8.0	Potassium phosphate	8 6	Tris HCl
		8 9	Tris HCl

The conductivity was then reduced with seven washes of 25 mls of diluted buffer (1x) As much buffer as possible was removed by pasteur pippette 2 mls of buffer was added (1x)

RPMI 2650 CM concentrated 14x by ultrafiltration was dialysed against ultrapure water to reduce salt concentrations 2 mls of this CM was added to each tube of equilibrated gel The tubes were mixed for one hour with gentle inversion. The gel was allowed to settle and the supernatnant removed (4 mls approximately). Each sample was dialysed against distilled water for 24 hours and against unconditioned medium for 48 hours

Each sample was then filter sterilised and assayed in the RPMI 2650 autocrine assay

2 10 2 Hydrophobic Interaction chromatography

A 4 ml phenyl sepharose CL4B (Pharmacia, Cat No 17-0810-02) column was equilibrated for 1 hour with MEM ultrafiltered to 5x with a 1,000 MW cut-off membrane 10 mls of a R1(10x) samples was applied and then eluted with 50% glycerol. 4 ml fractions were collected Each fraction was dialysed against PBS for 24 hours and MEM for 12 hours.

2.10.3 Heparin sepharose chromatography

CM was fractionated by heparin sepharose chromatography using Heparin Sepharose CL-6B (Pharmacia, Cat No 17-0467-01) which is stored in a dry powdered form which must be swollen in buffer before use 1 g of dry powder gives approximately 4 mls of gel The gel must be extensively washed in buffer before the sample is applied to remove all preservatives It is recommended that the 1 g of powder is washed with 250 mls of buffer

2.10 3.1 Step elution

Step elution from heparin sepharose columns proved to be a successful method of CM fractionation.

100 mls of untreated CM was incubated at 4°C with 4 mls of heparin sepharose gel with constant stirring overnight The supernatant was removed by allowing the gel to settle and the gel was washed with 60 mls of 0 01 M potassium phosphate buffer before being loaded into a 10 ml column The column was rinsed through with a further 30 mls of buffer This 180 mls (approximately), 'UNBOUND' fraction, was ultrafiltered using a 1,000 MW cut-off membrane down to 10 mls (10x of its original concentration).

A NaCl solution (3M) was prepared in 0 01 M potassium phosphate buffer and diluted appropriately with buffer The bound activity was then eluted with increasing salt concentration

0 0, 0 25, 0 5, 0.8, 1 0, 1 2, 1 5, 2 and 3 M NaCl

Fractions were collected (4 mls) and absorbance (280nm) was read

2.10.3 2 Gradient Elution

The operation of the 10 ml heparin sepharose column was controlled by the 'BIOPILOT system' (Pharmacia) The control unit consists of a programmable controller linked to a series of pumps and valves and two monitors (UV, conductivity) and a chart recorder The programmable controller directs the flow of liquid through the system It also controls product stream monitoring

A program was set up so that a concentrated CM sample (10 mls of a R1(14x) sample) was applied to a 10 ml heparin sepharose column, pre-equilibrated in 0.01 M potassium phosphate buffer, pH 7 4 (Buffer A). The column was then washed with 70 mls of buffer A A

salt gradient was set up, such that the NaCl concentration is increased from 0 M to 3 M NaCl over 100 mls at a rate of 1 ml/min The column is washed with a further 50 mls of 3 M NaCl in 0 01 phosphate buffer, pH 7 4 (Buffer B)

Fractions (6 mls) were collected and filter sterilised before assay

2 11 STABILITY OF CM

2.11 1 Temperature Stability

Conditioned medium (10 mls), concentrated five fold by ultrafiltration, was dispensed into clean glass universals (30 mls) and placed in a covered waterbath set at 65°C for the required lenght of time (1 hour) Samples for boiling were placed in a beaker of boiling water over a flame, with lids slightly loosened When the temperature of the CM reached 98°C, the boiling was monitored for 3, 6 and 20 minutes Each sample was then centrifuged at 3,000 rpm/15 minutes to removed insoluble precipitates, and filter sterilised before assay

2.11 2 pH stability

Conditioned medium samples (5 mls) were dispensed out into universals. The pH of each sample was adjusted with different volumes of 5 M HCl and 5 M NaOH (see Table 3 5 8) and the samples were incubated at 4°C for two hours before the pH was re-adjusted Osmolarity was measured on the Osmomat 030 (Gonotec). Each sample was filter sterilised before assay

2 11.3 Protease stability

A concentrated CM sample was incubated for two hours at 37°C with 10μg/ml trypsin (Gibco) The reaction was stopped with 20μg/ml soyabean trypsin inhibitor (Sigma)

2 12 PURE GROWTH FACTORS

2.12 1 Source

A range of growth factors from commerical sources were assayed in the soft agar assay. The growth factors, their suppliers and sources are given in Table 2 7

TGF-α was a kind gift form Marjorie Winkler, Genentech

TABLE 2.7 Growth Factors used in course of project

Growth Factor	Source
Insulin	Sigma, Cat No. I-1882 Bovine pancreas
IGF-I-P	Promega, Cat No G5111 Human recombinant protein from *S.cerevisiae*
IGF-I-B	Boehringer Mannheim, Cat No BCL 1048-066 Human recombinant protein from E. *coli*
IGF-II	Sigma, Cat No. M-9145 BRL-3A cell CM
aFGF	Sigma, Cat No F-5267 Bovine brain
bFGF	Sigma, Cat No F-5392 Bovine pituitary glands
TGF-ß	Peninsula Lab Europe Ltd., Cat No IP-1085 Purifed from human platelets
TGF-α	Genentech, Cat No PD-1 Human recombinant
EGF	Sigma, Cat No E 4127 Mouse
IL-1α	British Biotechnology Ltd , Cat No BDP-15 Human recombinant protein from E *coli*
IL-1ß	British Biotechnology Ltd , Cat No. BDP-17 Human recombinant protein from E. *coli*
PDGF	Peninsula Labs Cat No IP-1080

2.12.2 Preparation

The growth factors were supplied in lyophilised form and required reconstitution in appropriate buffers (each containing 1 mg/ml BSA) before use After reconsitution, the factors were diluted in ATCC medium containing 1 mg/ml BSA Fraction V (Sigma, Cat No A9418) to give the indicated growth factor concentrations per ml of agar

TABLE 2 8 Reconstitution of Growth Factors

Growth Factor	Quantity	Reconstitution Buffer (+ 1mg/ml BSA)	Final Concentrations (/ml agar)
Insulin	100mg	0 5 ml 0 1M Acetic acid	50,5,1 ng
IGF-I-P	25μg	0 5 ml 0 1M Acetic acid	50,10,5,1 ng
IGF-I-B	50μg	0 5 ml 10mM HCl	50,10,5,1 ng
IGF-II	10μg	0 5 ml 10mM HCl	50,10,5,1 ng
aFGF	1μg	1 0 ml ATCC	50,10,5,0.5 ng
bFGF	1μg	1 0 ml ATCC	50,10,5,0 5 ng
TGF-ß	5μg	0 5 ml 0 1M Acetic acid	Various
TGF-α	100μg	1 0 ml PBS	50,10,5,1,0 5 ng
EGF	1μg	1 0 ml ATCC	50,10,5,1,0 5 ng
IL-1α	5μg	1 0 ml PBS	10,1,0 1 units
IL-1ß	10μg	1 0 ml PBS	10,1,0 1 units
PDGF	100U	4 0 mls 0 15M NaCl	2, 1 0 5 units

3 0 RESULTS

3 1 DEVELOPMENT OF THE SOFT AGAR ASSAY

In order to reduce variability betweeen experiments a number of factors involved in the autocrine assay were examined

the number of cells present in each dish,

the indicator cell pretreatment before the assay, and

the performance of different batches of serum

In these assays a common batch of conditioned media was tested This had been concentrated ten fold with a 1,000 molecular weight cut-off ultrafiltration membrane (R1) Unconditioned medium (MEM) was also assayed, serving as a negative control

3.1 1 Effect of cell loading

The aim of this experiment was to determine an optimum cell concentration per plate in the RPMI 2650 autocrine assay The cell concentration per well was varied from 10^3 cells to 10^5 cells per plate The results are presented as the average number of colonies per plate and the calculated percentage colony forming efficiency (%CFE) based on the number of indicator cells plated

TABLE 3 1 1 Effect of indicator cell loading in the RPMI 2650 autocrine assay

RPMI 2650 cells per dish	R1 (10x)		MEM	
	Count±SEM per dish	CFE±SEM (%)	Count±SEM per dish	CFE±SEM (%)
10^3	4 0± 1 0	0 40±0 10	0 3± 0 58	0 03±0 06
$5x10^3$	10 0± 2 7	0 20±0 05	1 7± 0 58	0 03±0 01
$3x10^4$	85 8± 10 2	0 29±0 03	3 3± 0 57	0 01±0 01
$5x10^4$	220 5± 47 0	0 44±0 10	12 0± 4 00	0 02±0 01
$1x10^5$	508 0±104 0	0 51±0 10	29 7± 1 55	0.03±0 01

The number of colonies formed per plate increased with the number of cells plated At lower cell loading densities the difference between stimulation and control levels depends on less than ten colonies which would make assessment of activity problematic Such cell loadings (less than 10^4 cell per dish) make statistically significant assessment difficult

As more cells are present, more colonies are formed The errors are quite high here due to inaccuracies arising from the counting of such large numbers of cells per plate As the cell loading was increased colonies grew closer together making it difficult to count and size individual colonies At higher cell loading levels it is necessary to be aware of the danger of saturating the plates, resulting in nutrient limitation occuring before the assay is read.

While varying the number of indicator cells loaded increased colony stimulation, it did not greatly affect the colony forming efficiency for the range examined

The optimum cell loading in the autocrine assay was chosen to be in between these two extremes at $3x10^4$ cells per plate

3 1 2 Effect of indicator cell pretreatment

The condition of the cells used is known to affect the reproducibility of the assay (Dooley, 1987, PhD Thesis) An experiment was designed to analyse the effect of cell pretreatments of a number of different flasks of cells

Four flasks of RPMI 2650 cells (passage 59), were set up on day 1, two at 2×10^4 (Flasks A and B) and two at 6×10^6 (Flasks C and D). Flasks A and C were fed on day 3, 24 hours before the assay All four flasks were trypsinised on day 4 and the cells were used to detect autocrine activity in a sample of R1(10x) (i e conditioned medium concentrated 10 fold by ultrafiltration using a YM2 ultrafiltration membrane (1,000 MW cut-off)) and a medium (MEM) control The results are presented in Table 3 1 2 in the form of % colony forming efficiency of the R1(10x) and MEM samples and the fold stimulation of the R1(10x) compared with the control

TABLE 3 1 2 **Effect of indicator cell pretreatment in the RPMI 2650 autocrine assay represented as (%)CFE±SEM and the average fold stimulation above that of the MEM control.**

Indicator cell Pretreatment	(%)CFE ± SEM		Fold Stimulation
	R1(10x)	MEM	
A Sub-confluent, fed	0 06±0 04	0 03±0 01	2 0
B Sub-confluent, not fed	0 18±0 02	0 07±0 01	2 6
C Confluent, fed	1 28±0 17	0 22±0 07	5 8
D Confluent, not fed	0 49±0 02	0 18±0 02	2 7

The confluency of the indicator cell appears to be a crucial factor in the stimulation observed in the assay Flasks C, in particular, was more responsive to the autocrine activity The two subconfluent flasks, A and B, were not as responsive, although stimulation of more than 2 0 fold was present

This experiment highlights the importance of the condition of the cells in the design of the autocrine assay Variability will be reduced somewhat if the pretreatment of the indicator cells is kept constant for every assay, although obviously other factors also play a role

3.1 3 Different serum batches

This experiment examined the behaviour of a range of sera from various animal sources A sample of conditioned media (R1(10x))

known to be active was included as a positive control, MEM as a negative control and MEM + 5% of the appropriate sera an internal control

A suitable serum for the autocrine assay is defined here as a batch that shows increased stimulation for the R1(10x) sample above that of the MEM control without high stimulation in the MEM + 5% serum sample

TABLE 3 1 3 Effect of sera from various sources in the RPMI 2650 autocrine assay

Serum Batch*		R1(10x)	MEM	MEM+5% FCS
1	FCS	1 14±0 15	0 03±0 02	0 28±0 08
2	FCS	0 47±0 07	0 04±0 01	0 13±0 01
3	FCS	1 29±0 11	0 32±0 07	1 15±0 23
4	FCS	1 61±0 29	0 37±0 10	0 83±0 03
5	FCS	2 62±0 27	0 28±0 04	1 59±0 08
6	NC	0 35±0 09	0 03±0 02	0 10±0 03
7	DH	2 21±0 15	0 06±0 02	0 50±0 08
8	FCS	0 03±0 14	0 02±0 02	0 28±0 15
9	FCS	0 65±0 10	0 08±0 01	0 44±0 04

*FCS = Foetal Calf Serum
NC = Newborn Calf Serum
DHS = Donor Horse Serum

In this experiment it was found that the sera could be put into the following categories

Good Batches 1,7
Fair Batches 2,5
Bad Batches 3, 6, 8, 9

The performance of this particular batch of donor horse serum (sample 7) was comparable with the best batch of foetal calf serum but due to availability, it was decided to use batch 1 FCS in subsequent autocrine assays

3 1 4 Reasons for improvements in assay reliability

The general conclusions that can be drawn from this set of experiments are limited The reliability of the assay was improved, however due to a number of technical changes with the assay procedure itself The main changes are listed below

1 MEM(2x) was made up on the day of assay as required using freshly autoclaved ultrapure water This eliminated the formation of precipitates that was frequently apparent in MEM(2x) stocks following storage at 4°C
2 Foetal calf serum and l-glutamine were not added to the agar medium until just before the agar was added This reduced the time that these heat sensitive components would have to be exposed to 44°C
3 Indicator cells were set up in new flasks for each assay This ensured that the cells were evenly spread in the flask and were more likely to be at the same stage of confluency Cells pretreated in this way were also easier to trypsinise
4 In order to avoid the possibility of evaporation if pre-dispensed water was autoclaved, it was decided to autoclave excess water for preparation of agar medium and to dispense out 76mls of water from it

3 2 STIMULATION OF RPMI 2650 CELLS IN SOFT AGAR BY PURE GROWTH FACTORS

The aim of this set of experiments was to see what classes and types of pure growth factors were capable of stimulating RPMI 2650 colony formation in the autocrine assay system Because of size limitations this experiment was split into a number of smaller, more manageable assays

3.2 1 Effect of bovine serum albumin (BSA) in the RPMI 2650 soft agar assay

The purified growth factors were dissolved in appropriate buffers as described in Materials and Methods and then diluted in ATCC medium supplemented with BSA The effect of BSA in the assay was compared to controls of ATCC without BSA to investigate whether a control of ATCC with 1mg/ml BSA was required in the assays

TABLE 3 2 1 Effect of BSA on colony forming efficiency of RPMI 2650 cells in soft agar

Assay	% CFE ± SEM		Fold stimulation
	ATCC	ATCC + 1 mg/ml BSA	
1	0 21±0 04	0 33±0 04	1 6
2	0 13±0 04	0 88±0 09	6 8
3	0 10±0 03	0 14±0 02	1 4

Table 3 2 1 illustrates that BSA does effect the growth of RPMI 2650 cells in soft agar The presence of BSA results in increased growth over a medium control Subsequent assays with purified growth factors must therefore include a ATTC supplemented with 1mg/ml BSA.

This set of experiments again illustrates the variablity between individual assays In each of these assays, cell pretreatment was identical, the agar medium employed the same batch of FCS and ATCC medium, and the BSA was prepared from a common stock solution

3 2.2 Insulin, Insulin-like growth factors I and II

In this experiment insulin-like growth factor I from two separate sources were compared IGF-IB (source=Boehringer Mannheim, derived from *E coli*) and IGF-IP (source=Promega, derived from *S cerevisae*) IGF-II (Sigma) and insulin (Sigma) were also assayed at

the concentrations indicated Dilutions of the growth factors were made in ATCC medium supplemented with 1 mg/ml BSA as described in the Materials and Methods The results are presented in Table 3 2 2

TABLE 3 2 2 **Effect of insulin-like growth factors I and II and insulin on colony forming efficiency in the RPMI 2650 autocrine assay The fold stimulation above the control is presented in brackets {sample/control}**

	Concentration per ml of agar medium			
Growth Factor	50ng	10ng	5ng	1ng
IGF-IB	0 57±0 05 {1 7}	0 31±0 09 {0 9}	0 29±0 03 {0 9}	0 26±0 08 {0 8}
IGF-1P	0 70±0 10 {2 1}	0 38±0 08 {1 2}	0 34±0 02 {1 0}	0 20±0 06 {0 6}
IGF II	0 40±0 02 {1 2}	0 30±0 03 {0 9}	0 27±0 06 {0 8}	0 22±0 04 {0 6}
Insulin	0 38±0 05 {1 2}	N D *	0 24±0 02 {0 7}	0 26±0 04 {0 8}
ATCC/BSA	0 33±0 04			

N D * = not determined

The two sources of IGF-I examined in this assay had comparable stimulatory activity and this activity was dilutable IGF-II and insulin were slightly stimulatory at 50 ng/ml but showed no significant activity at lower concentrations

These results were reproducible over 3 separate experiments There was some variablity between the assays, as illustrated in the table below, but the general trend of each individual assay was similar

TABLE 3 2 3 Illustration of the variability of three different assays calculated fold stimulation of 50 ng/ml of IGF-IB over the ATCC/BSA control

Assay	% CFE ± SEM		Fold stimulation
	50 ng/ml IGF-IB	ATCC + 1 mg/ml BSA	
1	1 17±0 17	0 88±0 09	1 3
2	0 57±0 05	0 33±0 04	1 7
3	0 33±0 09	0 14±0 02	2 3

3 2 3 Acidic and basic fibroblastic growth factors (aFGF and bFGF)

In this set of experiments dilutions of acidic and basic fibroblast growth factors were made up in ATCC medium supplemented with BSA (1 mg/ml) and assayed in the RPMI 2650 autocrine assay at the concentrations indicated The acidic FGF samples were supplemented with 10 ng/ml heparin Controls of ATCC + 1 mg/ml BSA and ATCC + 1mg/ml BSA supplemented with heparin were included

TABLE 3 2 3 Effect of acidic and basic fibroblast growth factors (aFGF and bFGF) in the RPMI 2650 autocrine assay The results are presented in CFE(%)±SEM The fold stimulation over the control is also shown in brackets {sample/control}

Growth Factor	Concentration per ml of agar medium			
	50ng	10ng	5ng	0 5ng
aFGF+heparin (10ng/ml)	2 53±0 73 {4 7}	1 40±0 42 {2 6}	1 05±0 19 {1 9}	0 66±0 07 {1 2}
bFGF	1 46±0 53 {2 6}	1 09±0 13 {2 0}	0 45±0 13 {0 8}	0 42±0 17 {0 8}
5ng/ml aFGF -heparin	0 65±0 33 {1 2}			
Heparin (10ng/ml)	0 54±0 22			
ATCC+1mg/ml BSA	0 56±0 11			

Both acidic and basic FGF were stimulatory above the ATCC/BSA control Acidic FGF retained activity at 5 ng/ml while basic FGF was not active below 10 ng/ml The aFGF activity was not augmented by the presence of heparin

3 2 4 Transforming growth factor beta (TGF-ß)

The effect of TGF-ß was variable in a number of assays The variability in response to TGF-ß is illustrated in Tables 3 2 4-6 for 7 separate experiments with similiar indicator cell pretreatment looking at 3 different concentration ranges of TGF-ß

TABLE 3 2 4 Illustration of variability of response to TGF beta in three similar RPMI 2650 autocrine assays (range 0 002-0 2 ng/ml)

TGF-ß (ng/ml)	Assay 1	Assay 2	Assay 3
0 002	0 58±0 06	0 28±0 06	0 16±0 02
0 01	0 64±0 08	0 40±0 08	0 24±0 01
0 02	1 20±0 27	0 21±0 02	0 19±0 06
0 1	0 97±0 18	0 46±0 14	0 17±0 04
0 2	0 74±0 18	0 36±0 05	0 30±0 13
ATCC/BSA	0 88±0 09	0 33±0 04	0 14±0 02

The concentration of TGF-ß was increased in subsequent experiments and the counts per dish are presented below

TABLE 3 2 5 Illustration of variability of response to TGF beta in three similar RPMI 2650 autocrine assays (range 0 01-2 0 ng/ml)

TGF-ß (ng/ml)	Assay 1	Assay 2
0 01	0 12±0 01	0 06±0 01
0 02	0 06±0 02	0 03±0 01
0 10	0 04±0 01	0 05±0 02
0 20	0 04±0 01	0 03±0 01
0 50	0 06±0 02	0 02±0 01
1 00	0 03±0 01	0 03±0 01
1 50	0 02±0 00	0 02±0 00
2 00	0 06±0 01	0 04±0 01
ATCC/BSA	0 06±0 01	0 04±0 01

TABLE 3 2 6 Illustration of variability of response to TGF beta in three similar RPMI 2650 autocrine assays (range 0 5-50 0 ng/ml)

TGF-ß (ng/ml)	Assay 1	Assay 2
0 5	0 42±0 05	0 88±0 09
1 0	0 93±0 17	1 03±0 14
5 0	0 58±0 08	1 39±0 24
10 0	0 75±0 05	1 35±0 23
50 0	0 73±0 14	1 43±0 21
ATCC/BSA	0 64±0 04	0 97±0 07

As can be seen from the tables above, RPMI 2650 cells do not respond significantly to TGF-ß in a predictable manner over a range of concentrations Even at high levels of TGF-ß only control levels (or slightly above) were observed In some cases slight inhibition was detected

3 2 5 Interleukin 1-alpha and beta (IL-1α and IL-1ß)

In this set of experiments dilutions of IL-1α and IL-1ß were made up in ATCC medium supplemented with BSA (1 mg/ml) and assayed in the RPMI 2650 autocrine assay at the concentrations indicated

TABLE 3 2 7 Effect of IL-1α and IL-1ß on RPMI 2650 colony forming efficiency in soft agar The fold stimulation above the control is shown in brackets

	Concentration per ml of agar medium		
Growth Factor	10 Units	1 Unit	0 1 Unit
IL-1α	0 07±0 04 {0 4}	1 38±0 24 {0 72}	1 16±0 23 {0 60}
IL-1ß	0 10±0 03 {0 1}	0 26±0 10 {0 4}	0 38±0 08 {0 6}
ATCC/BSA	1 92±0 25		

The interleukins exhibited dilutable inhibition at the concentrations examined The experiment was repeated with similar trends

3 2 6 Epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α)

EGF and TGF-α were made up in medium containing ATTC + 1mg/ml BSA and assayed in the RPMI 2650 autocrine assay The results are presented in Table 3 2 8

TABLE 3 2 8 Effect of EGF and TGF-α on RPMI 2650 colony forming efficiency in soft agar The fold stimulation above control level is shown in parenthesis

	Concentration per ml of agar medium				
Growth Factor	50ng	10ng	5ng	1ng	0.5ng
EGF	0 94±0 03 {1 0}	0 87±0 33 {0 9}	0 67±0 16 {0 7}	0 59±0 03 {0 6}	1 13±0 07 {1 2}
TGF-α	0 96±0 11 {1 0}	0 76±0 19 {0 8}	1 19±0 19 {1 2}	0 85±0 11 {0 9}	1 11±0 32 {1 1}
ATCC/BSA	0 97±0 07				

Neither EGF or TGF-α significantly increased colony forming efficiency for the concentrations examined The results indicated inhibition by intermediate concentrations of TGF-α and EGF, with slight stimulation at lower concentrations and no effect at 50 ng/ml This experiment was repeated with similar results

3 2 7 Platelet-derived growth factor (PDGF)

PDGF was made up in ATCC medium containing 1mg/ml BSA in the RPMI 2650 soft agar assay at the concentrations indicated The results of two independent experiments are presented in the table below

TABLE 3 2 9 Effect of PDGF on colony forming efficiency of RPMI 2650 cells in soft agar The fold stimulation is presented in brackets

	Concentration per ml of agar medium			Control
	0 5 units	1 0 unit	2 0 units	
Assay 1	1 16±0 08 {5 3}	1 08±0 13 {4 9}	0 98±0 11 {4 5}	0 22±0 06
Assay 2	N D	N D	5 61±1 20 {1 5}	3 64±0 94

Although some PDGF can elicit some stimulation, the stimulation does not appear to be dilutable This would indicate that a wider range of concentrations should be examined

3 3 PRESENCE OF GROWTH FACTORS IN THE RPMI 2650 CONDITIONED MEDIA

3 3.1 Standard curve of TGF-α activity in the NRK soft agar assay

The presence of TGF-α-like activity in the RPMI 2650 CM was determined by comparing two fractions of CM to a standard curve of TGF-α in the NRK soft agar growth assay (see Section 3 3 3 1, below) CM from another cell line (HEP-2) was also assayed

A standard curve of TGF-α activity was set up as follows TGF-α (Genentech) was dissolved in PBS + 1mg/ml BSA and diluted in ATCC/BSA as before From a stock concentration of 10μg/ml of TGF-α, a range of dilutions was prepared to give the final concentrations of test sample indicated (per ml) A control containing ATCC supplemented with 1 mg/ml BSA was assayed along with the prepared TGF-α standards

RPMI 2650 conditioned medium was concentrated 10x using a 1,000 MW cut-off ultrafiltration membrane (R1) and diluted back to 1x with MEM

Conditioned medium from the cell line HEP-2, collected in Ham's F12, was included in the assay This CM was concentrated 10x using a 30,000 MW cut-off membrane (R30) The filtrate was concentrated 10 fold with a 10,000 MW cut-off (R10-30) These samples were assayed along with neat CM and an unconditioned medium control

The results are presented in Table 3 3 1 as % colony forming efficiency of NRK cells in soft agar The activity of the standards is graphed in Figure 3 3 1 against their log(concentration) Comparison of the activity of the samples against this standard curve gives an indication of the levels of TGF-α-like activity present

TABLE 3 3 1 Standard curve of TGF-α activity in the NRK soft agar assay

TGF-α (ng/ml)	% CFE ± SEM	Conditioned Medium	% CFE ± SEM
250	20 66±0 86		
		RPMI 2650	
125	18 89±0 73	R1(10x)	1 18±0 19
		R1(5x)	0 39±0 06
50	16 10±0 62	R1(1x)	0 00±0 00
25	14 26±1 93	MEM	0 04±0 05
5	6 68±0 84		
2 5	5 89±0 73	HEP-2	
		R10-30(10x)	0 00±0.00
0 25	0 12±0 00	R30(10x)	0 00±0.00
		neat (1x)	0 04±0 05
		HF 12	0 00±0 00
ATCC	0 04±0 05		

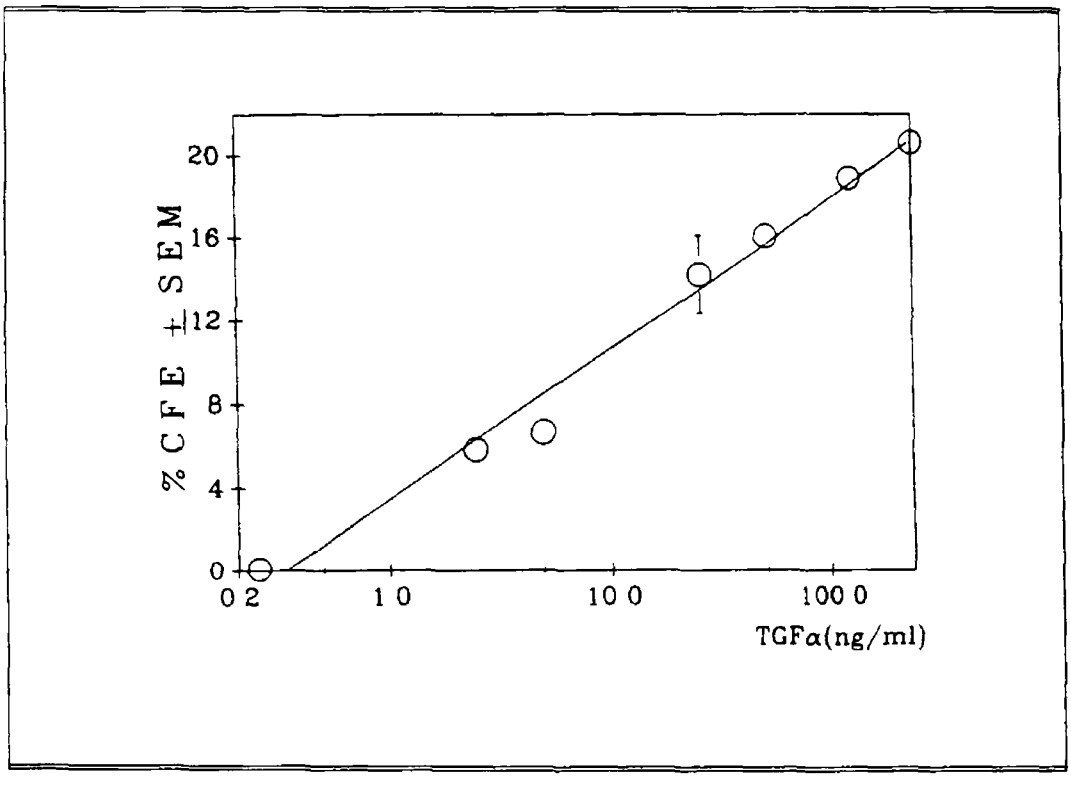


FIGURE 3 3 1 Standard curve of TGF-α activity

The estimated TGF-α-like activity present in the CM samples was determined by comparison with the standard curve and are shown below in Table 3 3.2

TABLE 3 3 2 Estimated levels of TGF-α in neat CM from different cell lines

Cell line	TGF-α-like activity (ng/ml)
RPMI 2650 R1(10x)	0 50
HEP-2 R30(10x)	0 00
R10-30(10x)	0 00
Unconcentrated	0 00

3 3 2 Standard curve of TGF-ß activity in the NRK-49F soft agar assay

The presence of TGF-ß biological activity in the RPMI 2650 CM was determined (using the NRK-49F bioassay, see Section 3 3 3 2, below) by comparing two fractions of CM to a standard curve of TGF-ß CM from other cell lines (BSC-1 and MSV-3T3) were also assayed A standard curve of TGF-ß activity was set up as follows TGF-ß was dissolved in DME supplemented with 4 mmol HCl/1 mg/ml BSA Two stock concentrations were made up (100 ng/ml and 10 ng/ml) with DME and from these Samples 1-4 and 5-7 (see Table 3 3 3) were prepared The appropriate controls containing HCl/BSA at the concentration of the stock solutions were assayed along with the prepared TGF-ß standards

RPMI 2650 conditioned medium was concentrated 10x using a 30,000 MW cut-off ultrafiltration membrane (R30) The filtrate was then concentrated 10x using a 5,000 MW cut-off membrane (R5-30) Both these concentrates were diluted back to 1x with MEM Conditioned medium from the cell line BSC-1 (a known producer of TGF-ß), collected in MEM, was included as a positive control This CM was concentrated 10x using a 10,000 MW cut-off membrane and dilutions were made as indicated MSV-3T3 conditioned medium, collected in DME, and concentrated 10x using the 1,000 MW ultrafiltration membrane was also assayed

Activity in the absence of EGF did not exceed control levels (0.02±0 02) for any sample and so is not included This assay was performed twice and the trends were comparable Table 3 3.3 shows the results obtained from a single assay

TABLE 3 3 3 Standard Curve of TGF-ß activity in the NRK-49F soft agar bioassay

Sample	TGF-ß (ng/ml)	% CFE ± SEM	Sample	TGF-ß (ng/ml)	% CFE ± SEM
1	2 0	3 92±0 09	5	0 2ng	1 22±0 03
2	1 5	3 81±0 05	6	0 1ng	0 88±0 03
3	1 0	4 02±0 08	7	0 05ng	0 50±0 02
4	0 5	3 06±0 06			
Control		0 66±0 01	Control		0 36±0 02

TABLE 3 3 4 TGF-ß activity of CM from three different cell lines

	RPMI 2650		BSC-1	MSV-3T3
	R30	R5-30	R10	R1
10x	3 69±0 13	1 49±0 02	2 85±0 06	0 04±0.01
5x	2 47±0 03	0 94±0 01	N D	0 32±0 02
1x	1 65±0 01	0 21±0 01	0 43±0 01	0 13±0.01
Control	0 18±0 01		0 18±0 01	0 31±0 01

The relationship between the log(TGF-ß) concentration and the colony forming efficiency of NRK-49F cells in soft agar is shown in Figure 3 3 2, which illustrates its linearity Comparison of the activity of the different CM samples against this standard curve gives an indication of the TGF-ß-like activity The calculated values are shown in Table 3 3 3, above

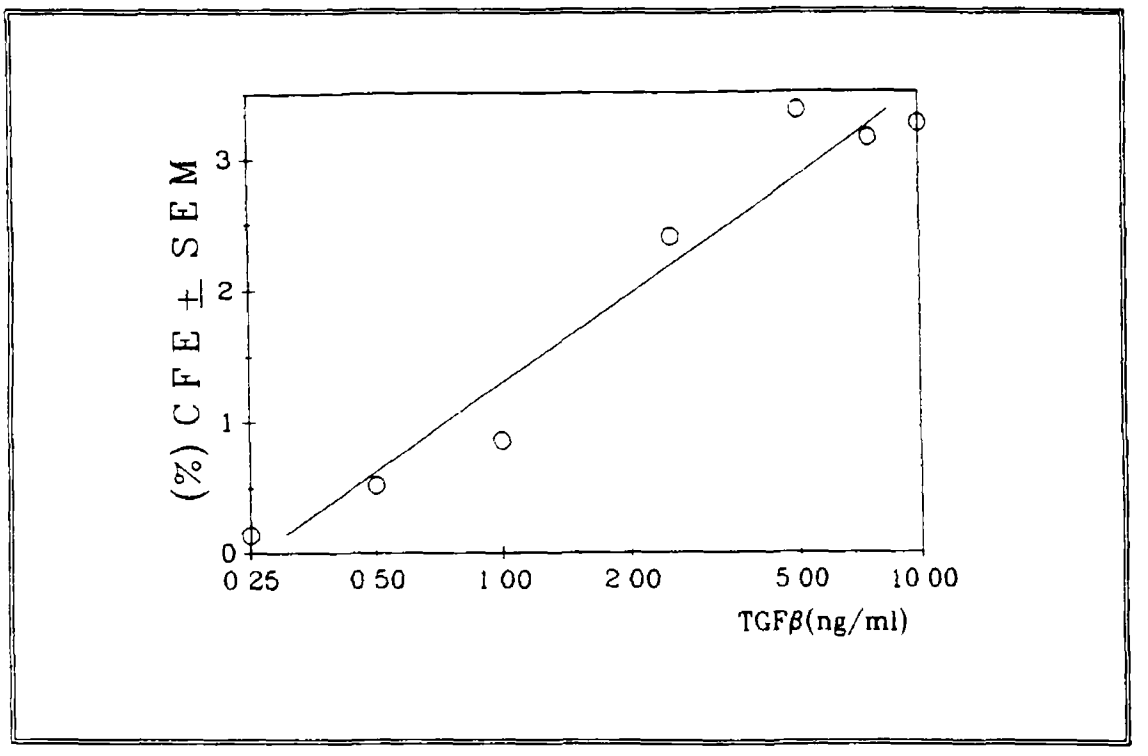


FIGURE 3 3 2 Standard Curve of TGF-ß Activity in the NRK-49F soft agar bioassay in the presence of EGF (2ng/ml)

TABLE 3 3 5 Calculated levels of TGF-ß in CM from different cell lines

Conditioned Media	TGF-ß-like activity (ng/ml)
RPMI 2650	
R30	0 91
R5-30	0 11
BSC-1	0 37
MSV 3T3	0 00

3.3.3 Effect of pure growth factors in the transforming growth factor α and ß soft agar assay systems

3.3 3 1 TGF-α Assay

Stimulation of colony forming efficiency of NRK cell in soft agar is a standard procedure for the measurement of TGF-like activity in conditioned media (Todaro and deLarco, 1978) As demonstrated in Table 3 3 2, the conditioned medium from RPMI 2650 cell exhibited this activity, but TGF-α did not cause stimulation in the autocrine assay system (see Table 3 2 8) The purpose of this experiment was to determine what other growth factors besides TGF-α can elicit a stimulatory response in the TGF-α bioassay

The pure growth factors were made up as described in Materials and Methods and assayed at the concentrations indicated Each experiment was repeated and the results were comparable The data for each repeat experiment are presented in Tables 3 3 6 and 3 3 7

TABLE 3 3 6 Effect of EGF, TGF-α, Bombesin, IGF I and IGF II on colony formation in the NRK soft agar assay

Growth Factor	conc /dish	% CFE ± SEM	
		ASSAY 1	ASSAY 2
EGF	4 0ng	4 71±0 24	16 88±2 02
	2 0ng	6 85±1 01	17 61±0 67
	1 0ng	5 77±1 27	14 80±0 91
TGF-α	50 0ng	7 40±1 75	17 66±0 43
	5 0ng	6 81±1 84	17 10±1 44
	0 5ng	1 51±0 43	8 10±0 34
Bombesin	100 0ng	0 08±0 00	0 00±0 00
	10 0ng	0 00±0 00	0 00±0 00
	1 0ng	0 00±0 00	0 00±0.00
IGF I	100 0ng	0 16±0 00	0 47±0 17
	50 0ng	0 08±0 08	0 10±0 09
	10 0ng	0 00±0 00	0 00±0 00
	1 0ng	0 00±0 00	0 00±0 00
IGF II	100 0ng	0 00±0 00	0 00±0 00
	50 0ng	0 00±0 00	0 00±0 00
	10 0ng	0 00±0 00	0 00±0 00
ATCC+1mg/ml BSA		0 00±0 00	0 05±0 09

TABLE 3 3 7 Effect of EGF, TGF-ß, PDGF, aFGF, bFGF and Insulin on colony formation in the NRK soft agar assay for TGF-like activity

Growth Factor	Conc/ml	% CFE ± SEM	
		ASSAY 1	ASSAY 2
EGF	4 0ng	5 49±1 02	10 93±1 08
	2 0ng	3 80±0 23	7 82±0 93
	1 0ng	1 28±0 28	4 71±1 19
TGF-ß	2 0ng	0 03±0 03	0 03±0 03
	0 2ng	0 03±0 03	0 03±0 03
	0 02ng	0 00±0 00	0 03±0 03
PDGF	2 units	0 00±0 00	0 00±0 00
	control	0 00±0 00	0 00±0 00
aFGF+heparin	50 0ng	0 00±0 00	0 00±0 00
	5 0ng	0 00±0 00	0 00±0 00
	0 5ng	0 00±0 00	0 00±0 00
bFGF	50 0ng	0 00±0 00	0 57±0 39
	5 0ng	0 00±0 00	0 00±0 00
	0 5ng	0 00±0 00	0 00±0 00
Insulin	500 0ng	0 00±0 00	0 00±0 00
	50 0ng	0 00±0 00	0 00±0 00
	5 0ng	0 00±0 00	0 00±0 00
ATCC		0 00±0 00	0 00±0 00
ATCC+1mg/ml BSA		0 00±0 00	0 00±0 00
Heparin		0 00±0 00	0 00±0 00

As expected TGF-α and EGF stimulate NRK cells in agar IGF I was also stimulatory No other growth factor assayed could support NRK colony formation

3 3 3 2 TGF-ß assay

Conditioned medium from RPMI 2650 cells is capable of stimulating NRK-49F cells in the presence of EGF The assay procedure is standard for determination of the presence of TGF-ß It was shown in Table 3 3 5 that RPMI 2650 CM contains levels of TGF-ß-like activity The stimulation due to pure TGF-ß in the autocrine assay was variable (see Tables 3 2 4-6) The aim of these experiments was to determine other growth factors that may cause a similar stimulation to TGF-ß in the NRK-49F bioassay

The growth factors were made up as before and assayed in the NRK-49F soft agar assay with and without 2 ng/ml EGF The results are presented in Table 3 3 8

TABLE 3 3 8 Effect of EGF, TGF-α, bombesin, IGF I and II in the NRK-49F soft agar assay

Growth Factor	conc /dish	% CFE ± SEM	
		+ EGF	- EGF
EGF	4 0ng	1 80±0 67	1 72±0 72
	2 0ng	1 50±0 21	1 18±0 02
	1 0ng	1 62±0 14	1 07±0 28
TGF-α	50 0ng	1 10±0 26	1 03±0 08
	5 0ng	1 52±0 29	0 83±0 08
	0 5ng	1 27±0 01	0 02±0 02
Bombesin	100 0ng	1 17±0 09	0 01±0 01
	10 0ng	1 51±0 17	0 00±0 00
	1 0ng	1 33±0 26	0 00±0 00
IGF I	100 0ng	4 81±0 55	0 47±0 17
	50 0ng	3 33±0 62	0 10±0 09
	10 0ng	2 28±0 55	0 00±0 00
	1 0ng	1 99±0 38	0 00±0 00
IGF II	100 0ng	1 67±0 38	0 00±0 00
	50 0ng	0 98±0 08	0 00±0 00
	10 0ng	1 30±0 29	0 00±0 00
ATCC		0 71±0 26	0 00±0 00
ATCC+1mg/ml BSA		1 28±0 24	0 00±0 00
Control*		1 23±0 14	0 00±0 00

* Control for IGF I and II=1/20 dilution (10 mmol HCl+1 mg/ml BSA) in ATCC+1 mg/ml BSA

TABLE 3 3 9 Effect of EGF, TGF-ß, PDGF, aFGF, bFGF and insulin in the NRK-49F soft agar assay

Growth Factor	conc /dish	% CFE ± SEM	
		+ EGF	- EGF
EGF	4 0ng	0 03±0 00	0 01±0 01
	2 0ng	0 00±0 00	0 00±0 00
	1 0ng	0 02±0 02	0 00±0 00
TGF-ß	2 0ng	2 33±0 25	0 00±0 00
	0 2ng	0 33±0 04	0 00±0 00
	0 02ng	0 02±0 02	0 00±0 00
PDGF	2 units	1 28±0 20	0 01±0 01
	control	Contaminated	0 00±0 00
aFGF+heparin (10ng/ml)	50 0ng	0 00±0 00	0 00±0 00
	5 0ng	0 00±0 00	0 00±0 00
	0 5ng	0 01±0 01	0 00±0 00
bFGF	50 0ng	0 02±0 02	0 57±0 39
	5 0ng	0 00±0 00	0 00±0 00
	0 5ng	0 00±0 00	0 00±0 00
Insulin	500 0ng	0 00±0 00	0 00±0 00
	50 0ng	0 08±0 05	0 00±0 00
	5 0ng	0 08±0 05	0 00±0 00
ATCC		0 00±0 00	0 00±0 00
ATCC+1mg/ml BSA		0 00±0 00	0 00±0 00
Heparin		0 00±0 00	0 00±0 00

Only TGF-ß showed additional stimulation above the control in the presence of EGF The effect of IGF I was further examined with IGF from two separate sources (c f Section 3 3 2) The results are presented in Table 3 3 10

TABLE 3 3 10 Effect of IGF I (from 2 separate sources), IGF II, insulin and TGF-ß in the NRK-49F soft agar assay.

IGF I-P=recombinant IGF from S *cerevisae* (Promega)

IGF I-B=recombinant IGF from E *coli* (Boehringer Mannheim)

Growth Factor	conc /dish	% CFE ± SEM	
		+ EGF	- EGF
IGF I-P	50 0ng	0 48±0 01	0 02±0 02
	10 0ng	0 25±0 01	0 02±0 00
	5 0ng	0 22±0 04	0 01±0 01
	1 0ng	0 08±0 01	0 01±0 01
IGF I-B	50 0ng	0 17±0 06	0 01±0 01
	10 0ng	0 12±0 04	0 00±0 00
	5 0ng	0 14±0 04	0 00±0 00
	1 0ng	0 04±0 04	0 00±0 00
IGF II	50 0ng	0 18±0 03	0 00±0 00
	10 0ng	0 05±0 02	0 00±0 00
	5 0ng	0 12±0 04	0 00±0 00
	1 0ng	0 07±0 02	0 00±0 00
Insulin	50 0ng	0 07±0 00	0 00±0 00
	5 0ng	0 10±0 03	0 00±0 00
	1 0ng	0 07±0 02	0 00±0 00
TGF-ß	0 2ng	0 19±0 13	0 00±0 00
	0 1ng	0 18±0 09	0 00±0 00
	0 02ng	0 20±0 05	0 00±0 00
	0 002ng	0 10±0 06	0 00±0 00
ATCC		0 00±0 00	0 00±0 00
ATCC+1mg/ml BSA		0 04±0 00	0 00±0 00

As in Table 3 3 8, IGF I appears to be stimulatory above the controls in the presence of EGF in this experiment The TGF-ß samples were not particulary stimulatory due to the low concentration assayed, here

3 4 STIMULATION OF RPMI 2650 CELLS IN SOFT AGAR BY CONDITIONED MEDIA FROM VARIOUS CELL LINES

Conditioned media from cell lines that have been shown to possess autocrine activity were tested in the autocrine assay to investigate their effects on the stimulation of colony formation of the RPMI 2650 cells in soft agar

The activity was compared with that obtained using a R1(10x) fraction of RPMI 2650 CM CM from SCC9 and HEP2D cell lines were assayed neat and dilutions were made up with the appropriate media The results are presented in table 4 1

TABLE 4 1 Effect of conditioned media from different cell lines in the RPMI 2650 autocrine assay

RPMI 2650		SCC 9		HEP2D	
R1(10x)	4 46±1 13	Neat	1 81±0 11	Neat	3 10±0 77
R1(5x)	4 15±0 50	½	2 20±0 35	½	2 29±0 63
MEM	2 13±0 57	ATCC	3 24±0 43	DME	2 05±0.35

RPMI 2650 conditioned media stimulated the soft agar growth by more than two fold above the medium control Neat CM from SCC 9 cells was inhibitory in the assay and this inhibition was dilutable CM from HEP2D cells exhibited some stimulation and again this effect was dilutable

3 5 CHARACTERISATION OF THE RPMI 2650 AUTOCRINE STIMULATORY ACTIVITY

3 5 1 Concentration of the autocrine activity

Neat conditioned medium (CM) was sometimes active in the autocrine assay but the level of stimulation was normally quite low and made determination of activity and losses in activity particularly difficult to determine This is illustrated in Table 3 5 1, where the percentage colony forming efficiency of neat and concentrated CM are compared for four independent assays with different batches of CM The CM was concentrated by ultrafiltration with a YM2 membrane (1,000 MW cut-off) to one tenth of its original volume (R1(10x))

TABLE 3 5 1 Comparison of autocrine activity between neat unconcentrated and concentrated conditioned media Fold stimulation is shown in parenthesis

	MEM control	Neat CM	R1(10x)
CM A	0 05±0 12	0 37±0 01 {7 4}	1 48±0 48 {29 6}
CM B	0 04±0 01	0 33±0 07 {8 3}	0 92±0 11 {23 0}
CM C	0 01±0 01	0 12±0 03 {12 0}	0 73±0 06 {73 0}
CM D	0 16±0 04	0 23±0 11 {1 4}	1 45±0 31 {9 1}

Table 3 5 1 illustrates how concentrating the CM can significantly increase the percentage stimulation of the RPMI 2650 cells in soft agar

This table also gives an indication of the variability of both the assay system and the activity levels of the CM over a random number of assays and batches of CM
The backround level varied from minimal growth (CM C) to quite high levels (CM D) This may be indicative of variation in the cells used for the assay, since all other parameters were held constant For each of these assays the cells were pretreated in a similar manner and fed with the same medium type supplemented with the same batch of FCS The passage numbers of the cells were also similar and only varied from passage 39 to 42

The following table illustrates the relationship of concentration of CM to autocrine activity Conditioned medium (CM) was concentrated to the indicated concentrations through a 1,000 MW cut-off ultrafiltration membrane (R1) to 10 times its original concentration (10x) This CM was diluted with medium, as indicated, to show the linearity of autocrine activity in this batch of CM before stability studies were carried out The results are shown in Table 3 5 2 in % C F E ± S E M and the fold stimulation of each sample above the MEM control is also presented

TABLE 3 5 2 Activity curve of conditioned medium Fold stimulation is calculated by dividing by the % CFE of medium (MEM)

	% CFE ± SEM	Fold Stimulation
10x	4 117±0 405	32
8x	3 627±0 865	28
6x	1 941±0 676	15
4x	1 697±0 514	13
2x	0 821±0 257	6
1x	0 459±0 074	4
0 1x	0 181±0 018	1
MEM	0 128±0 064	

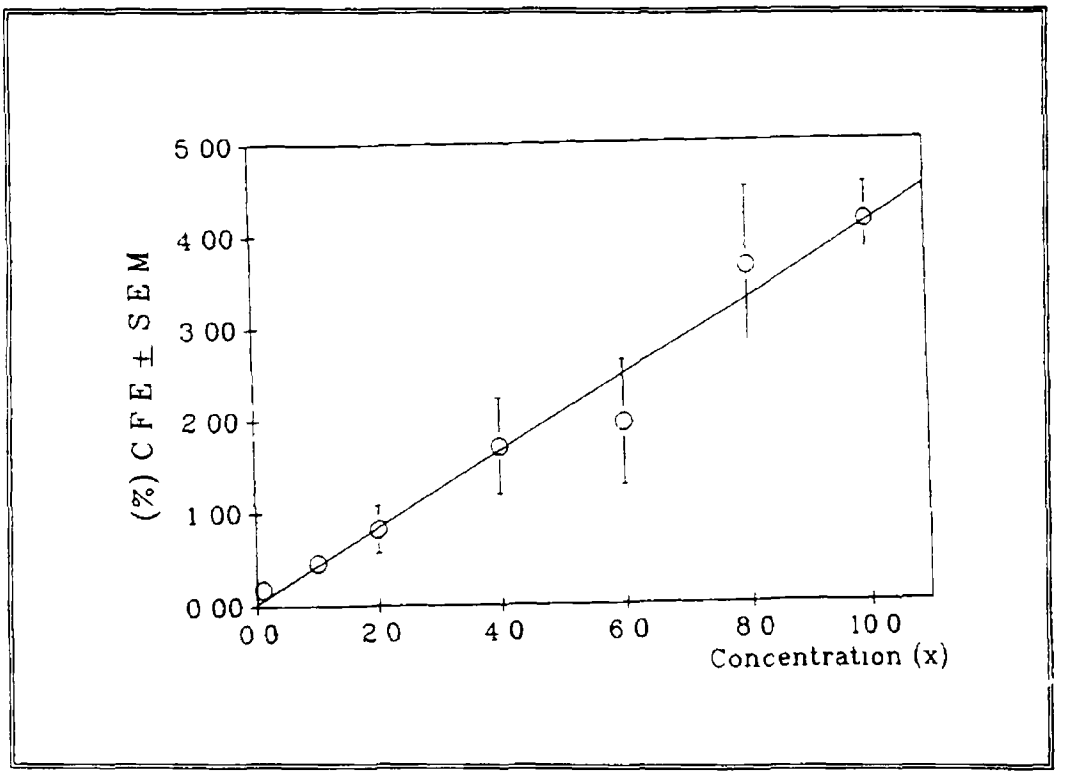


FIGURE 3 5 1 Linearity of conditioned medium activity with concentration

This CM was a very active batch where a 1/10 dilution of CM still retained activity The CM could be diluted down to 7-5x without reducing its activity below 10 fold of the MEM control This CM was used for subsequent stability work

3 5 2 Collection of conditioned medium

The following experiment was set up to determine the optimum collection method of medium conditioned by RPMI 2650 cells

A number of 75cm² flasks were set up with 4 x 10^6 cells on day 1 and fed on day 4 On Day 6 the flasks were rinsed well with PBS and fed with medium without serum The flasks were divided into two sets, Set A and Set B

Set A - 24-hour collection for one week.

The conditioned medium was removed and replaced with fresh serum-free medium on a daily basis for seven days (CM1-7) At each collection one flask of cells was trypsinised and counted The viability of the cells was determined by trypan blue exclusion

Set B - Collection at 24, 48, 72 and 96 hours

The medium was left conditioning for increasing periods of time 48, 72 and 92 hours At each collection (CM 48 hrs-92 hrs) one flask of cells were counted and viability was estimated by trypan blue exclusion

The pH of each lot of CM was measured and the CM was concentrated by ultrafiltration using a CX-10 immersible unit (with a molecular weight cut-off of 10,000) to a concentration of 5x The results are presented in the table below (Table 3 5 3)

TABLE 3 5 3 Comparison of different protocols for collecting CM

	pH	Total Count ($\times 10^7$)	% viability	% CFE±SEM	Fold Stimulation
Set A					
CM 1	7 38	1 81	97	1 28±0 32	12 8
CM 2	7 26	0 91	97	2 36±0 44	23 6
CM 3	7 21	1 48	98	0 97±0 21	9 7
CM 4	6 83	2 76	96	2 08±0 47	20 8
CM 5	6 99	1 83	98	1 88±0 11	18 8
CM 6	7 12	1 67	93	1 31±0 15	13 1
CM 7	6 99	2 31	92	1 27±0 63	12 7
Set B					
CM 48 hrs	7 07	1 17	95	1 85±0 48	18 5
CM 72 hrs	7 03	2 75	96	1 95±0 63	19 5
CM 96 hrs	6 95	1 64	96	0 67±0 24	6 7
MEM				0 10±0 02	

The activities of the CM collected at different times do not appear to vary significantly over a seven day period of collection After the first day of collection, the pH drops below 7 3 while the cell numbers per flask and their viabilities change remain fairly constant The viability of the cultures do not drop

below 92% which is high for cells growing in serum free medium for one week, which presumably is a result of conditioning factors being released by the cells

The reduced activity in CM 96 hrs may be due to protease activity. The concentration of secreted proteases increases the longer the cells are in culture Many proteases may be released from dying cells as they lyse These proteases could act on proteins, including the autocrine factor(s), inactivating them

The pH of the conditioned medium drops below 7 0 in a number of samples, which may destroy acid sensitive autocrine growth factors in the CM (investigated in Section 3 5 4), and result in reduced activity

3 5 3 Temperature Stability

Conditioned medium, concentrated five fold by ultrafiltration using a 1,000 MW cut-off membrane (R1(5x)), was dispensed into glass universals and exposed to the indicated temperatures for the lengths of time shown The samples were then centrifuged, filter sterilised and assayed by the RPMI 2650 autocrine bioassay The results are presented in Table 3 5 4

TABLE 3 5 4 Temperature stability of the autocrine activity

Treatment	% CFE ± SEM	Average activity remaining (%)
4°C	1 230±0 250	100
65°C/1 hour	0 384±0 110	29
100°C/3 mins	0 530±0 280	41
100°C/6 mins	0 256±0 064	18
100°C/20 mins	0 352±0 160	26
MEM	0 043±0 015	

This experiment was repeated on 5 different occasions, each with similiar results Table 3 5 5 shows the average activity retained following heat treatment for the 5 sets of results It shows that 65% of the activity is lost after boiling for 6 minutes

TABLE 3 5 5 Average autocrine activity remaining following temperature treatment

Treatment	Average % Activity Remaining*
37 °C/1 hour	100
65 °C/1 hour	62
100°C/3 mins	61
100°C/6 mins	35

* Calculated by $\frac{R1(\text{treated}) - MEM(\text{treated})}{R1(\text{control}) - MEM(\text{control})}$

These results demonstrate that the autocrine activity is sensitive to heat treatment and that prolonged exposure to high temperatures can further reduce the activity Boiling for 20 minutes does not completely eliminate the activity, indicating that there is a heat stable component in the CM

Temperature stability of the TGF-α-like activity in the CM was also assessed The samples from one set of experiments were assayed in the NRK bioassay for TGF-α-like activity The results are presented in Table 3 5 6 as percentage colony forming efficiency of NRK cells in soft agar

TABLE 3 5 6 Temperature stability of the TGF-α-like activity in conditioned medium using the NRK soft agar assay

Treatment	% CFE ± SEM	Average activity remaining(%)
37°C/1 hour	4 77±0 34	100
65°C/1 hour	2 52±0 24	53
100°C/3 mins	2 41±0 31	51
100°C/6 mins	2 16±0 65	45
MEM	0 00±0 00	

It appears from this experiment that TGF-like activity is more heat stable than the autocrine activity Some TGF activity is lost in the R1 fraction on boiling, but not to the same extent as the autocrine activity

These results indicate that the activity remaining following heat treatment may be due to the presence of TGF-like molecules

3 5 4. pH stability

A R1 sample (concentrated as described previously througha 1,000 MW cut-off membrane to a concentration of 7x) was exposed to pH 3 and pH 9 for 1 hour at 4°C and then readjusted back to pH 7 4. Each sample was freeze-dried and reconstituted to the same volume in water An untreated sample was also freeze-dried and reconstituted as a control The results are presented in Table 3 5 7

TABLE 3 5 7 PH stability of RPMI 2650 autocrine activity

	% CFE ± SEM	
	R1 (7x)	MEM
Untreated	2 04±0 15	0 18±0 05
Freeze-dried	2 42±0 58	0 31±0 05
Acid treated	0 33±0 05	0 07±0 03
Base treated	0 52±0 12	0 07±0 04

This initial experiment indicated that the autocrine activity was somewhat acid and base sensitive, although residual activity remained following incubation at pH 3 and 9

The pH stability was then examined more closely by exposing concentrated CM (R1(5x)) to a range of pH levels from 2 8 to 12 2 5mls of the R1(5x) conditioned media were dispensed out and treated with 5M NaOH and 5M HCl (volumes indicated in Table 3 5 8) The pH of each sample was measured The samples were incubated at 4°C for 2 hours before the pH was readjusted to pH 7 4-7 7 Osmolarity (Osm/kg) was also measured The treatments are summarised in Table 3 5 8

TABLE 3 5 8 **PH stability of RPMI 2650 conditioned medium. Treatment with different volumes of 5M HCl and 5M NaOH**

Sample	Volume added	Test pH	Volume added	Final pH	Osm/kg
1	50µl HCl	2 8	50µl NaOH	7 6	0 325
2	20µl HCl	4 3	15µl NaOH	7 7	0 291
3	10µl HCl	6 8	10µl NaOH	7 5	0 276
4	5µl HCl	7 1	5µl NaOH	7 5	0 288
5	no addition	7 2	no addition	7 2	0 298
6	5µl NaOH	7 6	5µl HCl	7 5	0 294
7	10µl NaOH	8 5	9µl HCl	7 6	0 295
8	20µl NaOH	8 8	12µl HCl	7 6	0 287
9	35µl NaOH	11 0	30µl HCl	7 6	0 325
10	50µl NaOH	12 2	45µl HCl	7 5	0 460

The osmolarity only reached excessively high levels for sample number 10 due to overshooting pH 7 5 when re-adjusting the pH

Each of the samples was filter sterilised and assayed in the RPMI 2650 autocrine assay The resulting colony forming efficiency (%) and the percentage of activity remaining is presented in Figure 3 5 2 and Table 3 5 9

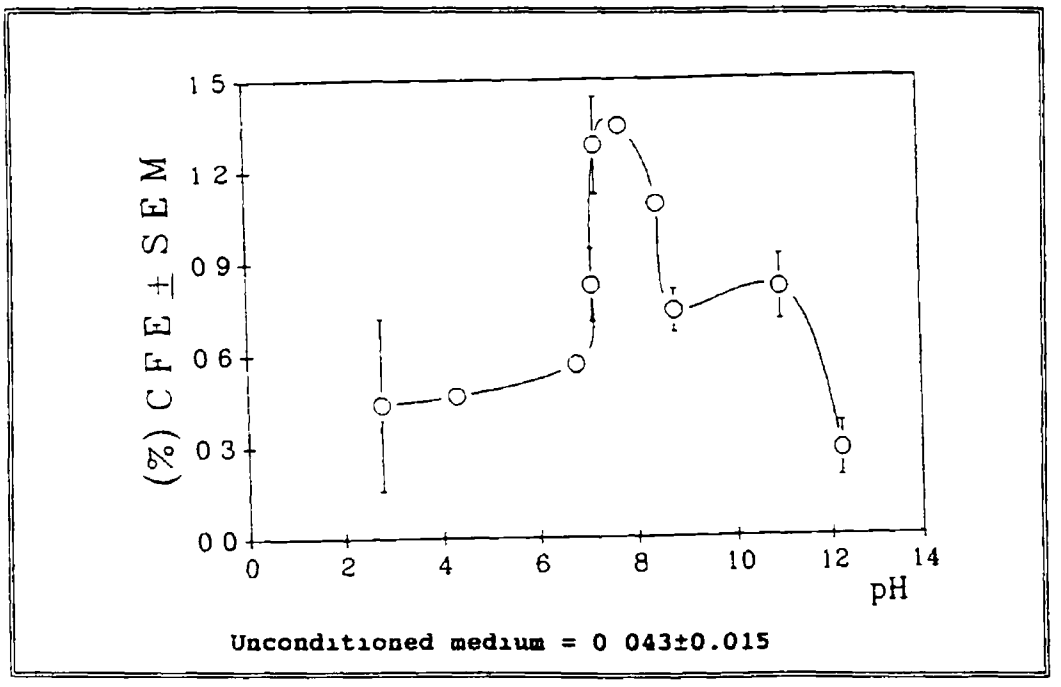


FIGURE 3 5 2 PH stability profile of autocrine activity

TABLE 3 5 9 Percentage of autocrine activity remaining following incubation for 2 hours at different pH levels. The percentage is calculated on the basis of sample 5 (untreated)

Sample	pH	Activity remaining (%)
1	2 78	32
2	4.32	34
3	6.79	42
4	7 13	63
5	7 24	100
6	7 75	105
7	8 47	85
8	8 79	56
9	10.97	62
10	12 24	19

These results show that residual activity is still present in the conditioned media exposed to extreme pH levels Acid treatment

resulted in a residual activity of 32% (based on the untreated sample, number 5), whereas slightly more activity remains following base treatment

The band, within which the autocrine activity is retained at high levels, is quite narrow, (between pH 7 24 and 8 24). This indicates that a proportion of the autocrine activity is particularly sensitive to extremes of pH. The high osmolarity levels in sample 10 may have contributed to its low activity (see Table 3 5 8)

3 5 5 PH stability of the heat stable activity

Conditioned medium was treated as in Section 3 5 3 Each of the boiled samples were exposed to high (pH 12 2) and low (pH 2 8) extremes pf pH for 2 hours (as described in Section 3 5 4) by addition of 30 μl of 5M NaOH or HCl The pH was re-adjusted to pH 7 24-8 24 and the samples were filter sterilised The results are presented in Figure 3 5 3 and in Table 3 5 10 as percentage of remaining activity following treatment

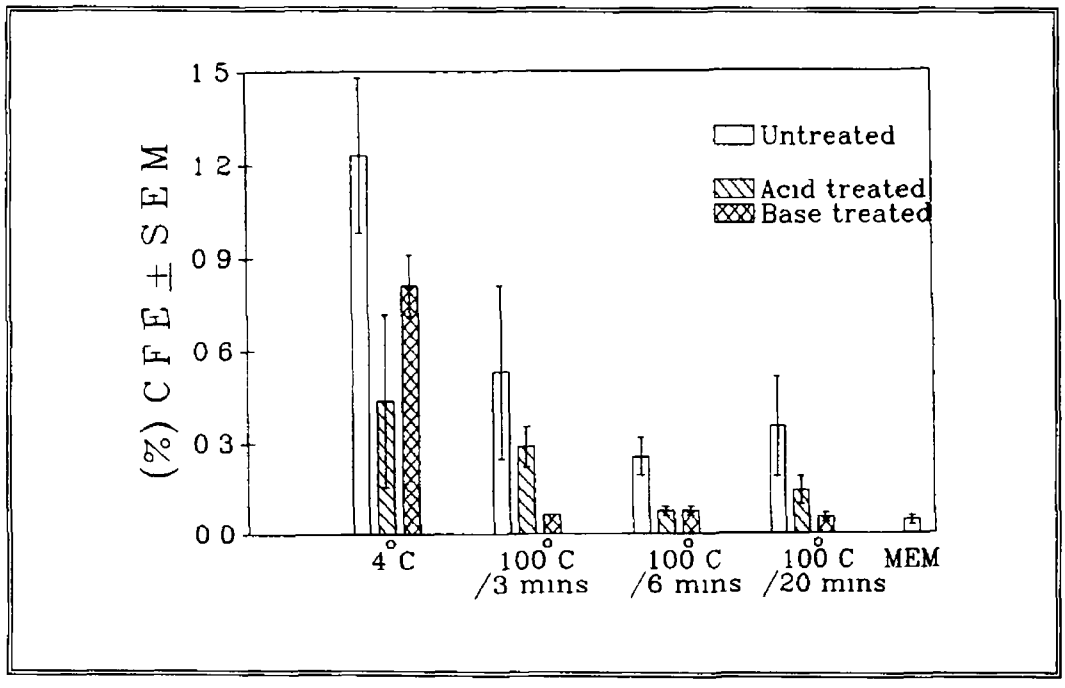


FIGURE 3 5 3 **pH stability of the heat stable activity in RPMI 2650 CM (R1(5x))**

TABLE 3 5 10 pH Stability of heat treated RPMI 2650 conditioned medium (R1(5x)) (% activity remaining following treatment calculated as a percentage of untreated activity, based on the average % CFE for each sample)

	Average % activity remaining		
Sample	Untreated	Acid treated	Base treated
4°C	100	40	65
100°C/3 mins	49	21	2
100°C/6 mins	18	3	3
100°C/20 mins	26	6	1

It can be seen from these results that exposure of the heat treated samples to extremes of pH eliminates the autocrine activity, reducing its activity down to control levels The experiment was repeated with comparable results

3 5 6 Heat stability of the acid- and base- stable activities

Samples 1, 2 and 3 (acid treated) and 8, 9 and 10 (base treated) (from Table 3 5 8) were pooled, boiled for 6 minutes, filter sterilised and assayed in the RPMI 2650 autocrine assay. The resulting % CFE ± SEM and the percentage of remaining activity are shown in Table 3 5 11

TABLE 3 5 11 Heat stability of the autocrine activity in the acid and base treated RPMI 2650 CM (R1(5x)) (% colony forming efficiency ± SEM, % activity remaining following treatment)

	% CFE ± SEM		% Activity Remaining	
Sample	Untreated	100°C /6 mins	Untreated	100°C /6 mins
4°C	1 23±0 25	0 26±0 06	100	18
Acid (Ave)	0 44±0 28	0 25±0 05	38	18
Base (Ave)	0 55±0 36	0 28±0 08	27	24
MEM	0 04±0 01			

The results in Table 3 5 11 indicate that the acid- and base-stable activities are heat stable, as boiling for six minutes does not significantly reduce the activity further

The errors in this experiment were quite large with nearly 50% error This was due to the design of the experiment The values for acid (average) and base (average) samples (untreated) were calculated from the values from samples 1-3 and 8-12 in Figure 3 5 9 These samples had been exposed to a range of pH levels from 6 8-2 8 and 8 8-12 2, respectively Acid and base sensitive factors in the CM may not be inactivated completely by exposure to pH 6 8 or 8 8, and so this experiment was not a true indication of the heat stability of the acid and base stable factors

A second experiment was set up in which the CM was exposed to two extremes of pH before the boiling step
The same batch of R1(10x) was brought to pH 12 2 and 3 4 with 5M NaOH and HCl The samples were incubated for 2 hours at 4°C before being readjusted to physiological levels as before The pH

treated samples were then boiled for 6 minutes The results of the unboiled acid and base treated samples and the boiled samples are presented in the table and figure below

TABLE 3 5 12 Heat stability of the acid and base treated RPMI 2650 CM (R1(5x)) (% activity remaining following treatment)

	% Activity Remaining	
Sample	Untreated	100°C /6 mins
4°C	100	43
Acid treated	54	-1
Base treated	54	23

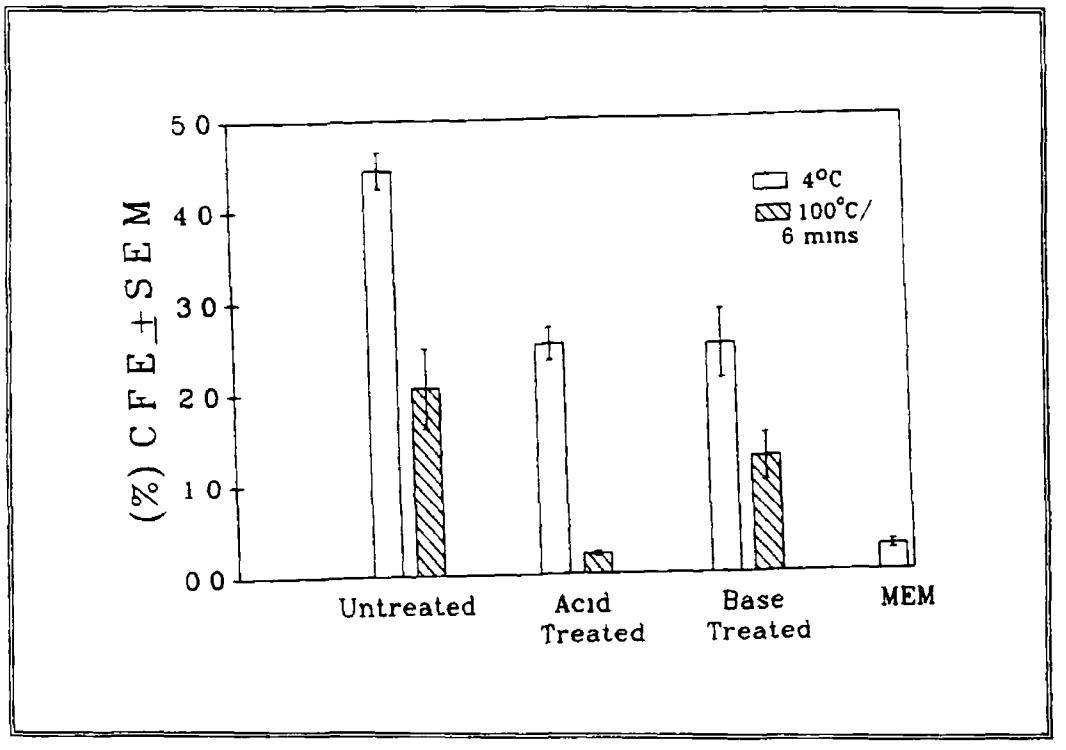


FIGURE 3 5 4 Heat stability of the acid and base stable activities in RPMI 2650 CM (R1(5x))

These results illustrate that the acid- and base- stable entities are sensitive to high temperatures. In both experiments, however, base treated CM is still partially heat stable

The results above also indicate that molecules which retain activity at pH 6 8 are partially heat stable and on their removal (by acid treatment) the remaining activity in the CM can be eliminated by heat treatment

3 5 7 Protease stability of the autocrine activity

The stability of the autocrine effect following treatment by trypsin was examined Trypsin is a specific protease, acting on polypeptide chains by catalysing the hydrolysis of peptide bonds at either lysine or arginine residues

R1(7x) and MEM were exposed to 10 μg/ml trypsin for 2 hours at 37°C before addition of 20 μg/ml trypsin inhibitor Controls for this experiment were a R1 sample incubated with trypsin plus trypsin inhibitor for 2 hours (T + TI) and a R1 sample incubated only with trypsin inhibitor (TI) for 2 hours The results are presented in % CFE ± SEM in Figure 3 5 5 and as the % of activity remaining following treatments in Table 3 5 15

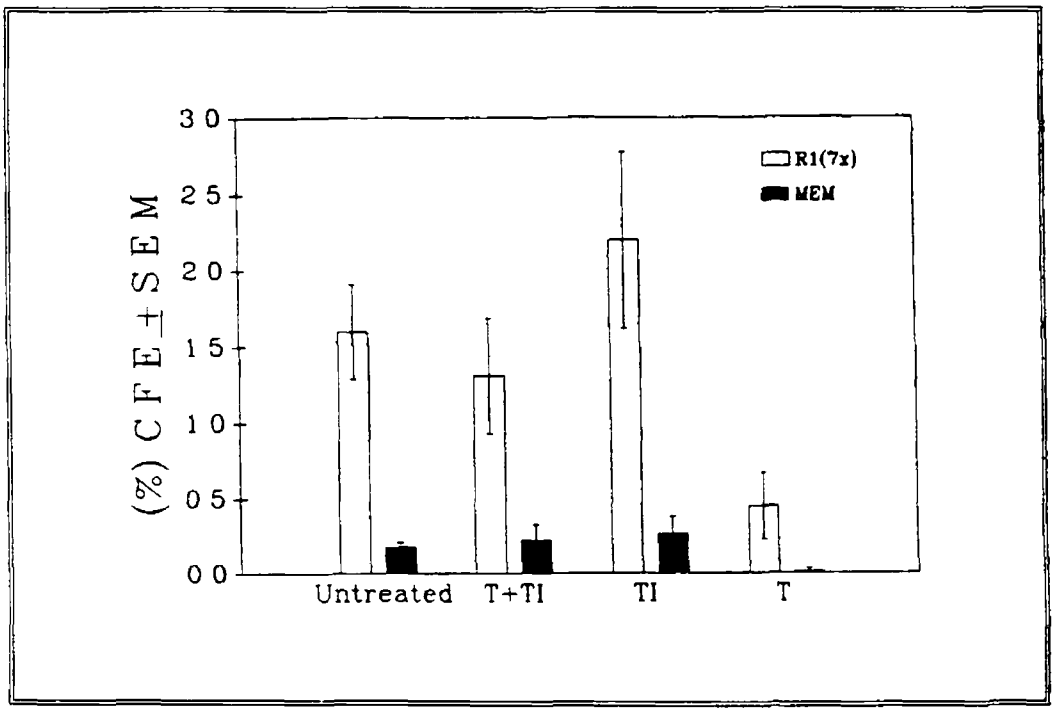


FIGURE 3 5 5 Protease stability of autocrine activity

T = Trypsin (10μg/ml)

TI = Soyabean trypsin inhibitor

(see text for details of controls)

TABLE 3 5 15 Protease stability of autocrine activity

Treatment	% Activity remaining
37°C/2 hours	100
T + TI	76
TI	137
Trypsin	30

In an average of 4 similar experiments the activity lost by exposure to trypsin amounted to 57% of the controls and so it can be considered that the active fraction is at least partially due to protein material

3 6 ULTRAFILTRATION OF THE CONDITIONED MEDIUM

3 6 1 Fractionation of the autocrine activity by ultrafiltration

As shown previously (Section 3 5 1) the autocrine activity can be concentrated using ultrafiltration with a 1,000 MW cut-off membrane Initial size determinations were carried out on conditioned media using different molecular weight cut-off ultrafiltration membranes The conditioned medium was concentrated ten fold and diluted down to 7x, 5x, 3x, and 1x with unconditioned medium (MEM) The results are presented in Table 3 6 1

Notation used

R1, R30 concentrated CM using the 1,000 and 30,000 MW cut-off membranes

F1, F30 filtrates from ultrafiltration of CM through the 1,000 and 30,000 MW cut-off membranes

R1-30 filtrate from the 30,000 MW membrane concentrated through the 1,000 MW membrane (i e concentrated molecules between 1,000 and 30,000 MW)

TABLE 3 6 1 **RPMI 2650 autocrine activity in conditioned medium following ultrafiltration through 1,000 and 30,000 MW cut-off membranes**

Sample	10x	7x	5x	3x	1x
R1	1 25±0 14	0 77±0 09	0 48±0 07	0 22±0 05	0 10±0 05
R1-30	0 25±0 07	0 19±0 05	0 07±0 02	0 07±0 02	0 05±0.02
R30	1 02±0 22	0 52±0 09	0 26±0 03	0 15±0 02	0.03±0.03
F1	0 05±0 02				
F30	0 01±0 02				
MEM	0 02±0 02				

Autocrine activity is present in each of the fractions analysed. R1 and R30 concentrates have comparable activities, while the R1-30 fraction in this particular assay has a lower activity The

relative activities of the R30 and R1-30 fractions seem to be a function of the batch of conditioned medium itself (see also Figures 3 6 1 and 3 8 3)

The R1-30 fraction could be further fractionated into molecules between 1,000 and 10,000, and 10,000 and 30,000 using an ultrafiltration membrane with a 10,000 MW cut-off The results are presented in Table 3 6 2, below

TABLE 3 6 2 **RPMI 2650 autocrine activity in conditioned medium following ultrafiltration through 1,000, 10,000 and 30,000 MW cut-off membranes**

Sample	10x	7x	5x	3x
R1	0 41±0 11	0 62±0 18	0 41±0 03	0 47±0 09
R1-10	0 47±0 13	0 36±0 04	0 14±0 05	0 02±0 02
R10-30	0 38±0 09	0 30±0 08	0 13±0 05	0 09±0 05
MEM	0 04±0 02			

It appears from these results that activity is derived from 3 sets of molecules, One set greater than 30,000 MW, a second set between 10,000 and 30,000 MW and a third set between 1,000 and 10,000 MW The R1 fraction would therefore contain a concentration of each of these entities The previous stability work, therefore, examined physiochemical properties of the bulk conditioned medium

In this section the properties of fractionated CM is investigated. To simplify the problem only two fractions were examined· R30 (containing concentrated molecules over 30,000 MW) and R1-30 (containing concentrated molecules in the range 1-30,000 MW approximately)

3 6 2 Temperature stability of fractionated CM

A batch of conditioned medium was ultrafiltered through a 30,000 MW cut-off membrane down to a concentration of 7x (R30) The filtrate from this ultrafiltration step was then concentrated with a 1,000 MW membrane to 7x (R1-30) This fraction contains molecules between 1,000 and 30,000 MW The same batch of CM was ultrafiltrated with a 1,000 molecular weight cut-off membrane (R1) This concentrated all the molecules greater than 1,000 MW seven fold

Each of these fractions was exposed to a temperature of 100°C for 3 minutes and 20 minutes The samples were then filter sterilised and assayed in the RPMI 2650 autocrine assay The results are presented in Figure 3 6 1 in the form of % CFE ± SEM and in Table 3 6 3 in the form of percentage of activity remaining following the described treatments

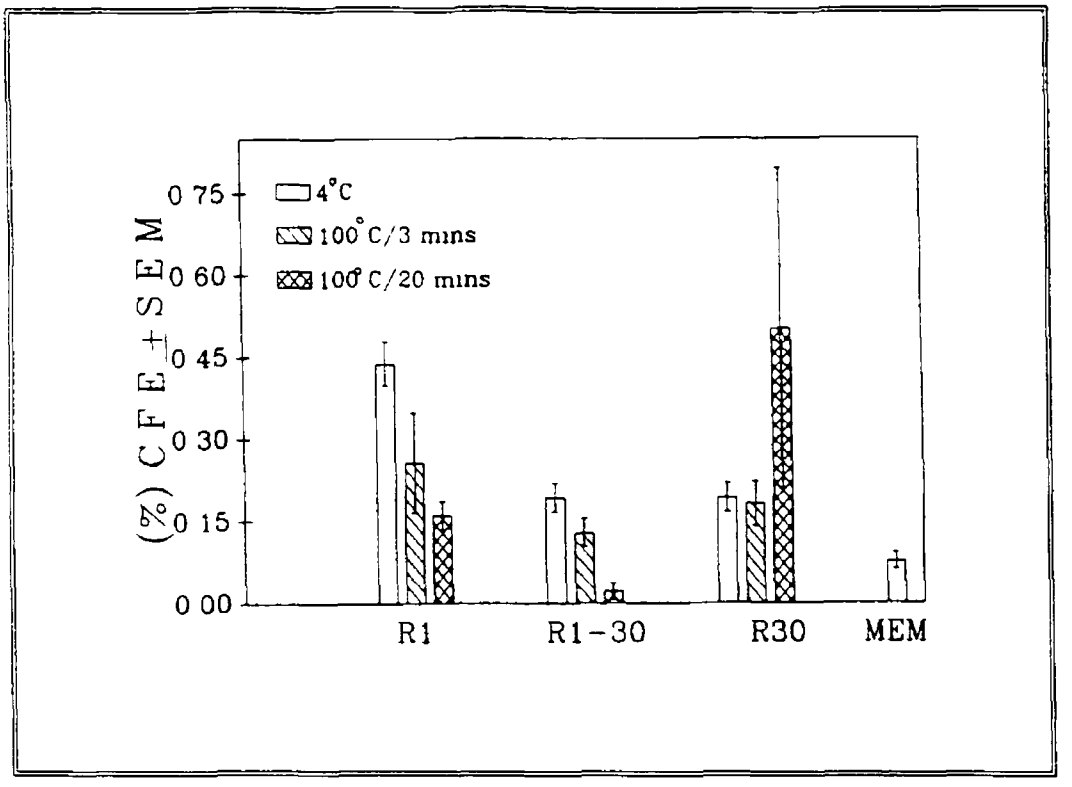


FIGURE 3 6 1 Heat stability of conditioned medium fractionated by ultrafiltration into molecules greater than 30,000 MW (R30), molecules between 1,000 and 30,000 MW (R1-30) and molecules greater than 1,000 MW The results are presented in the form of % colony forming efficiency

Boiling the R1 and R1-30 retentates for 3 minutes resulted in the loss of about half of the activity compared with the untreated samples Prolonged boiling (20 minutes) reduced the remaining activity further in both of these samples

The physiochemical profile for R30 was different Boiling for 3 minutes did not significantly reduce the activity below the untreated sample Further boiling resulted in stimulation of growth, more than doubling the activity found with the untreated control (although the % error in this sample was quite large) In repeat experiments autocrine activity of boiled R30 samples were also higher than control activities

TABLE 3 6 3 Heat Stability of conditioned medium fractionated to 7x by ultrafiltration into molecules greater than 30,000 MW (R30), molecules between 1,000 and 30,000 MW (R1-30) and molecules greater than 1,000 MW (% remaining activity)

Sample	% activity remaining		
	R1	R1-30	R30
4°C	100	100	100
100°C/3 mins	50	45	91
100°C/20 mins	23	-46	364

3 6 3 Acid and base stabilities of the fractionated CM

A similar batch of CM was fractionated into R1-30 and R30 fractions as before Each sample, along with a R1 concentrate, were acid and base treated as in Section 3 5 4 The treated samples were filter-sterilised and assayed in the RPMI 2650 soft agar assay The results are presented in Figure 3 6 2 and in Table 3 6 4

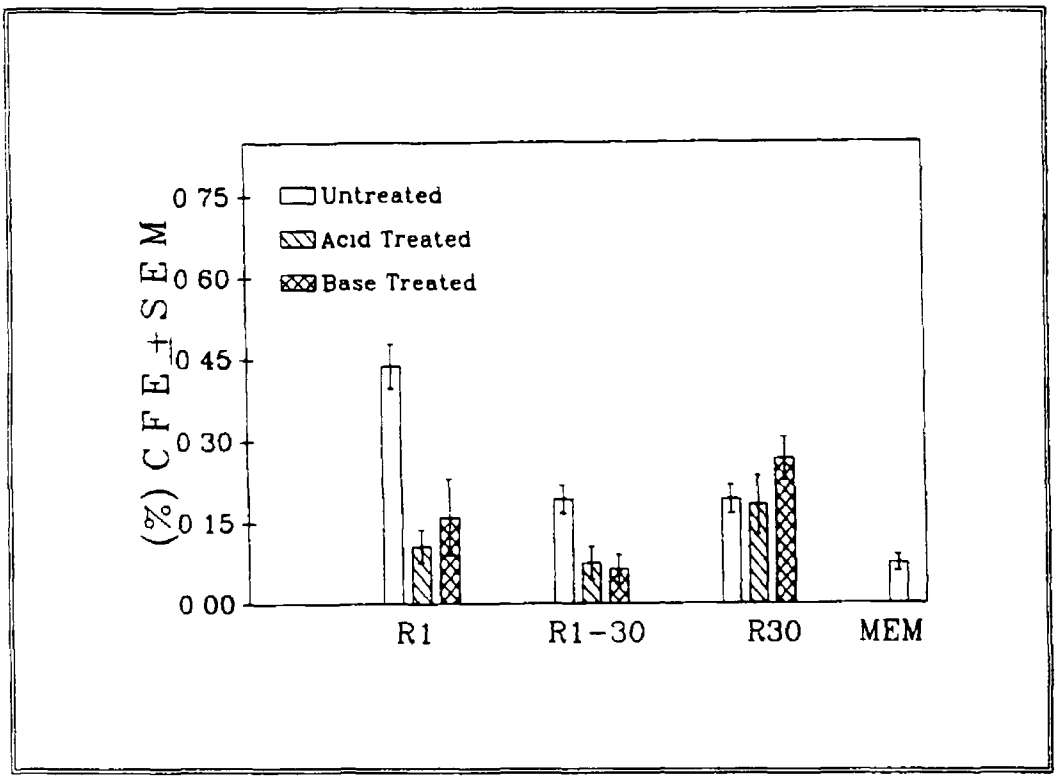


FIGURE 3 6 2 pH stability of conditioned medium fractionated to 7x by ultrafiltration into molecules greater than 30,000 MW (R30), molecules between 1,000 and 30,000 MW (R1-30) and molecules greater than 1,000 MW The results are presented in the form of % colony forming efficiency

TABLE 3 6 4 pH Stability of conditioned medium fractionated 7x 7x by ultrafiltration into molecules greater than 30,000 MW (R30), molecules between 1,000 and 30,000 MW (R1-30) and molecules greater than 1,000 MW (% remaining activity)

	% activity remaining		
Sample	R1	R1-30	R30
Untreated	100	100	100
Acid Treated	9	0	91
Base Treated	23	-9	164

Acid and base treatment significantly reduced activity in the R1 fraction and eliminated the autocrine activity in the R1-30 sample While acid treatment had no significant effect on the R30 fraction, base treatment appeared to enhance it slightly

3 6 4 Boiling R30 fraction at low pH

Samples of R30(7x) and unconditioned medium (MEM) were acid treated (pH 3) and heat treated (boiled for 20 minutes) as before. An identical sample was boiled at pH 3 before being readjusted back to pH 7 4 A fourth sample was acid treated and then boiled for 20 minutes The results are presented in Table 3 6 5

TABLE 3 6 5 Stability of the autocrine effect to boiling under acidic conditions

Sample	% CFE ± SEM	
	R30	MEM
Untreated	0 38±0 03	0 02±0 02
Heat treated	0 33±0 03	0 02±0 03
Acid Treated	0 17±0 05	0 02±0 02
Heat treated at pH 3	0 02±0 02	0 02±0 02
Acid treated, heat treated	0 05±0 02	0 03±0 00

Again in this experiment, the autocrine activity is retained following boiling for 20 minutes Acid treatment, here, however appears to reduce autocrine activity

Boiling the R30 fraction at low pH resulted in complete elimination of activity in a one step procedure

The acid stable activity in the R30 sample is destroyed by boiling, this effect is similar to that observed in the bulk CM (Section 3 5 6) suggesting that the stable activity in the R1 CM may be as a result of the acid stable entity in the R30 fraction

3 6 5 Effect of boiling on the protease stability of the R30 fraction

The unusual heat stability profile of the CM was examined with regard to protease stability Samples of R30 (untreated) and R30 (boiled for 20 minutes) were prepared as before Each sample, along with a medium control (MEM) and medium boiled for 20 minutes, was subjected to the same treatments as described in Section 3 5 7 The results are presented in Figure 3 6 3 and in Table 3 6 6 as the average percentage of activity remaining following treatment

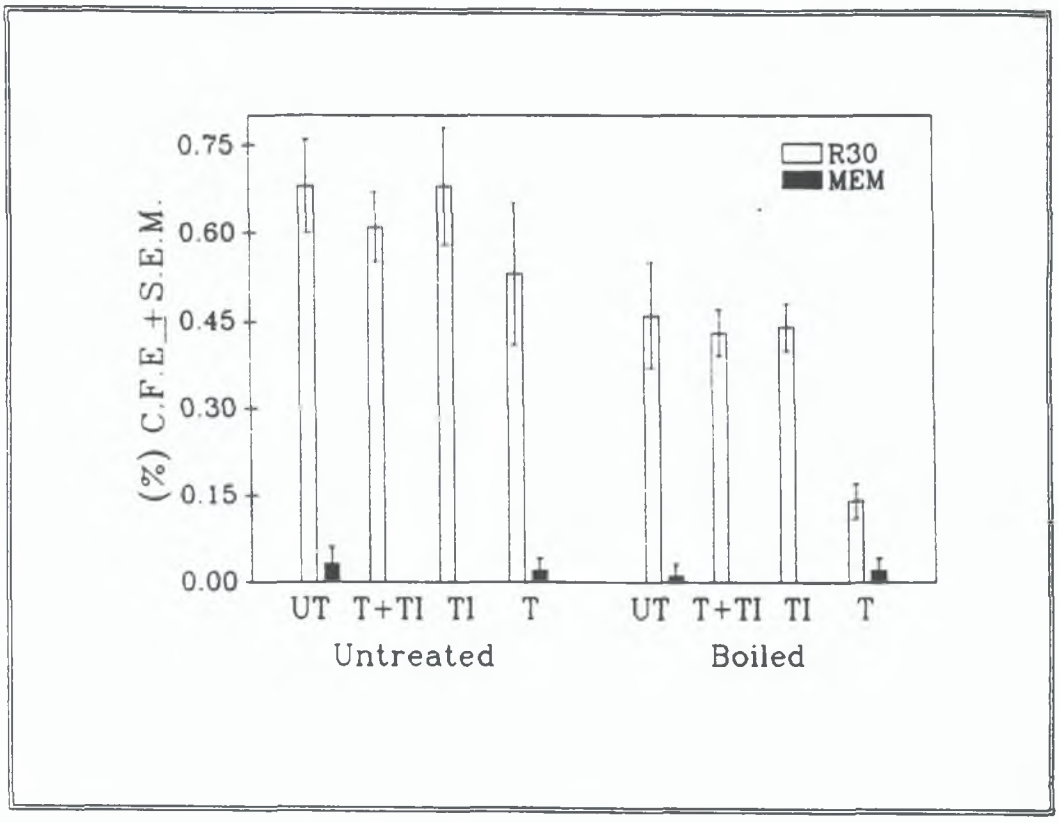


FIGURE 3.6.3 **Comparison of the protease stabilities of R30 fractions before and after boiling for 20 minutes**

TABLE 3.6.6 **Comparison of the protease stabilities of R30 fractions before and after boiling for 20 minutes**

Treatment	% activity remaining	
	R30	R30 boiled
Untreated	100	100
Trypsin (T)	78	27
T + T inhibitor	90	96
T inhibitor	100	98

This experiment was repeated with comparable results.

No activation above the level of stimulation of the R30 sample was observed on boiling, but most of the activity was retained

Unboiled R30 is not particulary sensitive to trypsin, this might account for the residual 30 % activity on protease treatment of the bulk R1 CM (Section 3 5 7)

Boiled R30 is trypsin sensitive, but not all its activity is removed Trypsin acts by hydrolysing the peptide bonds at either lysine or arginine This result would suggest that either boiling removes a binding moiety that blocks these residues leaving the molecule more susceptible to protease attack or boiling, results in loss of a conformationally stable form, exposing sites in the molecule where the trypsin can act

3 6 6 Diafiltration of the R30 and boiled R30 samples

The aim of this experiment was to investigate the hypothesis that a large binding molecule is removed from the R30 autocrine factor on boiling resulting in an active fraction with a lower molecular weight

In this experiment R30 and a boiled sample of R30 were diafiltered using a 30,000 MW cut-off membrane Their filtrates were concentrated with the 1,000 MW cut-off (Filt-R1-30) In this way, if the R30 fraction is broken down into smaller active components the activity will be concentrated in Filt-R1-30 fraction, rather than the R30 fraction The results are shown in Table 3 6 7

TABLE 3 6 7 Diafiltration of the R30 and boiled R30 fractions. All samples were concentrated to 10x

Treatment	% CFE ± SEM	
	R30	R30 boiled
Untreated	0 38±0 03	0 33±0 03
diafiltered R30	0 14±0 04	0 11±0 05
Filt-R1-30	0 25±0 02	0 16±0 03
Control (MEM)	0 02±0 02	

Again in this experiment, while no activation occurs, the results demontrates that the R30 activity is retained following extensive boiling

Diafiltration reduces the activity of both the boiled and unboiled R30 samples This may be due to the presence of molecules in the range 1,000-30,000 MW being removed from the R30 fraction by diafiltration where they are present at a 1x concentrations These molecules were then concentrated in the Filt-R1-30 fraction, where their activity was detected

The boiled R30 fraction exhibited lower levels of activity in the Filt-R1-30 concentrate This is due to the inactivation of the smaller (temperature sensitive) molecules by boiling for 20 minutes

It does not appear from these results that boiling causes a large molecule to break down to smaller sized molecules (less than 30,000 MW), since the trend of the activity remains the same before and after boiling (except for the loss in activity of the heat labile R1-30 fraction) It is possible however that removal of a binding moiety may result in active molecules greater than 30,000 MW which will not be revealed by this experiment

It is unlikely, therefore, that boiling alters the R30 fraction in a way that would render the molecule similar to the R1-30 fraction It may be assumed therefore that at least two distinct entities are involved in the autocrine activity of the CM

3 6 7 Fractionation of the TGF-α-like activity

The samples from the fractionation experiment (Table 3 6 1) were also assayed in the NRK soft agar assay for the detection of TGF-α-like activity The results are presented in Table 3 6 8 as % colony forming efficiency of NRK cells

TABLE 3 6 8 TGF-α-like activity in conditioned medium following ultrafiltration through 1,000 and 30,000 MW cut-off membranes

Sample	10x	7x	5x	3x	1x
R1	N D	0 53±0 15	0 21±0 15	0 11±0 08	0 00±0 00
R1-30	0 08±0 08	0 00±0 00	0 00±0 00	0 00±0 00	0 00±0 00
R30	0 00±0 00	0 00±0 00	0 00±0 00	0 00±0 00	0 00±0 00
F1 (1x)	0 00±0 00				
F30(1x)	0 00±0 00				
MEM	0 00±0 00				

N D = Not Determined

Activity appears to manifest only in the R1 fraction but it is lost when the R1 is fractionated into large and small molecules. This would make it seem that the activity is dependant on the presence of both fractions and so is a more complicated event than simply the presence of TGF-α-like molecules

3 6 8 Fractionation of the TGF-ß-like activity

The samples from the fractionation experiment (Table 3 6 1) were also assayed in the NRK-49F soft agar assay for the detection of TGF-ß like activity The results are presented in Table 3 6 9 as % colony forming efficiency of NRK-49F cells in the absence of EGF and Table 3 6 10 in the presence of 2 ng/ml EGF

TABLE 3 6 9 Stimulation of NRK-49F in soft agar without EGF by conditioned medium following ultrafiltration through 1,000 and 30,000 MW cut-off membranes

Sample	10x	7x	5x	3x	1x
R1	0 13±0 05	0 00±0 00	0 00±0 00	0 00±0 00	0 00±0 00
R1-30	0 00±0 00	0 00±0 00	0 00±0 00	0 00±0 00	0 00±0 00
R30	0 00±0 00	0 00±0 00	0 00±0 00	0 00±0 00	0 00±0 00
F1 (1x)	0 00±0 00				
F30(1x)	0 00±0 00				
MEM	0 00±0 00				

N D = Not Determined

TABLE 3 6 10 Stimulation of NRK-49F in soft agar with 2 ng/ml EGF by conditioned medium following ultrafiltration through 1,000 and 30,000 MW cut-off membranes.

Sample	7x	5x	3x	1x
R1	47 92±9 34	39 78±13 21	N D	7 03±1 69
R1-30	0 00±0 00	0 00±0 00	0 00±0 00	0 00±0 00
R30	25 38±2 49	19 57±0 00	14 02±3 09	2 91±0 29
F1 (1x)	N D			
F30(1x)	N D			
MEM	0 00±0 00			

N D = Not Determined

TGF-β activity in the R1 fraction appears to be derived from molecules greater than 30,000 MW, and this activity is dilutable The reduction in activity between R1 and R30 however, maybe due to some synergy with factors in the R1-30 fraction This result supports the results presented previously in Section 3 3 2

3 7 PURIFICATION OF THE RPMI 2650 AUTOCRINE ACTIVITY

A number of strategies were employed to attempt to partially purify the RPMI 2650 autocrine activity Purification methods included ion exchange chromatography, hydrophobic interaction chromatography and heparin sepharose chromatography

3 7 1 Effect of dialysis on the autocrine activity

In the course of this part of the thesis a number of purification procedures were examined which required dialysis of concentrated conditioned media Comparison between the activity of dialysed and undialysed concentrated CM (to the indicated levels by ultrafiltration through a 1,000 MW cut-off membrane) is presented in Table 3 7 1 for four independent assays

TABLE 3 7 1 **Effect of dialysis on different batches of CM in a number of different assays**

Assay	R1	% CFE ± SEM			% Activity Remaining after dialysis
		Undialysed	Dialysed	Control	
1	14x	5 08±0 38	2 90±0 10	0 40±0 04	53%
2	10x	2 17±0 35	0 36±0 07	0 13±0 06	11%
3	10x	1 48±0 84	0 47±0 43	0 05±0 12	29%
4	7x	1 72±0 32	0 37±0 25	0 05±0 12	20%

As can be seen from these results, substantial loss in activity occurs on dialysis This may be due to a number of factors

(a) Autocrine Activity may "stick" to the dialysis bags Following dialysis the tubing had adsorbed quite an visible amount of phenol red. It is possible that the autocrine activity may adsorb to the membrane in a similar manner

(b) Inadaquate washing of dialysis tubing The dialysis tubing is stored at 4°C in benzoate preservative Before use, the tubing is boiled extensively in 10mM EDTA and then rinsed well in ultrapure water Insufficient rinsing of these compounds could perhaps result in reduced cell growth

(c) Removal of molecules less than the cut-off MW of the membrane. The MW cut-off of the tubing used was 1,200 daltons This should ensure that most of the active molecules greater than 2,000 MW will be contained in the dialysis bags

3 7 2 Effect of dialysis on TGF-α activity

Dialysis is a common procedure in published purification protocols for TGFα To investigate if the failure to dialyse the autocrine activity may be due to either material or technical problems, TGF-α activity levels were detemined before and after dialysis for a similar sample of CM

CM concentrated to 10x by ultrafiltration with a 1,000 MW cut-off membrane was dialysed using the same dialysis tubing as in Section 3 7 1 (1,200 MW cut-off) Dilutions of the dialysed and undialysed samples were assayed in the TGF-α soft agar bioassay to determine if dialysis would cause a reduction in activity The results are presented in Table 3 7 2

TABLE 3 7 2 Dialysis of TGF-α activity in RPMI 2650 CM

R1	% CFE ± SEM	
	Undialysed	Dialysed
10x	2 85±0 71	2 77±0 00
5x	1 34±0 29	1 98±0 36
1x	0 04±0 05	0 00±0 00
MEM	0 00±0 00	

This result shows that CM can be safely dialysed without loss of TGF-α activity This experiment was carried out independently from the assays in Table 3 7 1, with different batches of CM and dialysis tubing, the procedure, however, was identical This result suggests that the washing procedure of dialysis tubing is adequate, discounting possibility (b) in Section 3 7 1, although it does not rule out the possibility that there may be a fault in the particular batch of dialysis tubing used for the autocrine assays

3 7 3 Ion-Exchange Chromatography

Conditioned media concentrated 14x by ultrafiltration with a 1,000 MW membrane were incubated with anion exchange (Cellex-D) and cation exchange (Cellex-CM) gels Fractions were equilibrated with different pH buffers Each fraction was dialysed before being filter sterilised and assayed in the autocrine assay The results are presented in Table 3 7 3

TABLE 3 7 3 Autocrine activity following treatment with ion-exchange resins (% CFE ± SEM)

Controls		Anion exchanger (DEAE)		Cation Exchanger (CM)	
		pH	% CFE±SEM	pH	% CFE±SEM
R1(14x)	5 08±0 38	5 0	0 19±0 09	4 0	0 13±0 01
R1(7x)	4 58±0 42	5 6	0 20±0 06	4 4	0 02±0 01
R1(14x) dialysed	2 90±0 10	6 0	0 30±0 10	4 8	0 08±0 03
		6 4	0 22±0 03	5 2	0 09±0 02
MEM	0 40±0 04	6 8	0 21±0 07	5 6	0 05±0 03
		7 1	0 10±0 02	6 0	0 02±0 01
		7 4	1 91±0 40	6 4	0 04±0 03
		7 8	0 11±0 03	6 8	0 03±0 01
		8 2	0 06±0 04	7 2	0 07±0 06
		8 6	0 03±0 01	7 9	0 06±0 02
		8 9	0 04±0 02	8 0	0 04±0 03

The conditioned media used in this experiment was particularly active A 14x concentrate exhibited high autocrine activity (12 7 fold above the control) Diluting this activity down to 7x showed comparable activity This apparently is the upper limit of the linearity of this CM sample

Dialysing the R1(14x) concentrate in the same way as the samples from the ion exchange column resulted in a reduction of autocrine activity of (on average) 53% This is discussed in Section 3 7 1.

Activity was below control levels for most of the samples assayed The one exception to this was the sample, DEAE(7 4), where 32% of the activity was eluted

The inability of this procedure to concentrate activity may be due to a number of reasons Firstly the autocrine activity is particulary pH sensitive and exposure to the pH extremes used in the ion exchange chromatography, would undoubtedly eliminate the activity Although from the stability experiments it would be expected that the activity would be reduced by about 20% of the untreated sample In these samples from the ion-exchange column the activity is less than the MEM control This indicates that the loss in activity in the CM is not due solely to the adverse pH conditions Secondly, each of the samples were dialysed It was shown in section 3 7 1 that dialysis does have a detrimental effect on the autocrine activity A combination of these two effects may result in complete inactivation

3 7 4 Hydrophobic Interaction Chromatography

A 4ml phenyl sepharose column was equilibrated for 1 hour with MEM that had been concentrated to 4x by ultrafiltration using a YM 2 (1,000 MW cut-off) membrane 10 mls of R1(10x) was applied and then eluted with 50% glycerol in 5x MEM 4ml fractions were collected Each fraction was dialysed against PBS for 24 hours and MEM for 12 hours In some samples this resulted in a volume change The experimental details of the diaysis step and the optical densities measured at 280nm are shown in Table 3 7 4 The samples were then assayed in the RPMI 2650 soft agar autocrine assay The activities are presented in % CFE ± SEM in Table 3 7 5

TABLE 3 7 4 Dialysis of fractions from hydrophobic interaction column and a CM control OD(280) are also presented.

Sample	Initial Volume	Final Volume	OD 280
Wash			
1	3 7	3 7	0 939
Sample Breakthrough			
2	4 0	3 7	0 804
3	3 9	3 5	0 879
4	3 8	3 5	0 736
Elution with 50% Glycerol			
5	3 9	3 4	0 825
6	3 9	3 4	0 603
7	3 0	7 0	0 531
Control			
R1(10x) dialysed	3 5	3 5	1 125

TABLE 3 7 5 Autocrine Activities (% CFE ± SEM) of fractions 1-7 (see Table 3 7 4) from the hydrophobic interaction (phenyl sepharose) column

Controls	% CFE ± SEM	Sample	% CFE ± SEM
R1 (10x)	2 17±0 35	1	0 09±0 04
R1 (2x)	0 54±0 03	2	0 10±0 03
R1 (10x) dialysed	0 36±0 07	3	0 11±0 02
R1 (2x) dialysed	0 34±0 08	4	0 05±0 04
		5	0 14±0 05
MEM	0 13±0 06	6	0 08±0 04
MEM(4x)	0 18±0 02	7	0 20±0 05

The conditioned media used in this experiment was quite active giving on average a 12 fold stimulation above that of the MEM (4x) control Again following dialysis this activity is reduced to approximately 2 fold stimulation

It appears from this experiment that some activity has an affinity for the column and is retained after extensive washing, although the eluted activity is not as high as the CM controls This, however, may be due to the dialysis step

3 7 5 Heparin Sepharose Chromatography

3 7 5 1 Step Elution

100 mls of conditioned medium was incubated overnight with 4mls of heparin sepharose gel at 4°C (stirred) The heparin sepharose gel was allowed to settle for 30 minutes and the supernatant was removed (approximately 90 mls) The gel was washed with 60mls of 0.01M Potassium Phosphate buffer and loaded into a column The column was washed through with a further 30mls of buffer This 180 mls (approximately) constituted the unbound sample and was ultrafiltered down to 10 mls with a 1,000 MW cut-off membrane to give a unbound fraction concentrated 10 fold from its original volume (100 mls)

Batches of 20mls of buffer containing 0, 0 25, 0 5, 0 8, 1 0, 1 2, 1.5, 2 and 3M NaCl were passed sequentially through the column 4 ml fractions were collected and OD(280) was read Each fraction was filter sterilised before assaying in the RPMI 2650 autocrine assay. The results are presented in Table 3 7 6 and Figure 3 7 1

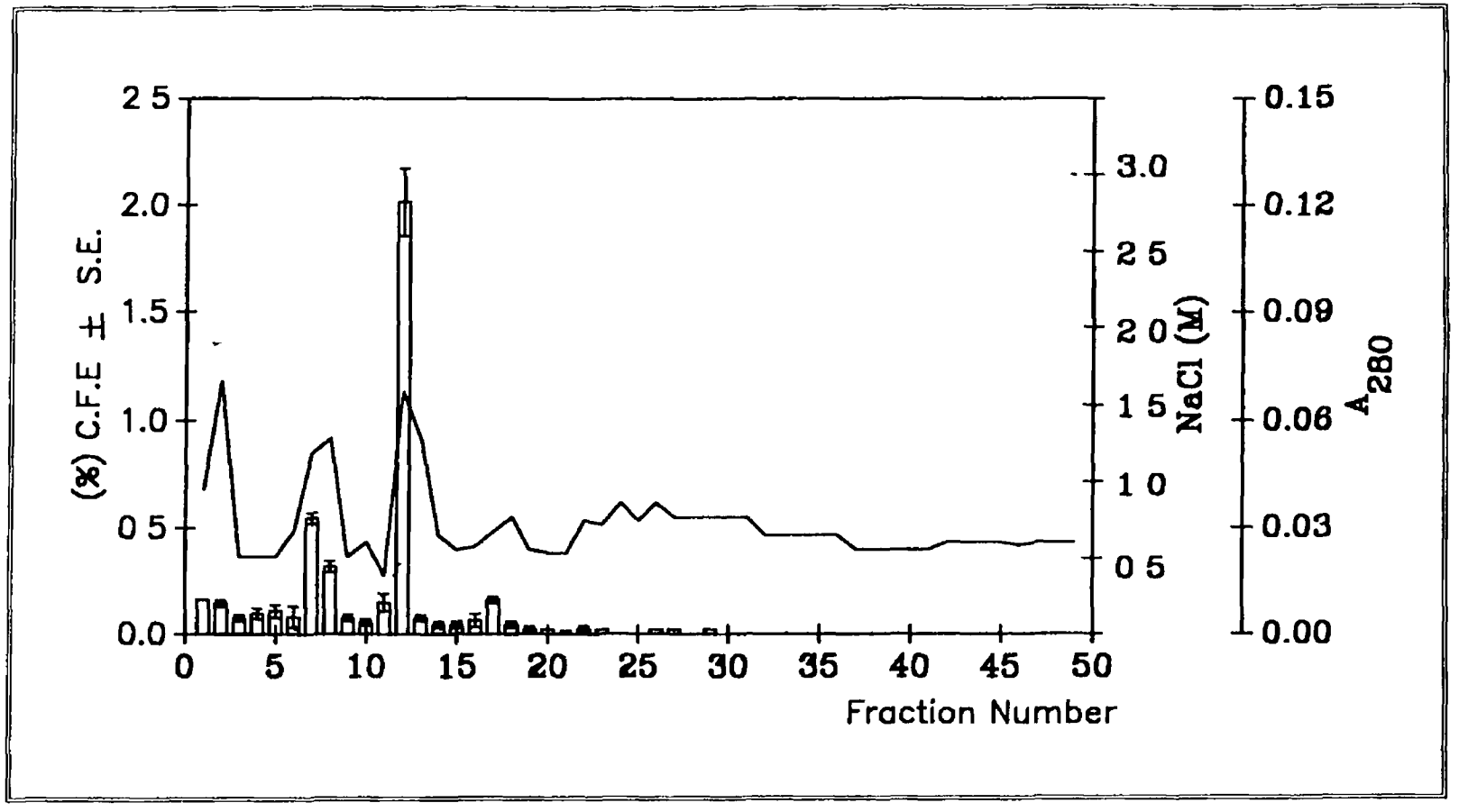


FIGURE 3 7 1 Heparin Sepharose Chromatography of RPMI 2650 CM by step elution (bar= % CFE ± SEM, dotted line= NaCl (M), Continous line = OD280) The optical density (280 nm) for neat CM was equal to 0 858 and unbound fraction (10x), 1 154)

TABLE 3 7 6 Autocrine activity of non-heparin binding autocrine activity (dilution curve), concentrated CM (R1), unconcentrated CM (CM(neat)), and unconditioned medium (MEM) The bound activity was eluted stepwise and its activity is presented in Figure 3 7 1

Controls	% CFE ± SEM	Unbound	% CFE ± SEM
R1(7 5x)	1 429±0 454	(10x)	0 288±0 045
R1(2 5x)	0 192±0 032	(7 5x)	0 288±0 064
R1(1x)	0 171±0 033	(5x)	0.171±0 037
CM(neat)	0 139±0 018	(2 5x)	0 064±0 032
MEM	0 000±0 000	(1x)	0 032±0 032

Conditioned medium which had no affinity for heparin sepharose was found to exhibit autocrine stimulation when concentrated with a 1,000 MW cut-off membrane The level of stimulation was not as high as the conditioned medium that had been concentrated 7 5x by ultrafiltration , indicating the possiblility of some autocrine activity being retained on the column

Eluting the column with increasing salt concentration resulted in a number of activity peaks A small peak of autocrine activity was isolated at a salt concentration of 0 25M NaCl and a larger peak was eluted at 0 5M NaCl Above 1M NaCl little or no growth was observed Similar results were obtained over four repeat experiments

Control levels of growth in this assay was particularly low and so discriminating between inhibition and lack of stimulation was difficult

3 7 5 2 Diafiltration of fractions eluted from heparin sepharose

To see if any autocrine activity was eluted from the heparin sepharose gel at salt concentrations higher than 1M NaCl, but whose activity was obscured due to the high salt concentration, the following diafiltration experiment was undertaken

150mls of RPMI 2650 conditioned medium was incubated overnight at 4°C (stirred) with 8mls of heparin sepharose gel The gel was allowed to settle and 150mls of supernatant was drawn off This was then concentrated by ultrafiltration to 15mls (10x) The remaining supernatant was removed and the gel washed well with buffer Two 15ml fractions were collected following elution with 15 mls of 0 5M NaCl and 3M NaCl 150 mls of the same batch of conditioned medium was also concentrated down to 15 mls by ultrafiltration using a 1,000 MW cut-off membrane

10 mls of each of the above samples (R1, Unbound, 0 5M and 3M eluants) were added to 100mls of MEM and ultrafiltrated down to 10 mls This was repeated resulting in a overall reduction in salt concentration of 1/121 Each sample was filter sterilised and assayed in the RPMI 2650 autocrine bioassay The results are presented in Table 3 7 7

TABLE 3 7 7 Effect of diafiltration on autocrine activity of samples eluted from the heparin sepharose chromatography column Each sample is a 10x concentrate (see text)

Sample	% CFE ± SEM	
	Untreated	Diafiltrated
R1	5 55±0 93	5 27±0 41
Unbound	1 05±0 21	1 05±0 10
0 5M fraction	1 03±0 42	1 06±0 27
3M fraction	0 00±0 00	0 46±0 29
MEM	0 12±0 04	

It can be seen from these results that the high salt concentration does effect the autocrine activity of the fraction eluted at 3M NaCl

The untreated 3M eluate showed considerable inhibition below the level of the control But diafiltrating this sample resulted in growth equivalent to control levels (when SEM is taken into account) This would suggest that little or no autocrine stimulation was eluted at salt concentrations higher than 0 5M NaCl Alternatively it might mean that the fraction tightly bound to the heparin requires some entity in either the 0 5M fraction or the unbound fraction to elicit an autocrine response

This experiment also shows that the autocrine activity can be safely diafiltered without loss in activity In cases where desalting is essential, diafiltration may be considered a viable alternative to dialysis

3.7 5 3 Gradient elution

Heparin sepharose chromatography of the CM was further examined by applying a similar sample (10 mls) of concentrated CM (R1(14x)) to a 10 ml heparin column using the automated Biopilot System (FPLC) The column was pre-equilibrated in phosphate buffer (pH 7 4) as before A program was set up (described in detail in Material and Methods) so that, following extensive column washing, a gradient from 0 to 1 5 M NaCl in phosphate buffer eluted off bound activity 6 ml fractions were collected and filter sterilised before assay

The column was run at room temperature so a CM control (R1(14x)) was left at the same temperature for the duration of the run

The activity was low in all the samples assayed Phenol red was eluted over 3 fractions in a volume of approximately 18 mls, this is equivalent to a 1/1 8 dilution Presuming the unbound fraction is diluted in a similar manner, it is possible that, at this concentration level, its autocrine activity could not be detected

The bound fraction is eluted by a salt gradient, but no detectable autocrine activity was measured It is possible that either the activity was diluted out below detection level or else running the Biopilot at room temperature inactivated the labile component of the autocrine activity (see later)

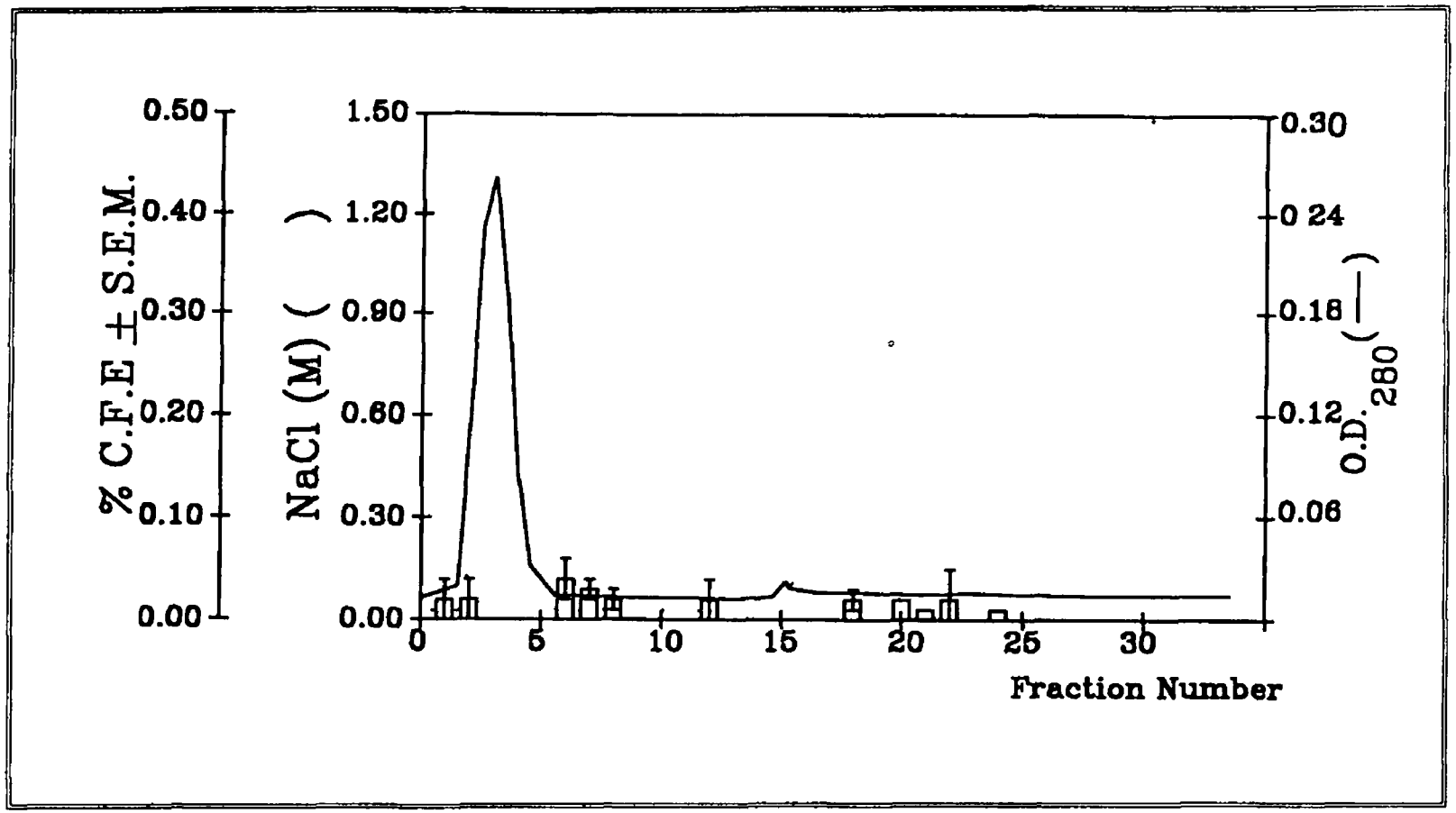


FIGURE 3 7 2 Heparin sepharose chromatography of RPMI 2650 CM eluted by gradient elution

3 8 FURTHER CHARACTERISATION OF HEPARIN BINDING AND NON-HEPARIN BINDING FRACTIONS

3 8 1 Temperature stability of fractions eluted from heparin sepharose

Samples of unbound and 0 5M eluate were prepared by step elution These samples, along with conditioned medium concentrated by ultrafiltration with a 1,000 MW cut-off membrane (R1(10x)) were exposed to boiling for 3 and 20 minutes (as described in section 3 5 3) Each fraction was then filter sterilised and assayed in the RPMI 2650 autocrine bioassay The results are presented in Figure 3 8 1 and Table 3 8 1

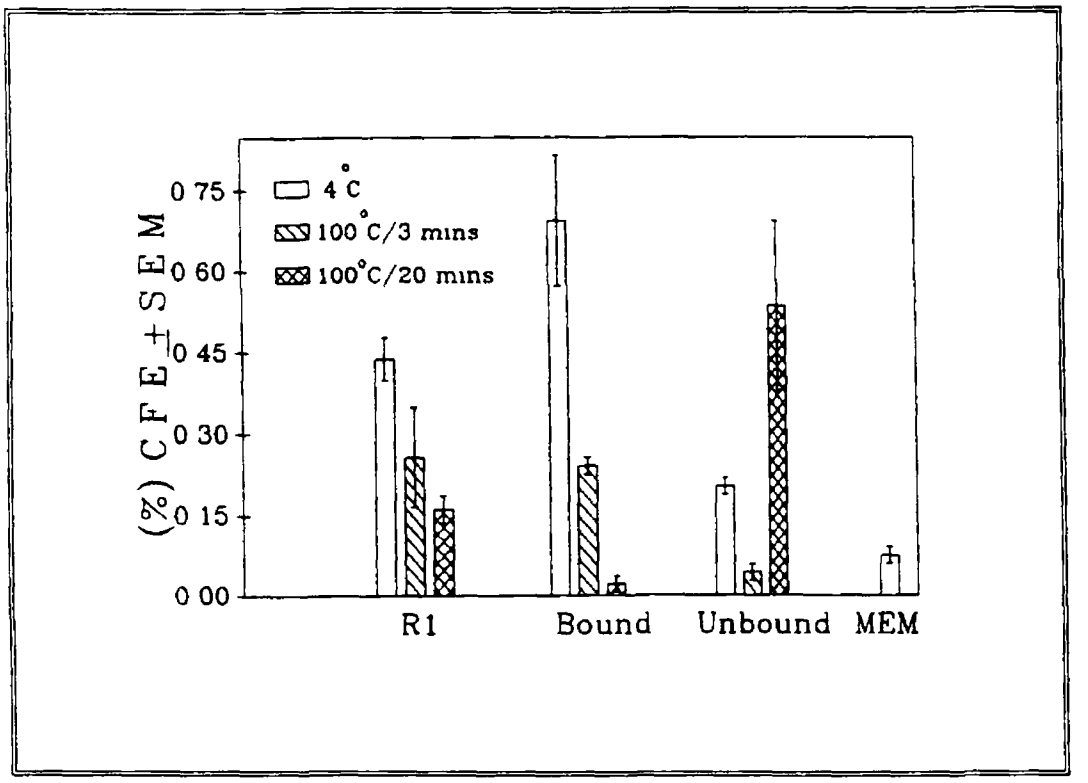


FIGURE 3 8 1 Heat stability of the fractions eluted from the heparin sepharose column (presented in % CFE ± SEM).

TABLE 3 8 1 Heat stability of the fractions eluted from the heparin sepharose column (presented as % activity (average) remaining)

Sample	% activity remaining		
	R1	unbound	0 5M Eluate
4°C	100	100	100
100°C/3mins	50	-25	27
100°C/20mins	23	358	-9

The results obtained for the R1(7x) sample correlate with results obtained previously for the stability of the autocrine activity (see Section 3 5 3) Boiling for 20 minutes further reduces the autocrine activity by about 50% of that observed when the same fraction was boiled for 3 minutes

The bound fraction (0 5M eluate) also exhibits heat sensitivity. Boiling for 3 minutes reduces the autocrine activity while boiling for 20 minutes reduces it further (to control levels) This is similiar to the results obtained for the 1-30,000 MW ultrafiltration fraction (see Figure 3 6 1 and Table 3 6 3)

The results obtained from the unbound fraction were not as easy to interpret Boiling for 3 minutes actually eliminated the activity of the unbound fraction, while prolonged boiling appeared to activate it A similiar activation was also apparent when the R30 fraction from ultrafiltration was boiled for extended periods (see Figure 3 6 1 and Table 3 6 3)

The loss of activity in the unbound fraction following boiling for three minutes is unexpected Due to time constraints, further investigation of this effect was not undertaken

3 8 2 pH Stability of fractions eluted from heparin sepharose

Samples of unbound and 0 5M eluate were prepared (as for Section 3 7 5 2) These samples, along with conditioned medium concentrated by ultrafiltration with a 1,000 MW cut-off membrane (R1(10x)) were exposed to pH 2.5-3 0 and 11 0-12 4 for two hours (as described in section 3 5 4) Each fraction was then filter sterilised and assayed in the RPMI 2650 autocrine bioassay. The results are presented in Figure 3 8 2 and Table 3 8 2

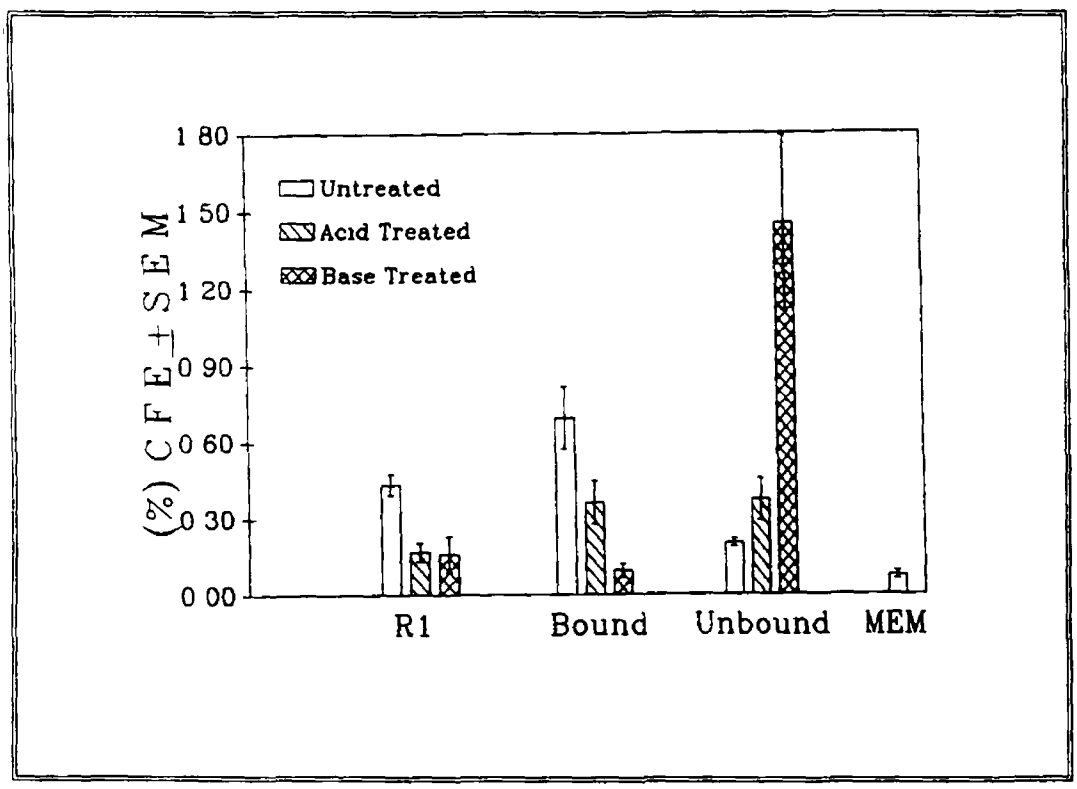


FIGURE 3 8 2 pH stability of the fractions eluted from the heparin sepharose column (presented in % CFE ± SEM).

TABLE 3 8 2 pH stability of the fractions eluted from the heparin sepharose column (presented as % activity (average) remaining)

Sample	% activity remaining		
	R1	unbound	0 5M Eluant
Untreated	100	100	100
Acid treated	26	223	47
Base treated	23	1075	3

Again, the results for the R1 sample correlate with profiles obtained previously (Section 3 5 4) Both acid and base treatments eliminate over 70% of the autocrine activity

The bound fraction (0 5M eluate) is pH sensitive Acid treatment results in a loss of more than 50% of the activity while base treatment eliminates the activity almost completely This again follows the trends of the results from the R1-30 fraction where the autocrine activity was lost by both acid and base treatment

The unbound fraction is more pH stable than the bound fraction Acid treatment resulted in no reduction of activity (the acid treatment seems to be even somewhat stimulatory compared with the untreated control) From these results it can be seen that treatment with NaOH resulted in a quite definite stimulation in activity above the untreated control The R30 fraction from ultrafiltration experiments showed comparable trends (Figure 3 6.2 and Table 3 6 4)

3 8 3 Affinity of autocrine activity (fractionated by ultrafiltration) for heparin sepharose

The similarity in reponse to treatments between the R1-30 and the bound fraction (0 5M eluate) and the R30 fraction and unbound fraction was further investigated in this experiment

Conditioned medium was fractionated into three fraction (R1, R1-30 and R30) A sample of each concentrate was incubated overnight with heparin sepharose The unbound fraction was taken off and the bound fraction was eluted with 0 5M NaCl in phosphate buffer as before Each resulting fraction was a ten fold concentrate of the original CM (see Materials and Methods) Medium (MEM), also concentrated 10x by ultrafiltration, was incubated in the same way with heparin sepharose and bound and unbound fractions collected as controls

The results in % CFE ± SEM are presented in Figure 3 8.3 and in Table 3 8 3 as the distribution of autocrine activity between bound and unbound forms

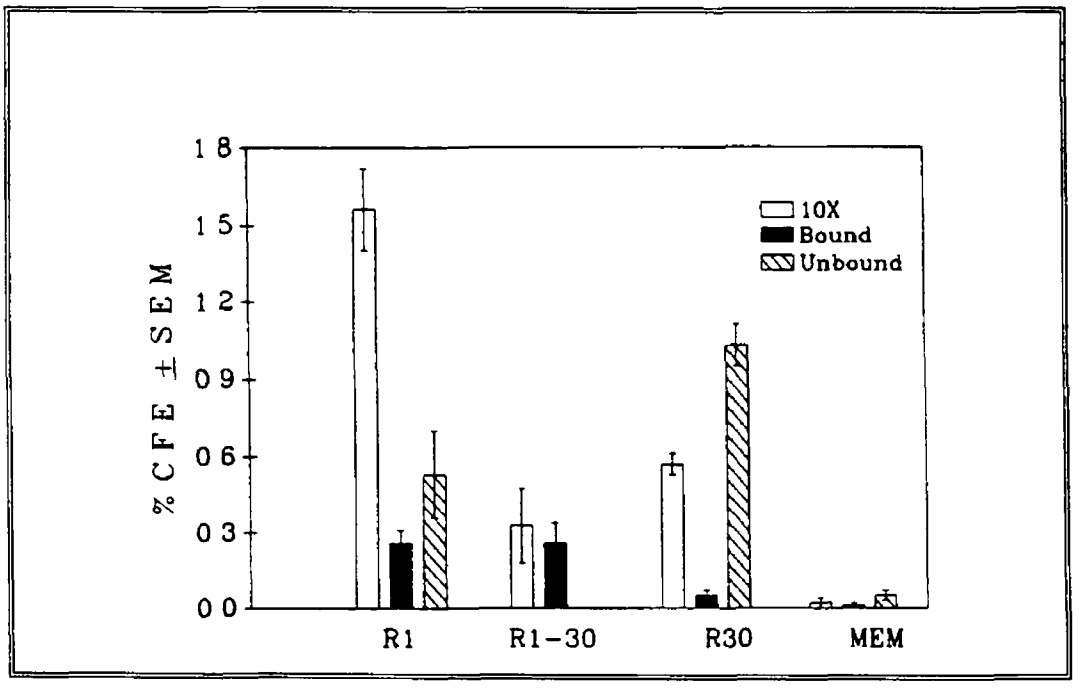


FIGURE 3 8 3 Affinity of ultrafiltration fractions for heparin sepharose There was no growth in the R1-30-Unbound sample

TABLE 3 8 3 Affinity of ultrafiltration fractions for heparin sepharose (expressed as % of activity remaining following heparin sepharose chromatography)

	% Activity		
Sample	R1	R1-30	R30
10x	100	100	100
Bound	16	81	7
Unbound	31	0	172

The results presented in Figure 3 8 3 and Table 2 8 3 show that no activity was eluted in either the bound or the unbound fractions when unconditioned medium was incubated with heparin sepharose This indicates that all the eluted activity could be attributed to the CM only Since MEM was the applied to the heparin sepharose after the CM samples, this result shows that no activity is retained on the column which can be leeched off in subsequent runs.

The activity in the R1 sample divides between bound and unbound fractions in a similar manner to heparin sepharose chromatography of the bulk CM, but the ratio of bound unbound depends on the batch of CM (analogous to the ratio of R1-30 R30, see Section 3 6 1).

It is clear from these results that the R1-30 activity is heparin binding, while the R30 fraction is predominately non-binding

This experiment was repeated on three different occasions with comparable results

3 8 4 Heparin sepharose affinity of TGF-ß activity fractionated by ultrafiltration

The samples assayed in Section 3 8 3 were also tested in the NRK-49F soft agar bioassay for detection of TGF-ß-like activity. The results, in the form of % CFE (± SEM) of NRK-49F cells in the presence of 2ng/ml EGF, are presented in Figure 3 8 4 and Table 3 8 4 Activity in the absence of EGF did not exceed control medium levels (0 02±0 02) and so are not presented

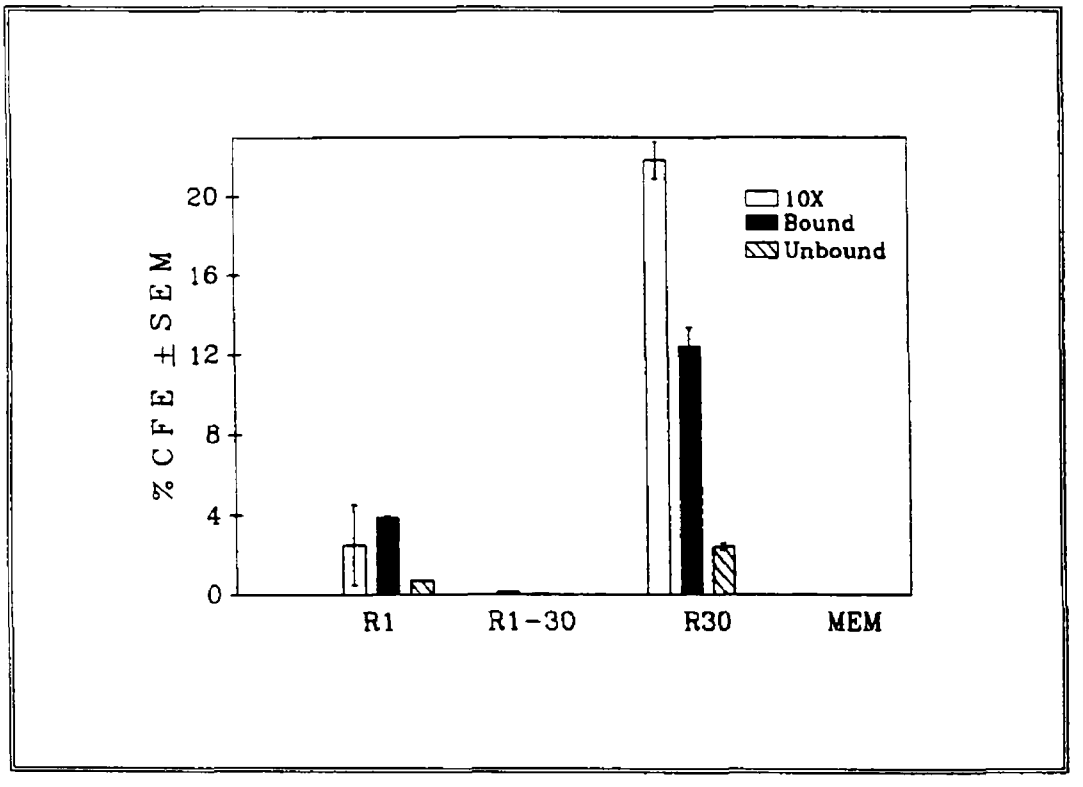


FIGURE 3 8 4 Affinity of ultrafiltered TGF-ß activity for heparin sepharose.

TABLE 3 8 4 TGF-ß activity remaining following heparin sepharose chromatography of conditioned medium fractionated by ultrafiltration

	% CFE ± SEM of NRK-49F cells		
Sample	R1	R1-30	R30
Concentrate	C	0 13±0 02	21 83±0 92
Bound	3 87±0 12	0 03±0 02	12 42±0 92
Unbound	0 71±0 01	0 00±0 00	2 42±0 15
MEM	0 02±0 02		

C = Contaminated

These results support the results shown earlier (Table 3 6 7 and Section 3 3 2) where TGF-ß-like activity in the R1 concentrate is predominately found in the R30 fraction

These figures demonstrate that the TGF-ß activity in both the R30 and the R1 fractions is heparin binding, although substantial activity is lost in the R30 fraction when eluted from heparin sepharose This may suggest a synergistic interaction between the bound and unbound forms

3 9 SCALE-UP OF GROWTH AND AUTOCRINE FACTOR PRODUCTION FROM RPMI 2650 CELLS

3 9 1 Growth of RPMI 2650 cells on different microcarrier bead types

Conditioned medium from RPMI 2650 cells was routinely collected from monolayer roller bottle cultures (as described in Materials and Methods) This set of experiments examined the suitability of using microcarrier beads coated with different materials for growing RPMI 2650 cells and their suitability for collection of autocrine activity Two different bead types were studied Biospheres (Whatman) and Cytodex (Pharmacia)

3 9 1 1 Glass, plastic and collagen coated Biospheres

Three types of microcarrier beads (Biospheres, Whatman) were compared in 100 ml spinner flasks containing 0 2 mg/100 mls of each of the beads and 4 x 10^6 RPMI 2650 cells The cultures were incubated at 37°C for 6 days with feeding on day 4 The microcarriers have a central core of plastic and are coated with either glass, plastic or collagen (Flasks A, B and C respectively) At intervals samples were removed and the number of free and attached cells were counted The growth curve for the cells in each flask is shown in Figure 3 9 1

The growth curves are similar for each of the bead types tested

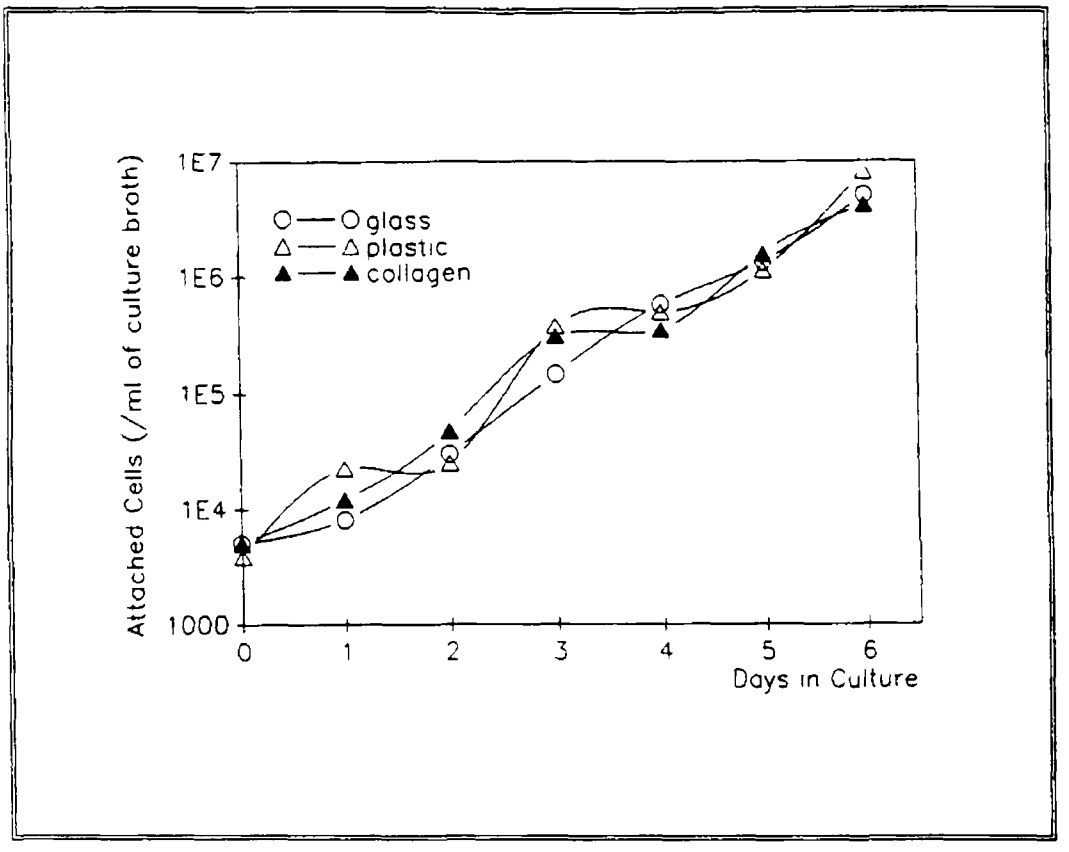


FIGURE 3 9 1 **Comparison of the growth of RPMI 2650 cells on three different types of microcarrier beads (Biospheres)**

Not all the cells attached to the beads The number of free cells in suspension was also monitored and the percentage of attached cells was calculated The results are presented in Figures 3 9 2. and 3 9 3

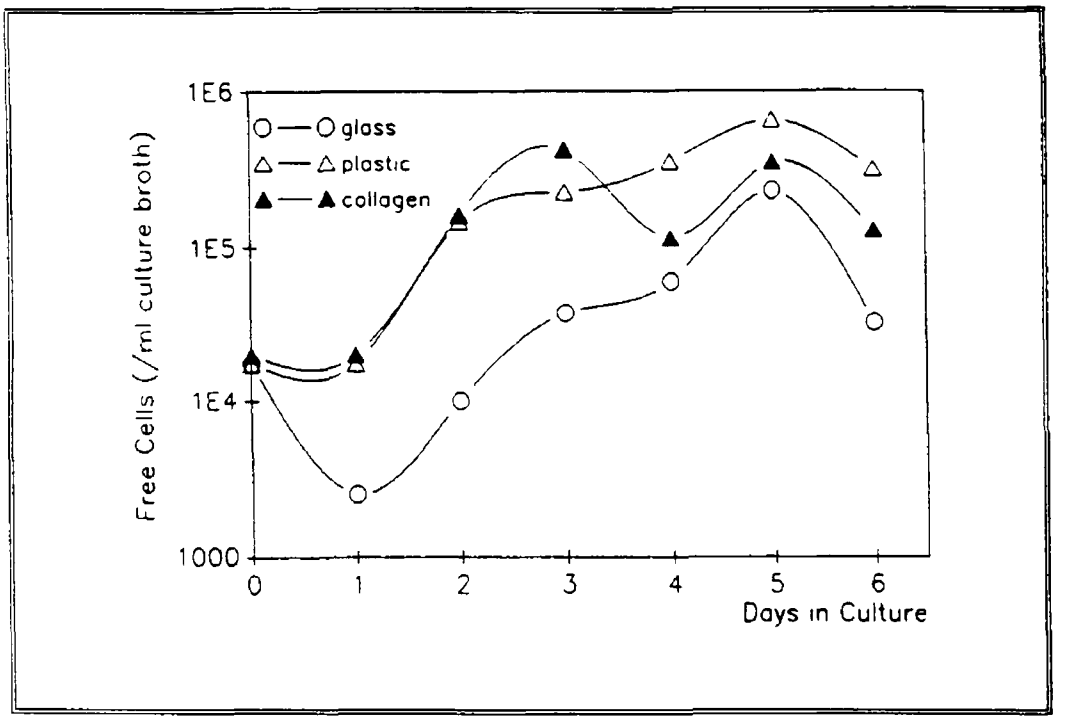


FIGURE 3 9 2 Comparison of the growth of RPMI 2650 cells in suspension in three microcarrier cultures.

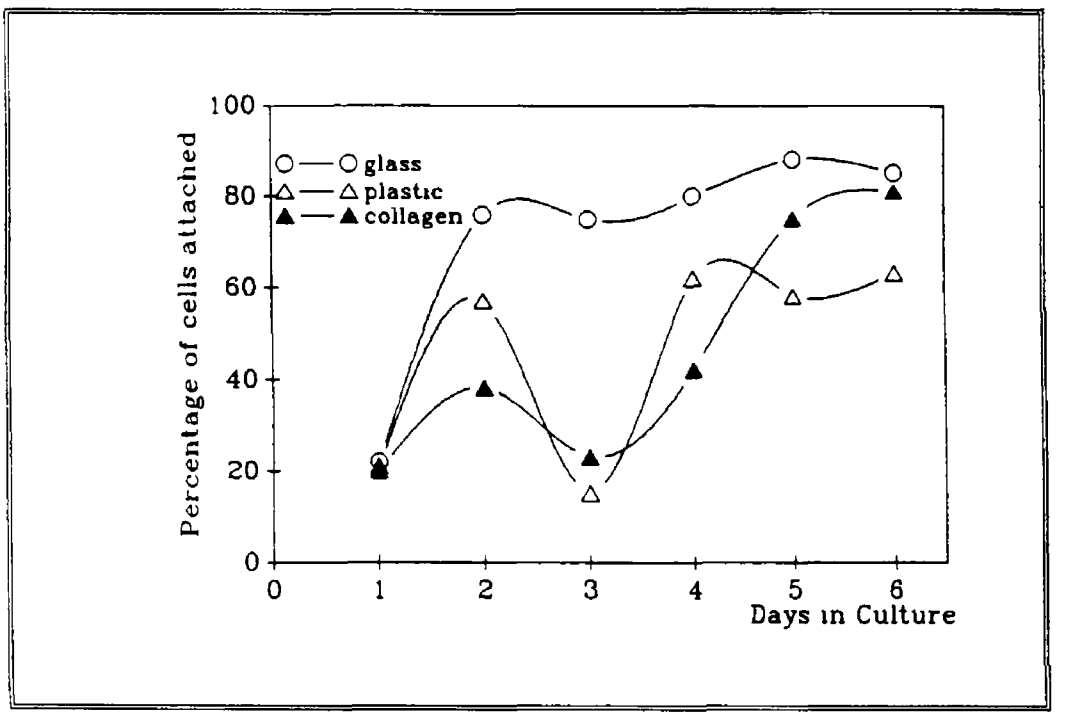


FIGURE 3 9 3 Percentage of cells attached to each microcarrier bead (Biospheres).

These results show that while initially the cells attached quickly to the glass beads, more than 50% of the cells were in suspension in the plastic and collagen cultures after 3 days By the end of the experiment, however, most of the cells were attached in each

of the systems The cells in suspension grew, but apparently not as the same rate as the attached cells This is the reason why the percentages of cell attachment were so high after 6 days

These beads, while providing a good substrate for the cells to grow, were not translucent and so did not allow adequate visual monitoring of the attached cells Because it was difficult to determine the physical state of the attached cells, no further work was done on this bead type

3.9.1 2 Cytodex 1, 2 and 3

The performance of three different dextran bead types were examined (Cytodex, Pharmacia) These beads are translucent and allow observation with standard microscopy techiques Cytodex 1 is a dextran bead with positively charged DEAE distributed throughout the matrix Cytodex 2 has a dextran core surrounded by a layer of positive charges (N,N,N-trimethyl-2-hydroxy-aminopropyl groups). Cytodex 3 is a dextran bead coated with collagen which is said to promote cell adhesion

Three spinner flasks were set up with 6 x 10^5 RPMI 2650 cells per ml in 100mls of MEM + 5% FCS and 0 3 g/100 mls of each microcarrier bead type The cultures were monitored for 6 days, with complete feeding on day 4 Samples were taken after 42 hours, 72 hours and 6 days (144 hours) according to the procedure for microcarrier sampling The number of attached and free cells were monitored The number of attached cells per ml of culture is given in the Figure below The percentage of cells attached at the various sample times is given in Table 3 9 1

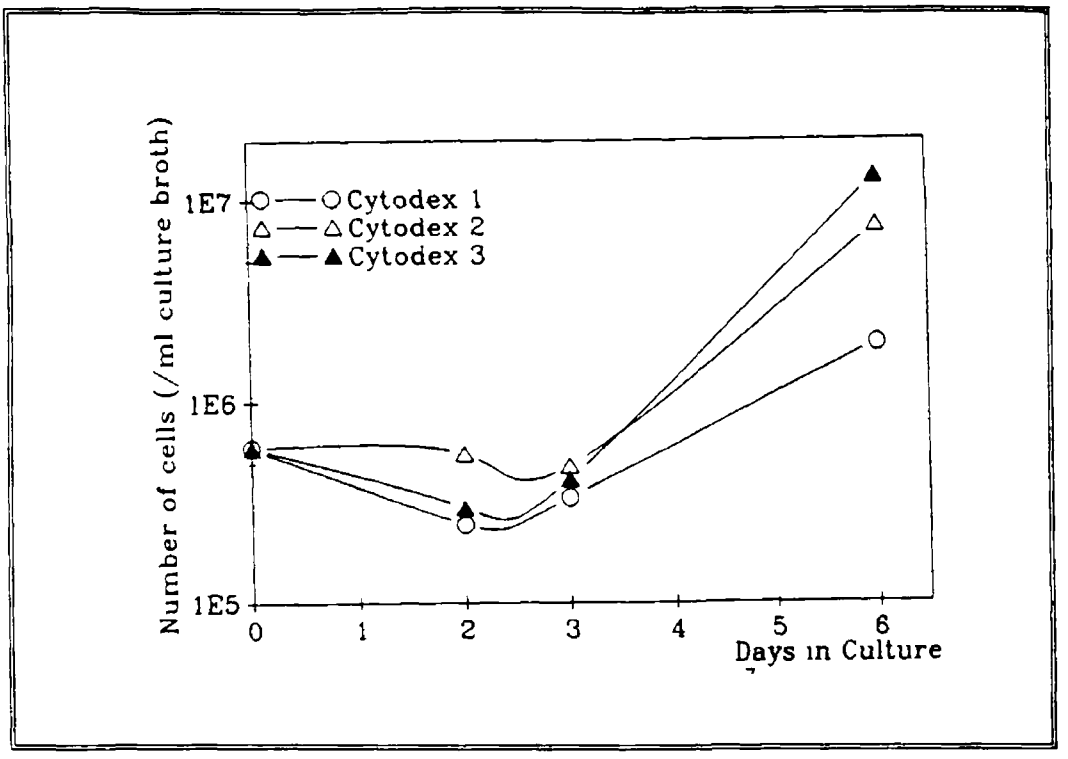


FIGURE 3 9 4 **Growth of RPMI 2650 cells on different microcarrier beads (Cytodex).**

TABLE 3 9 1 **Percentage of cells attached to the Cytodex microcarrier beads**

Sample	Cytodex 1	Cytodex 2	Cytodex 3
48 hrs	98%	51%	100%
72 hrs	100%	85%	100%
114 hrs	99%	99%	100%

The growth curves (in Figure 3 9 4) suggest that Cytodex 3 is the best microcarrier for the growth of cells The cells spread well on the beads with only minimal free cell growth. Cytodex 1 allowed better cell attachment than Cytodex 2, although the cells did not grow as well after attachment The reduced growth may be due to the high charge density in the Cytodex 1 beads.

The cells grown on Cytodex microcarrier beads in this experiments experiences a quite long lag phase, which was not seen in the cultures grown on Biospheres (Figure 3 9 1) This is not a characteristic of the bead-type, however, since a Cytodex 3 culture which was set up at the same time as the Biosphere cultures, exhibited no observable lag phase (Figure 3 9 5)

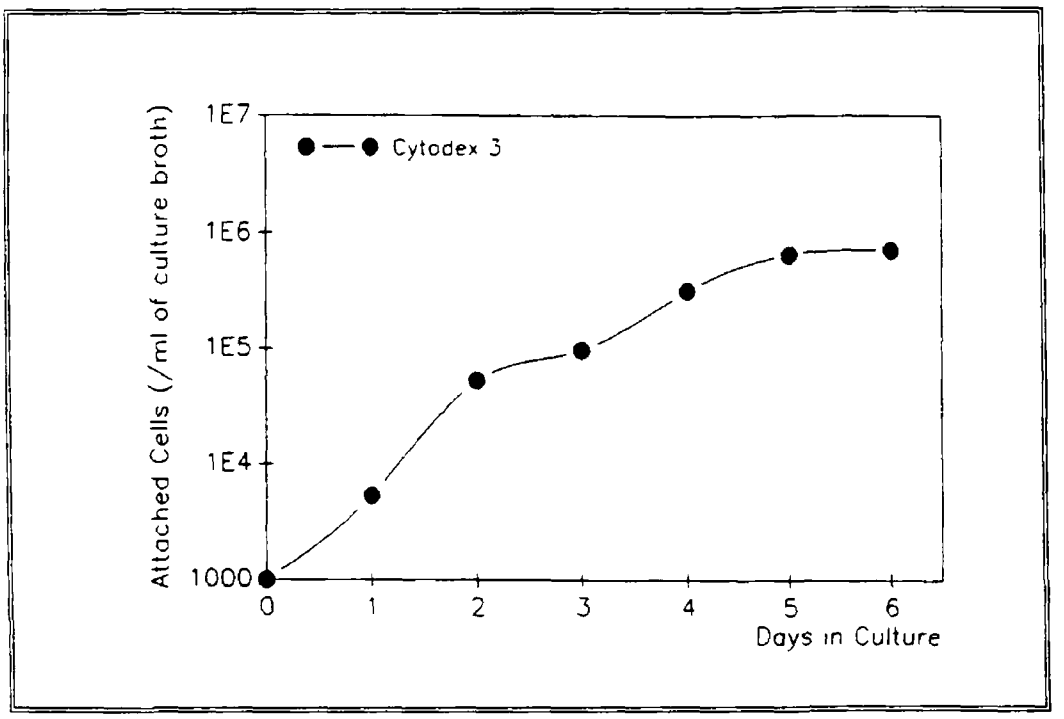


FIGURE 3 9 5 **RPMI 2650 cells grown on Cytodex 3 Microcarriers**

3 9 1 3 Autocrine Activity in CM collected from RPMI 2650 microcarrier cultures

The culture flasks in the previous section (3 9 1 2) were set up on day 6 in serum free medium Conditioned medium was collected after 3 days The CM was then ultrafiltered and concentrated ten fold through a 30,000 MW cut-off membrane (R1(10x)) The filtrate was then passed through a 5,000 MW cut-off membrane (R5-30(10x)) Each sample was assayed in the RPMI 2650 autocrine assay and the results are presented in Table 3 9 2

TABLE 3 9 2 **Autocrine activity of conditioned media collected on from cells grown different microcarrier beads.**

Bead	R30	R5-30
Cytodex 1	2 72±0 27	3 24±0 00
Cytodex 2	2 43±0 13	3 92±0 00
Cytodex 3	1 61±0 43	3 00±0 38
MEM	1 62±0 22	

By dividing the stimulation above the control by the number of cells present, an "Autocrine Index" can be calculated This is a measure of the fold stimulation per producer cell for each of the conditioned media collected and is equal to

$$\frac{\%\ \text{CFE}}{1\ 62} - \text{Total number of cells}$$

TABLE 3 9 3 Autocrine Index (see above) per cell {6 days growth}

Bead Type	Autocrine Index ($x10^6$)	
	R30	R5-30
Cytodex 1	0 86	1 03
Cytodex 2	0 20	0 33
Cytodex 3	0 07	0 14

Calculation of the Autocrine Index allows better comparison between each production system Although the Cytodex 1 bead did not appear to provide an optimum substrate for cell attachment, the relative amount of growth factor produced was higher than the other systems

Alternatively, Cytodex 3 was the best microcarrier for cell growth, but the autocrine activity was very low with barely detectable levels in the R5-30 fraction Cytodex 3 is coated with denatured Type I collagen It is possible that the heparin-binding moiety is adsorbed into the collagen layer and so is not released freely into the CM

The results emphasise the difference between an optimum system for cell growth and an optimum for production

3.9 2 Growth optimisation and autocrine activity production from RPMI cells 2650 in suspension cultures

3 9 2 1 Culture Medium

All the monolayer work with RPMI 2650 was carried out using MEM medium supplemented with a particular batch of FCS (No 48) The following experiment was set up to examine the performance of a modified MEM, MEMS (Flow) (See Appendix F) MEMS, designed especially for suspensions cultures has a low calcium content and is said to promote single cell suspensions

Two spinner flasks (100mls) were set up, one with MEM and one with MEMS Each flask was supplemented with 5% FCS Each flask was inoculated with 1 2x10^5 RPMI 2650 cells per ml and was incubated at 37°C/30 rpm Samples were taken at regular intervals The growth curves are presented in Figure 3 9 6

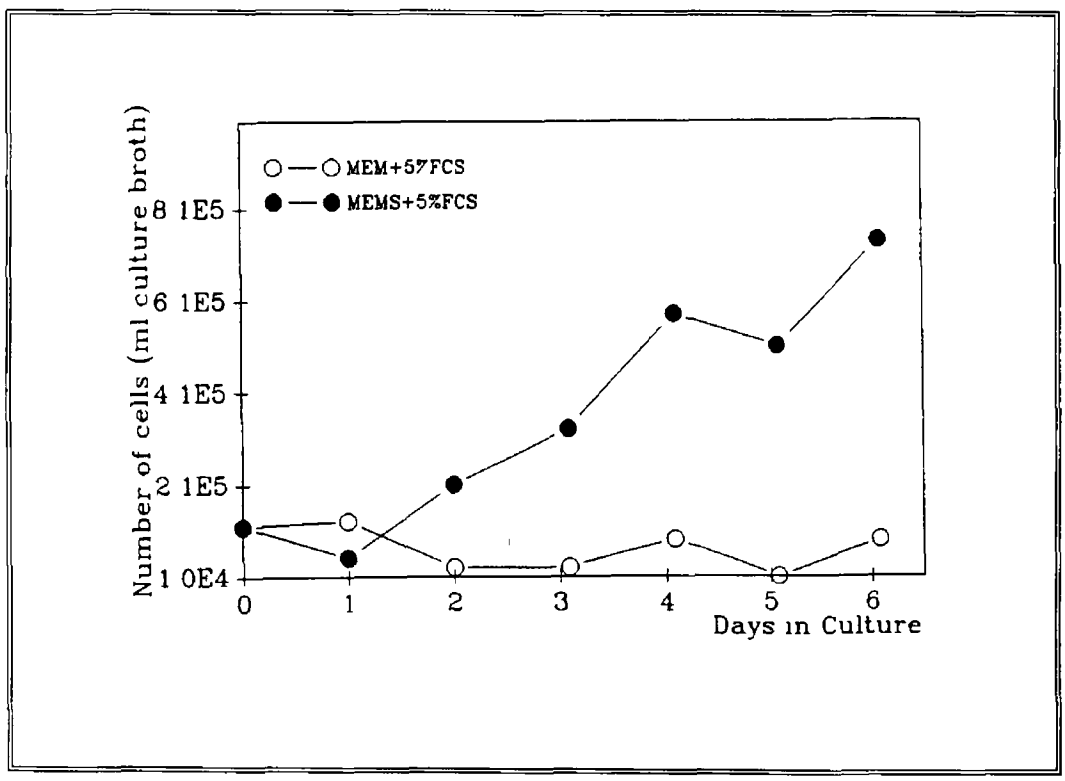


FIGURE 3 9 6 Comparison of the growth of RPMI 2650 cells in MEM and MEMS

The physiology of growth was very different between the MEM and the MEMS media The cells grew in very large clumps in MEM The cells were very tightly packed, perhaps resulting in decreased viability in the centre Because the clumps were so entrapped they were quite difficult to trypsinise so accurate counting was difficult

In the low calcium medium (MEMS), the clumps were much looser and could be trypsinised with less difficulty The viability was also improved

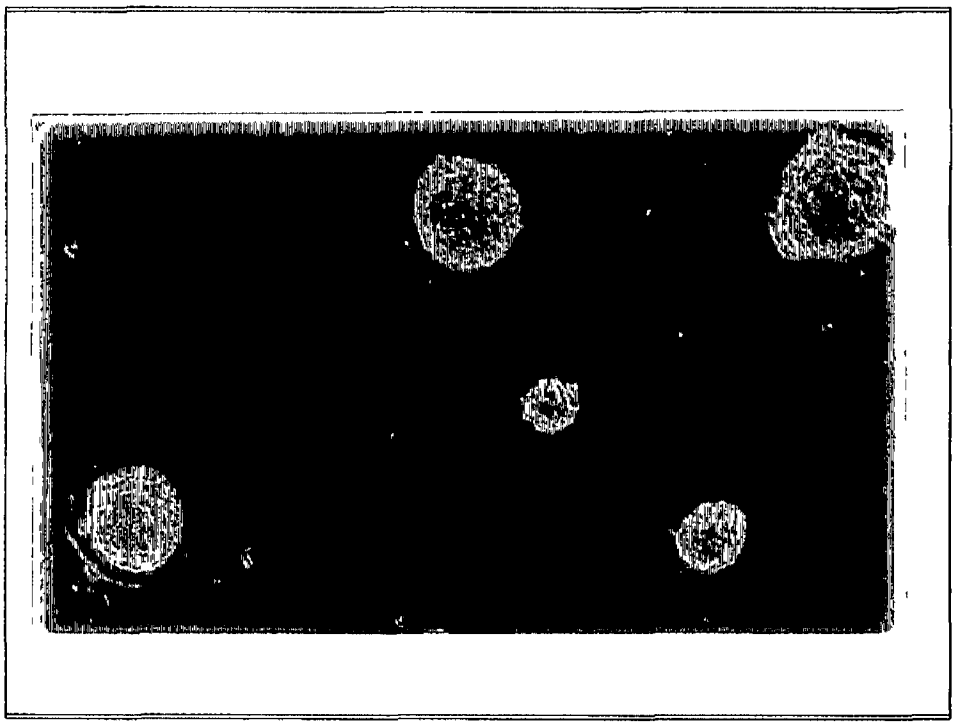


PLATE 3 RPMI 2650 cells grown in aggregate culture in MEM medium supplemented with donor horse serum (5%) Phase 1 (150X) magnification.

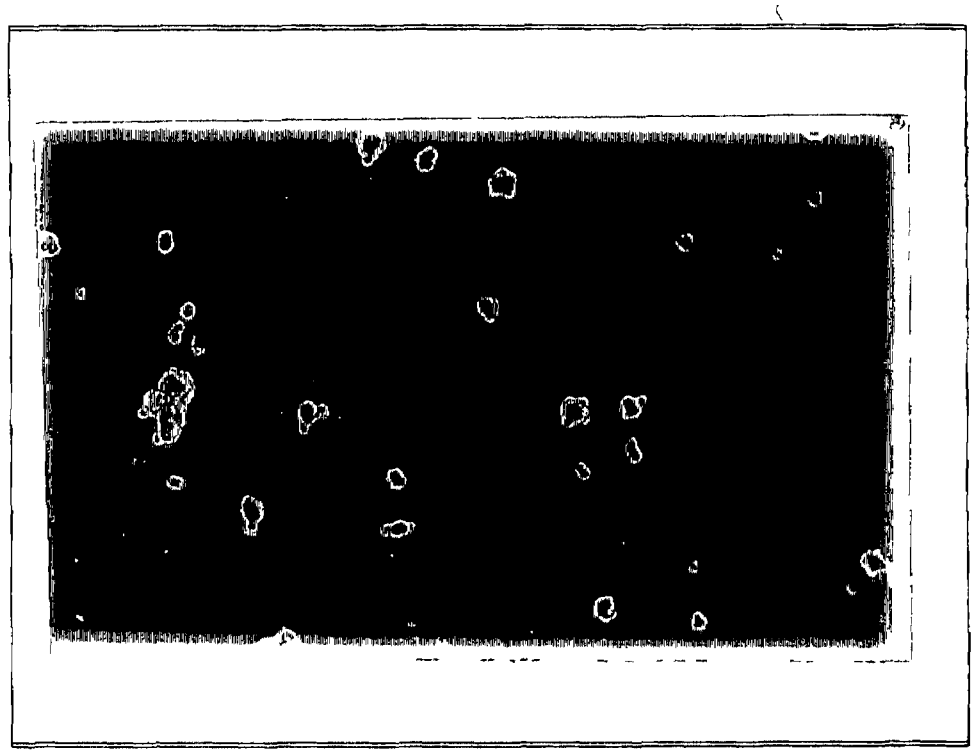


PLATE 4 RPMI 2650 cells grown in aggregate culture in MEMS medium supplemented with donor horse serum (5%) Phase 1 (150X) magnification

After 7 days culture the medium was removed and the cells were set up for CM collection CM was collected on a daily basis by removing 50mls of medium, centrifuging out the cells and resuspending the pellet in 50mls of fresh medium without serum.-- This procedure was repeated for three days These conditioned media samples were then ultrafiltered ten fold using a 10,000 MW cut-off immersible CX-10 filter (Millipore) The retentates were filter sterilised and assayed in the RPMI 2650 autocrine assay The results are shown in Table 3 9 4

TABLE 3 9 4 **Autocrine activity of cultures grown in MEM and MEMS for one week before set up for daily CM collections**

	MEM	MEMS
DAY 1	0 03±0 01	0 00±0 00
DAY 2	0 21±0 08	0 09±0 01
DAY 3	0 24±0 02	1 66±0 13
Control	0 09±0 05	0 09±0 05

This experiment demonstrated that RPMI 2650 cells produced auto-stimulatory activity in both types of media tested Very low levels of activity were detected in MEM after 2 days with no activity at all in the MEMS culture On day 3, the autocrine activity in the MEM culture had not significantly changed but high amounts of activity was present in the MEMS CM

The autocrine activity profile from the suspension cultures appears to be different to that seen in monolayer cultures (see Section 3 5 2) Following this experiment it was decided to collect CM from suspension cultures after 3 days rather than on a daily basis as with roller bottles

3 9 2 2 Serum batch testing in suspension cultures

Since scaling up the RPMI 2650 CM production system involves increased usage of foetal calf serum, it was decided to test donor horse serum (DHS) as a less expensive alternative to FCS. In this experiment, three different batches of donor horse sera were compared to a standard batch of foetal calf serum

Four spinner flasks were inoculated with 10^7 RPMI 2650 cells in 100 mls of MEMS 5 mls of 4 different batches of serum were added as follows

Flask A	MEMS +	5% No	48	FCS
Flask B	MEMS +	5% No	87	DHS
Flask C	MEMS +	5% No	62	DHS
Flask D	MEMS +	5% No	32	DHS

The cultures were incubated at 37°C/30rpm The growth curves are presented in Figure 3 9 7

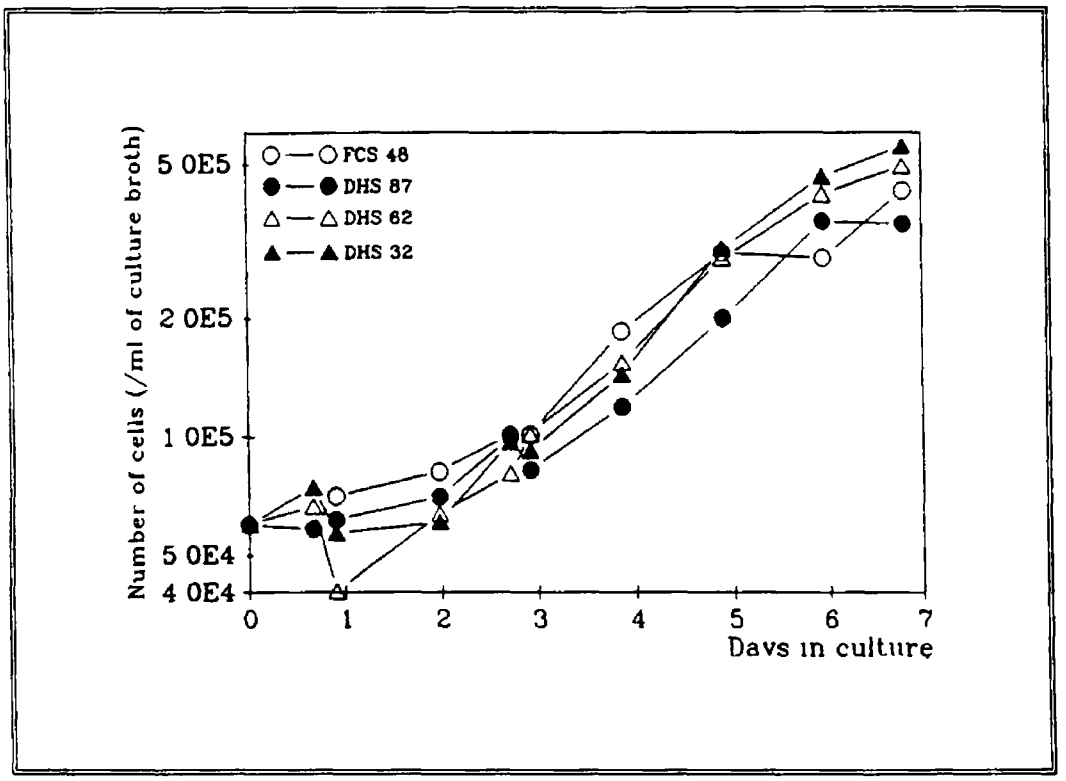


FIGURE 3 9 7 **Growth curves of RPMI 2650 cells grown in 100 ml spinner flasks with different serum batches**

Each of the batches tested performed quite well over the time period examined, with Flask D supporting the most growth From these results it was decided to use this donor horse serum batch for subsequent suspension work

3 9 2 3 Effect of agitation on growth factor activity

In this experiment, the effect of agitation on the production of autocrine, TGF-α and TGF-ß activities was examined

Two spinner flasks of RPMI 2650 cells were grown under identical conditions (37°C/ 30 rpm) in MEMS supplemented with DHS Samples were collected and counted on a daily basis as described previously After 4 days the cultures were centrifuged and the cells re-set up in MEMS without serum Flask A was set up at 20 rpm and flask B was set up at 60 rpm Samples continued to be taken for two more days after which the conditioned medium was collected

The growth curves for each flask is given in Figure 3 9 8

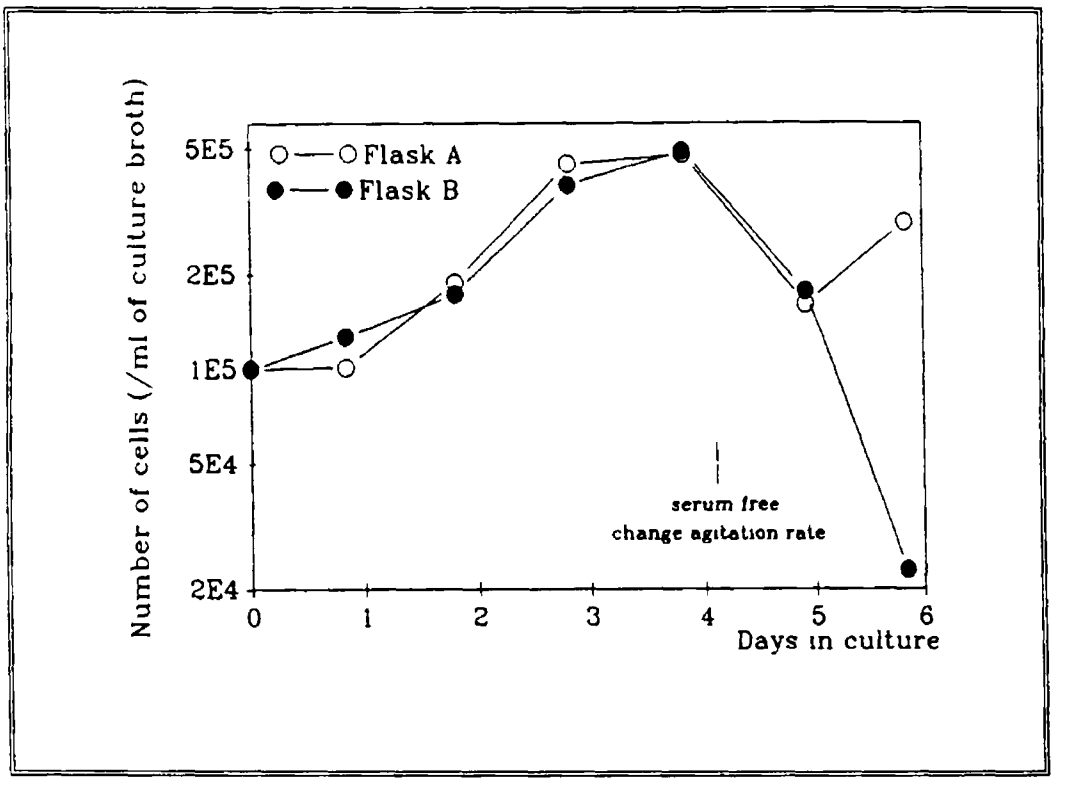


FIGURE 6 9 8 **Effect of different agitation rates on cell growth in serum free media 2 flasks of RPMI 2650 cells were incubated at 37°C/30rpm in MEMS supplemented with DHS On day 4 the cells were set up for CM collection. Flask A was agitated at 20 rpm, Flask B at 60 rpm.**

Growing the cultures at the same initial agitation rate ensured that the cells in each flask were in a similar condition when they were set up in serum-free media and the agitation rate changed This experiment, therefore, just looked at the effect of the agitation rate on growth factor production

Setting up the cultures for CM is in itself quite a distructive procedure Centifuging the cells and resuspending the pellet in medium without serum invariably causes some cell death, with viability dropping to about 60% in both flasks At high agitation rates the viable cells could not recover and after 2 days the viability had dropped to 8% At lower stirring speeds, however, recovery was possible and the viability had risen to 80% by the time the CM was collected

CM was collected on Day 3, as decided previously (Section 3 9 1 1) This CM was concentrated ten fold using a 1,000 MW cut-off membrane and retentate was filter sterilised and assayed in the RPMI 2650 bioassay

TABLE 3 9 5 Growth factor activity in CM collected at different agitation rates

	Flask A 20 rpm	Flask B 60 rpm	MEMS
Autocrine (5x)	3 13±0 33	3 06±0 13	0 33±0 05
TGF-ß (5x)	0 94±0 21	0 73±0 08	0 02±0 00
TGF-α (10x)	0 27±0 20	0 63±0 06	0 00±0 00

It would be expected that at such low levels of viability, a high number of dead cells lyse, releasing proteolytic enzymes These proteolytic enzymes would then act on the growth factors causing reduced activities This is not the case, however, in this system Comparable levels of autocrine and TGF-ß activities were measured in the CM collected from each flask, with increased levels of TGF-α-like activity at the higher agitation rate

These results would suggest that possibly the TGF-α-like activity is intracellular and is released on cell lysis It also suggests that the TGF-ß and autocrine activity may not be growth related and are produced consituitively

3 9 3 Scale-up of RPMI 2650 cell using a 2L Biostat MC

Three similar runs were set up on the Braun Biostat MC 2L bioreactor to examine the effect of dissolved oxygen levels on the growth factor activity of RPMI 2650 conditioned medium

The bioreactors were set up with 4 confluent 75cm² flasks of RPMI 2650 (passage 61-66) in 1 8L of MEMS supplemented with 5% DHS and 1% p/s The pH was controlled at pH 7 3 Each run was set up so that the oxygen concentration would not drop below 40% for the duration of the growth phase An upper oxygen limit was not imposed (which allowed calculation of metabolic oxygen consumption rates) The dissolved oxygen (DO) levels were monitored continuously and the measured readings (averaged every ten hours) are shown in Figure 5 9 9

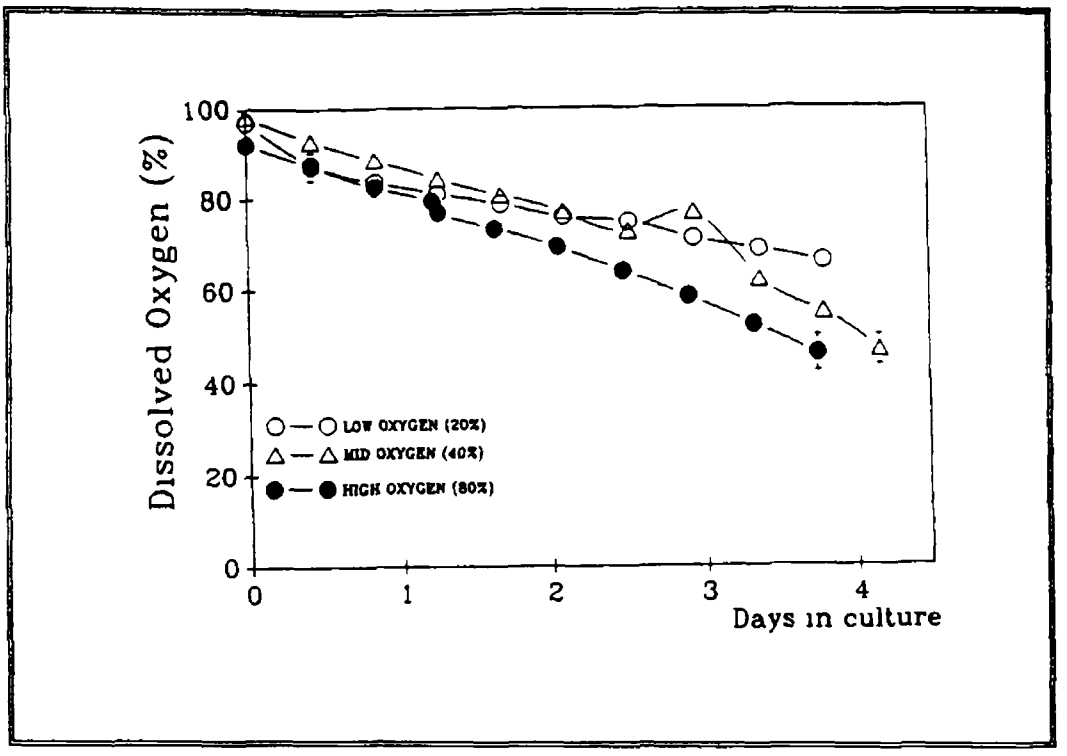


FIGURE 5 9.9 Dissolved oxygen levels in the Biostat MC. The cells were grown under identical conditions for four days in serum supplemented medium, without oxygen control. On day 4 the cultures were set up in serum free medium and the oxygen levels were set to the indicated levels. The graph represents 10-hourly averages for each run.

The reduction in dissolved oxygen levels (up to day 4) is most probably due to the metabolic activity of the cells It is unlikely that mass transfer would account for the decrease since the probe was calibrated for 100% air saturation. Calculation of oxygen depletion rates shows slight differences between each run. The values estimated are presented in Table 3 5.6

TABLE 3 9 6 Calculated oxygen consumption rates for each bioreactor run (see Appendix F) (for approximately 10^5 cells per ml in a 1 8L culture)

	Oxygen Consumption Rate (mmol O_2/hour)
Low Oxygen	0 0010
Mid Oxygen	0 0019
High Oxygen	0 0018

After four days the cell suspension was centrifuged and the cell pellet re-suspended in medium without serum The dissolved oxygen level was set to the appropriate value ±5% i e

Low oxygen = 20%,
Mid oxygen = 40%, and
High oxygen = 80%

The controllers maintained the oxygen levels at ±5% of the set point with nitrogen and oxygen Conditioned medium was collected after 3 days by centrifuging the culture at 3,500 rpm for 15 minutes The conditioned media was stored at 4°C until further processed

Cell counts were taken daily The growth curves of the three runs are shown in Figure 3 9 10

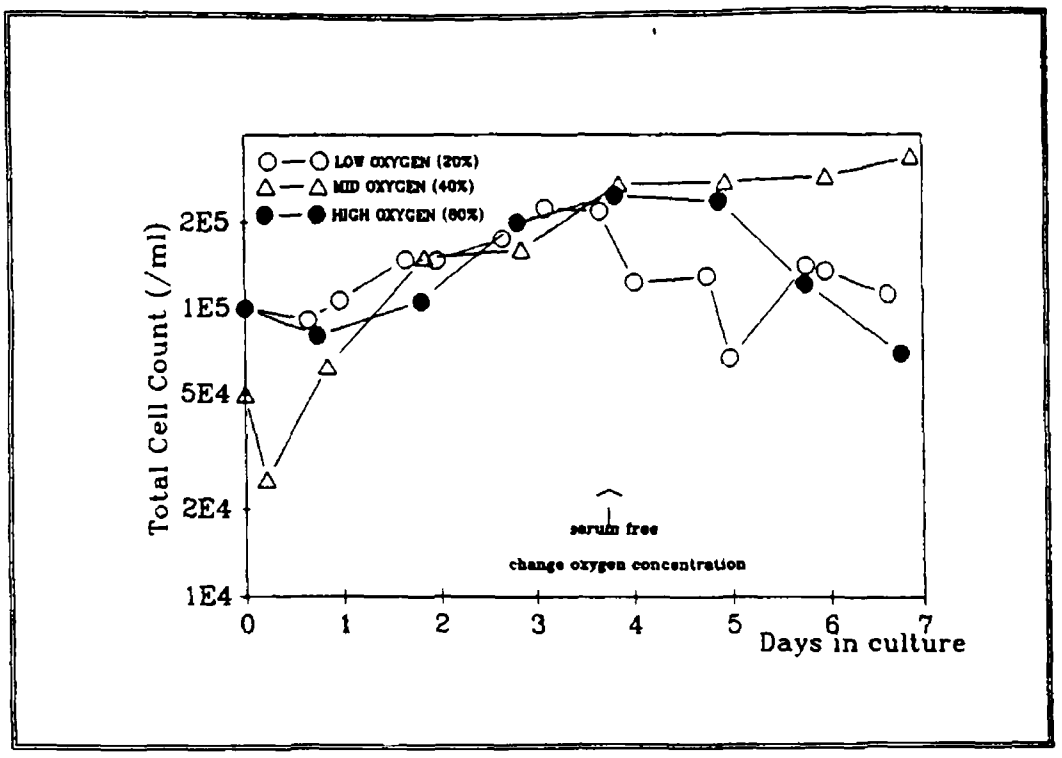


FIGURE 3 9 10 Growth curves of RPMI 2650 cells grown in the Biostat MC. The cells were grown under identical conditions for four days in serum supplemented medium. On day 4 the cultures were set up in serum free medium and the oxygen levels were set to the indicated levels.

Measurement of oxygen consumption rates is not necessarily a good marker for the growth of each culture. The cells in the "Low Oxygen" run grew at a slighly lower rate than the "High Oxygen" culture, but the oxygen consumption rate was almost half While the "Mid Oxygen" run growth rate was high compared with the "High Oxygen" run, but their oxygen consumption rates were similar.

The viability of each of the bioreactor cultures remained at over 95% for the duration of the growth phase. When the cells were set up in serum free medium and the oxygen level controlled, the viabilities dropped. The viabilities determined for the serum free samples in each of the runs are presented in Table 3.9 6

TABLE 3 9 6 Viability of RPMI 2650 cells at different oxygen levels (see text for details)

Culture	Viability (%)		
	Day 5	Day 6	Day 7
Low Oxygen	90	69	50
Mid Oxygen	94	97	94
High Oxygen	93	82	83

The growth of cells was comparable for each run Although the seeding inoculation for the "Mid-oxygen" run was lower than for the others, after 4 days growth the cell densities was the same for all three cultures

Cell numbers decreased dramatically due to experimental error when the "Low-oxygen" run was set up in serum free medium The viability of the remaining cells were initally high (90%) These cells maintained cell number but viability dropped to 50% after three days in serum free medium

Oxygen appears to have quite a dramatic effect at high levels on the growth and viability of the RPMI 2650 cells in suspension The number of cells was reduced by one-third over the three day collection period The viability also dropped, but only to 83% The loss in cell number must be attributed to cell lysis and substantial amounts of cell debris were found in the samples taken

These results suggest that an intermediate oxygen level is the best for serum free growth of RPMI 2650 cells

100mls of conditioned media from each run was ultrafiltered with a 1,000 MW cut-off membrane ten fold (R1(10x)) 100mls of each CM was also incubated with 4 mls of heparin sepharose and the bound

and unbound fraction were collected (as described in Materials and Methods) The resulting fractions were assayed in the RPMI 2650 autocrine assay, the NRK soft agar for TGF-α-like activity and the NRK 49F soft agar bioassay for TGF-ß activity The results are presented in Table 3 9 7

TABLE 3 9 7 Growth Factor activites in CM collected at different oxygen levels

Sample	% CFE ± SEM		
	Autocrine	TGF-α	TGF-ß
Low Oxygen			
R1	8 81±1 45	1 06±0 12	1 06±0 08
Unbound	5 21±0 49	0 27±0 22	0 13±0 02
Bound	1 54±0 50	0 04±0 05	0 47±0 13
Mid Oxygen			
R1	7 62±0 36	N D	0 62±0 00
Unbound	5 27±0 19	N D	0 32±0 04
Bound	1 13±0 43	N D	0 30±0 02
High Oxygen			
R1	7 45±0 66	2 36±0 12	0 58±0 15
Unbound	3 53±0 51	0 16±0 06	0 05±0.02
Bound	2 92±0 59	0 06±0 06	0 58±0 10
MEMS	0 37±0 12	0 00±0 00	0 02±0 02

N D = Not Determined

Autocrine activity

These results indicate that dissolved oxygen levels do not effect the overall autocrine activity of the cells Each of the concentrated CM (R1) gave equivalent activities These results were similar in four repeat assays

Most of the activity was non-heparin binding in the CM collected from low and mid oxygen While the CM collected at high oxygen levels demonstrated relatively less unbound activity, while the amount of heparin binding activity was higher than in the other runs

The total cell count at high oxygen levels had dropped dramatically during growth in medium without serum It is possible that a high number of dead cells lysed and released intracellular components which may include intracellular growth factors (e g FGF)

It is unlikely that the differences in bound activities is due to inactivation over time at 4°C since the sequence of runs was "Low", followed by "High" and "Mid" oxygen levels, i e the conditioned medium from the 'Low" run was longest in storage

TGF-α Activity

The results in Table 3 9 7 demonstrate an increase in TGF-α activity with increasing oxygen levels This activity was lost following heparin sepharose fractionation This correlates with previous results which showed that TGF-α activity was lost by ultrafiltration fractionation (Table 3 6 8)

Subsequently these samples were assayed with with a standard curve of pure TGF-α (see Figure 3 3 1) The calculated TGF-α-like activity present in the high and low oxygen CMs are presented in the table below The assay was repeated with similar results

TABLE 3 9 8 Equivalent TGF-α activity in conditioned media collected at high (80%) and low (20%) oxygen levels

Sample	Equivalent TGF-α Activity (ng/ml)
High oxygen	7 74
Low oxygen	0 47

The results (as in Section 3 9 3 3) demonstrate a correlation between viability and TGF-α levels This again may be an indication of that either the TGF-α activity is intracellular or it may be activated by extreme conditions

TGF-ß Activity

TGF-ß activity increases to a small degree as the oxygen level is decreased This effect is not reflected, however, in the activities of the bound and unbound fractions The assay was only performed once so the conclusions that can be drawn are somewhat limited

4 0 DISCUSSION

4.1 THE RPMI 2650 AUTOCRINE ASSAY SYSTEM

Transforming growth factors were originally identified by their ability to induce non-transformed cells to grow in semi-solid media, such as soft agar Growth in soft agar is generally considered to be the best *in vitro* correlate of tumorigenicity *in vivo* (Shin et al , 1975, Cinofe and Fidler, 1980) This is the system used to detect autocrine stimulatory factors in RPMI 2650 conditioned media (Dooley and Clynes, 1986).

In agar systems the number of cells forming colonies is low compared with agarose assay systems. One study comparing the growth of a range of different cell types in agar and agarose found that none of the 6 non-tumorigenic cell lines grew in agar or agarose, 10 out of 13 tumorigenic cell lines grew equally well in both systems while the remaining 3 cell lines grew in agarose but not in agar (Neugut and Weinstein, 1979) Pretreating the agar to remove mucopolysaccharides resulted in increased colony formation indicating that colony formation in soft agar is limited by the presence of acid polysaccharides (Sanders and Smith, 1970) These studies suggest that growth in agar is a more stringent criterion than growth in agarose and so may be indicative of a more advanced stage of transformation

The reason transformed cells can generally proliferate in semi-solid media, while non transformed cells can only do so after incubation with specific growth factors, is not well understood It has been suggested that proliferation in semi-solid media is dependent on either the interaction of cells with extracellular adhesion receptors (Allen-Hoffmann et al., 1990, Ignotz and Massagué, 1985) or the intracellular pH of the cells (Schwartz et al , 1990, Van Zoelen and Tertoolen, 1991).

Soft agar assay systems have a number of advantages over conventional anchorage dependent monolayer growth factor assays where the end point is established by determining the percentage area covered by cells Such a system can be responsive to factors present in serum which promote attachment and spreading of the cells Laminin, for example, has been found to be capable of promoting cell growth (Rizzino, 1984), while fibronectin was shown

previously to aid attachment to glass (Bradshaw et al , 1983) Such factors have been demonstrated to be unneccesary for growth of NRK cells in soft agar and their presence does not stimulate colony formation (Rizzino et al , 1984). Collagen, however, has been shown to stimulate colony formation of both transformed and non-transformed BHK cells in soft agar (Sanders and Smith, 1970)

The incorporation of ^{3}H-thymidine into acid insoluble materials has been widely used as a convenient method of assessing the stimulatory and inhibitory effects on the growth of many cell lines, including 3T3 cells However this technique not only measures effects on DNA synthesis, but can also reflect alterations in the transport of thymidine across the plasma membrane and in the phosphorylation of this nucleoside by thymidine kinase, which occurs before it can be incorporated into DNA In some systems previously thought to have anti-proliferative activity, have subsequently been shown merely to inhibit thymidine transport (Rozengurt, E , 1981).

The autocrine assay was set up in complete agar medium containing foetal calf serum It has been shown that serum contributes to the growth of the cells in agar (Kaplan and Ozanne, 1983; Wilder and Rizzino, 1991) Peehl and Stambridge (1981) found that increasing the serum concentration above 10% resulted in increased spontaneous colony formation. Also van Zoelen et al (1985) found that increasing FCS levels from 10% to 20% more than doubled the percentage of colonies formed in soft agar in the presence of 2 ng/ml EGF.

The serum concentration in this study (5 8%) supported a low background level of soft agar growth compared with published systems. Wilder and Rizzino (1991) grew murine epithelial cells in soft agar with 9% FCS, which resulted in background colony efficiency of less than 2%. Rizzino and Ruff (1986) suggested that a good serum batch would support the formation of less than 50 colonies in the presence of EGF and greater than 1,000 colonies in the presence of EGF plus TGF-ß (in 24-well plates with a cell loading of 2 5 x 10^{3} cells per well), which is equivalent to colony forming efficiencies of 2% in the absence of EGF, and 40% in its presence

Serum contains a range of polypeptide growth factors which have been shown to be capable of promoting anchorage independent growth It is generally recognised that anchorage independent growth is a function of the total concentration of growth factors in the medium to which these cells can respond (Kaplan and Ozanne, 1983) In most cases, response in soft agar is due to a combination of a number of different factors

For example maximal growth is achieved in the NRK-49F system (employing foetal calf cerum) in the presence of TGF-ß, EGF (or TGF-α) (Assoian et al., 1984), which are present in serum (Childs et al., 1982). Rizzino et al. (1986) have shown that in plasma supplemented media, PDGF can stimulate soft agar colony formation of NRK-49F cells in the absence of EGF (or TGF-α) and TGF-ß Under these conditions, FGF potentiates this response but is itself unable to induce soft agar growth of NRK-49F cells PDGF has also been implicated in a central role in the stimulation of growth of the mouse cell line AKR-2B in soft agar with TGF-ß In this system, TGF-ß stimulates the production of c-sis which codes for the PDGF B chain (Leof et al , 1986) TGF-α and ß, PDGF, and the FGFs have also been implicated in the soft agar growth of a range of cell lines (see below)

TABLE 4.1 **Cell type Factors implicated in soft agar stimulation**

NRK-49F	TGF-α, TGF-ß, PDGF, (potentiated by FGF) Rizzino et al , 1986
ARK-2B	TGF-ß, PDGF Tucker et al., 1984, Rizzino and Ruff, 1986
Human glioma (SNB-19)	bFGF Morrison et al., 1990
NR-6	bFGF, aFGF, PDGF (potentiated by TGF-ß) Rizzino and Ruff, 1986
Rabbit articular chondrocyte	TGF-ß, EGF Skantze et al , 1985
Murine epithelial JB6 cells	TGF-ß, potentiated by EGF Wilder and Rizzino, 1991

Serum normally is bought with a shelf life of six months This ensures that the activity of the sera can be considered to be optimum provided the bottles have been handled and stored correctly Three different bottles of batch number 46 were used for autocrine assays over a one year period. It was discovered that serum from one bottle from this batch could not support soft agar growth This was attributed to incorrect handling of the bottle, possibly due to too frequent freezing and thawing of the bottle during transfer from one freezer to another or during malfunction of freezer units.

Because the presence of specific growth factors is critical for the stimulation of colony formation, it is important to carefully batch test the serum before use. In the course of this work over 20 different batches of feotal calf serum (FCS), newborn calf serum (NBCS) and donor horse serum (DHS) were tested In general the greatest batch to batch variability was demonstrated with the FCS samples, some of which resulted in excellent stimulation while others were very poor The NBCS gave consistently poor results in

the soft agar assay, while the DHS was consistently average to good The criteria used to determine a good batch from a poor batch was the occurence of high stimulation of growth in the presence of conditioned media with a low backround in the absence of conditioned media.

In some published studies, NBCS is prefered to FCS for NRK soft agar growth and in growth factor defined systems, DTT-treated NBCS and FCS were found to have comparable backround levels of colony formation (van Zoelen, 1985) In our studies, it was found that NBCS only poorly supported soft agar growth This may be due to particulary bad batches being tested. In monolayer assays in 24-well plates (Dooley et al, unpublished) these batches of NBCS were found to be poor compared with 14 batches of FCS and DHS for the growth of RPMI 2650 cells, although it supported the growth of MSV-3T3 cells, 703D4 hybridoma cells and a primary human fibroblast cell line quite well

In general it is believed that the contribution to colony stimulation in soft agar from bovine sera is significantly less than from sera derived from other species (Rapp et al , 1983) Here, however, it was found that the background levels exhibited in the presence of DHS was less than that seen with many of the FCS batches examined The stimulation in the MEM+5%DHS sample, however, was high compared with the MEM+5%FCS and MEM+5%NBCS samples, which suggests that the background was more dependent on the level of serum compared with the FCS and NBCS batches

Because the serum batches used in the autocrine assay supported generally low background levels of growth compared to the stimulation in the presence of conditioned medium, it was decided to continue to use serum containing assay systems for the duration of this project, rather than investigating serum free systems The batches used also proved to allow for only minimal growth in the NRK-49F assay for TGF-ß activity in the presence of EGF while, on most occasions no colonies formed in the absence of EGF (for both NRK and NRK-49F cells).

To get an indication of the range of factors that might be involved in the stimulation of RPMI 2650 colonies in soft agar, several highly purified growth factors obtained from commerical sources were assayed in the RPMI 2650 soft agar assay at a number of different concentrations Insulin, insulin-like growth factors and two heparin binding growth factors (acidic and basic fibroblast growth factor) all appeared to stimulate colony formation at higher concentrations (50ng/ml of agar) and this activity was concentration dependent Interleukin 1α and 1ß exhibited dilutable inhibition at the concentrations used TGF-ß showed some stimulation at approximately 0 2 ng/ml but the extent of stimulation was small and variable TGF-α (50-0 5 ng/ml) did not stimulate or inhibit colony formation in the assay At 50 ng/ml and 10 ng/ml EGF illicted no response, but at 5 and 1 ng/ml the effect appeared to be slightly inhibitory.

The fact that the cells are responsive to a number of different growth factors suggests that RPMI 2650 cells express receptors for each of these factors and that any of the factors that elicit a response could be a possible paracrine or autocrine regulator of its growth Whether all of these factors operate through specific receptors or through a number of common receptors (like the IGF family) is uncertain. Each member of the insulin-like growth factor family has its own specific high affinity receptor,and it cross reacts to the other members' receptors with lower affinity (Perdue, 1984)

In Section 3 3, it was shown that TGF-like activity (equivalent to 0 05 ng/ml of TGF-α) and TGF-ß like activity (equivalent to 1 02 ng/ml of TGF-ß) were present in the conditioned medium of the RPMI 2650 cells These values correspond to 0 01 and 0 2 ng per ml of agar medium, respectively At 0 2 ng/ml, exogenous TGF-ß had no effect on the colony formation of RPMI 2650 cells While EGF showed very slight stimulation at 0 5 ng/ml, TGF-α was not active Concentrations below 0 2 ng/ml were not tested, and may be stimulatory

A lot of literature has been published on the ranges in which TGF-ß can elicit a response It appears that the response is dependent on a number of factors, including the end point used to establish TGF-ß activity The concentration of maximum response varies from the pico-gram per ml range in the case of stimulation of normal hepatocytes (Braun et al , 1988) to ngs/ml for inhibition of chondrocytes (Vivien et al , 1990) and BALB/ML keratinocytes (Coffey et al , 1988).

The presence of serum in the assay system can also cause a difference to the cell response to TGF-ß. Hoosein et al (1987) found that the human colon carcinoma cell, MOSER, exhibited maximum inhibition when it was exposed to about 5 ng/ml of TGF-ß in an assay system that contained 10% FBS A similar assay was set up under defined conditions without serum, using cells that had been adapted to growing in serum free medium In this assay TGF-ß was maximally inhibiting at 20ng/ml This may be due to other growth factors in the serum acting on the cells making them less responsive to TGF-ß

The TGF-ß response may also be a function of the confluency of the indicator cells Centrella et al (1987) looked at ^{3}H-thymidine incorporation in sparse, subconfluent and confluent cultures of osteoblasts They found that below a certain cut-off concentration, TGFß was stimulatory and above this cut-off it was inhibitory. They also showed that the actual cut-off concentration varied with the confluency of the culture being examined such that TGFß was stimulatory up to 15 ng/ml in confluent cultures but for sparse cultures the cut-off limit was only 0 15 ng/ml

Hamburger et al (1991) reported that the number of EGF receptors on the breast carcinoma cell line, MDA 468, decreases as the confluency of the cells increases This decrease is associated with reduced levels of mRNA transcripts.

These findings may be significant with regards to the variability of the autocrine assay Although the cells are plated at the same concentration for each individual assay, the level of the cells' response is quite variable In some assays the background is a lot

higher than in others In these assays, it is possible that because of the higher concentration of cells after a number of days the response to the particular growth factor may be different than when there is a lower overall background of cell growth.

The possibility that these purified growth factors (TGF-α, EGF and TGF-ß) were not biologically active is discounted by the fact that they were shown to be active in their specific soft agar assays i.e the NRK soft agar assay for TGF-α-like activity which showed that both the EGF and TGF-α were active and the NRK-49F soft agar assay in which TGF-ß was stimulatory

Morishige et al (1991) found that while TGF-α, at various concentrations (0 1, 1 0 and 10 mM), did not cause stimulation in monolayer cultures of ovarian cancer cells in serum free medium, monoclonal antibodies against TGF-α (but not EGF) could significantly inhibit their growth No explanation was provided for this result, but it may prove important for the RPMI 2650 system where neither TGF-α, TGF-ß nor EGF caused a significant effect, even though TGF-α and TGF-ß-like activity levels could be detected in CM samples

TGF-α-like and TGF-ß-like activity were detected in the RPMI 2650 CM by the NRK and NRK-49F assay systems respectively, but other pure growth factors, besides TGF-α and TGF-ß, have been reported to bring about similar stimulation

Normal rat kidney cells (clone 49F) have been widely used as a non-transformed indicator cell line for studying the role of polypeptide growth factors The original studies with this cell line in agar containing 10% FCS found that phenotypic transformation of this line requires the presence of TGF-ß in combination with EGF or TGF-α (Anzano et al , 1983) Subsequent work with plasma-containing media established an additional requirement for exogeneous PDGF (Assoian et al , 1984) which elicts a response in the presence of extracts of human platelets, but PDGF alone does not have an effect (stated by Miyagawa et al , 1991) Because of the contribution of serum to the stimulation of colony formation, growth factor defined and serum free methods were developed.

Van Zoelen et al (1985) employed a dithiothreitol treated foetal calf serum to eliminate spontaneous colony formation in soft agar in the presence of EGF in a growth factor defined assay system containing selenite, transferrin, BSA and insulin In such a system, insulin (in combination with EGF) was demonstrated to be strongly mitogenic for NRK cells in monolayer but insulin alone could support very little growth

A serum free medium was designed by Rizzino (1984), containing transferrin, EGF (or TGF-α), FGF and high density lipoprotein (HDL) which was capable of sustaining growth equivalent to that observed with 10% FCS in monolayer cultures while in agar systems, colonies formed in the presence of these components plus TGF-ß Due to problems with HDL preparations, 1mg/ml BSA was subsequently added (Rizzino et al., 1986) Because preparation of human HDL is time consuming, expensive and susceptible to batch to batch variation, a more readily prepared medium incorporating plasma was designed (Rizzino and Ruff, 1986)

An EGF-nonresponsive mutant N-3 from NRK cells has been isolated (Hamanaka et al , 1990) in which the anchorage independent growth of N3 cells is significantly stimulated by TGF-ß alone, without a requirement for TGF-α/EGF. It was suggested that TGF-ß enhances the secretion of EGF (or TGF-α) from N-3 cells into culture medium, which may support anchorage- independent growth in the presence of TGF-ß alone.

As mentioned before, because of the presence of serum growth factors in the assays used here, it is necessary to exercise caution when interpreting results with the NRK-49F cells. Other factors besides TGF-ß, EGF/TGF-α, PDGF and FGF cause a response in the NRK-49F assay.

Bovine brain derived growth factor, whose chemical and physical properties are similar to those of aFGF, also induces anchorage independent growth of NRK-49F cells in the presence of EGF (Huang et al , 1986)

More recently, a recombinant hst-1 protein produced in silkworm cells by a recombinant baculovirus, was found to have transforming growth factor activity stimulating anchorage independent growth of NRK-49F cells at a concentration as low as 1 ng/ml without a requirement for EGF (Miyagawa et al , 1991)

Beacause of the non-specificity associated with the NRK and NRK-49F assays, a range of purified growth factors from commerical sources were tested in each assay system.

Only the TGF-α-like molecules (TGF-α and EGF) showed high stimulation in the NRK assay. IGF I was slightly stimulatory at the highest concentration assayed (100ng/ml) This result would indicate that stimulation of NRK colony formation elicited by the RPMI 2650 CM was most likely to be TGF-α-like

In the NRK-49F assay system, however, both TGF-ß and IGF I (and to a lesser extent IGF II) were stimulatory in the presence of EGF. The IGF I used in this set of experiments was a human recombinant form from Boehringer Mannheim GmbH To ensure that this effect was not merely a result of this particular source of growth factor, IGF I from two different sources were examined A fresh vial of human recombinant IGF 1 derived from E coli (Boehringer Mannheim GmbbH) was compared with a recombinant IGF I derived from S cerevisiae (Promega). In this set of experiments, both these factors exhibited significant stimulation above that of the medium controls It is possible that insulin and IGF potentiate the colony formation, at the lower serum levels used in these but cannot induce it themselves (as with FGFs), and so the insulin and IGF-I effect was not documented before

Massagué et al (1986) also reported a IGF effect. They found that by treating BRL-3A conditioned medium with IGF binding protein, the formation of NRK-49F colonies in the presence of EGF and TGF-ß was inhibited, and this inhibition could be counteracted by the addition of excess IGF II. They suggested that sufficient IGF could be provided in the 10% FCS present in the agar so that exogeneous IGF II could not elicit a response

In the experiments set up here, IGF II did cause a small stimulation. The reason for this may be the lower level of FCS present (5 8%) resulting in decreased accessibility of the cells to individual growth factors in semisolid medium, rather than unique forms of synergism between TGF-α or IGFs and TGF-ß Massagué et al (1986) did not examine IGF I effects on TGF-ß stimulation of NRK-49F colony formation

Recently, Sushelnitsky et al (1991) studied the effect of EGF and insulin on the number of TGF-ß receptors on NRK-49F cells They found that while the affinity of the receptors for TGF-ß did not change, the number of receptors increased from about 5 x 10^4 to 8 x 10^4 per cell in the presence of insulin. A similar effect may be happening in the NRK-49F system presented in this thesis IGF-I may increase the number of TGF-ß receptors on the NRK-49F cells and so augment their response to TGF-ß present in the serum

It is reasonable to conclude from this that the NRK-49F assay system is not very specific for TGF-ß and that other factors can stimulate the NRK-49F cells in soft agar.

Transforming growth factors produced by one tumour cell line may be able to stimulate the anchorage-independent growth of one type of indicator cell but not that of another. Salomon et al (1984) showed that normal mouse or rat mammary epithelial cells are better than fibroblasts as target cells for TGF's isolated from CM of breast carcinomas. Such is the case in the NRK systems and the RPMI 2650 system, whereby a range of different factors stimulate the RPMI 2650 cells but only a limited number appear to stimulate NRK cells

In general, the effect of TGF-ß and other transforming growth factors are reversible (Shipely et al , 1986), but the soft agar growth stimulation of murine epithelial cells JB6 by the tumour promoter, TPA, has been shown to be irreversible (Colburn et al , 1979) Soft agar colonies formed in response to single and multiple exposures of TPA have been isolated and shown to retain the capacity for anchorage-independant growth in the absence of tumour promoters. EGF (Colburn et al , 1981) and TGF-ß (Wilder and

Rizzino, 1991, Hamel et al , 1988) have also been shown to have similiar irreversible long-term effects on these cells. It would be interesting to examine the reversibility of the autocrine response of RPMI 2650 cells and see if it is a characterisic of this cell line

4 2 THE AUTOCRINE ACTIVITY

From the initial work of Dooley and Clynes (1986) it had been established that RPMI 2650 cells produce a factor or factors into their conditioned medium that are capable of promoting colony formation of RPMI 2650 cells in culture. In this work I attempted to better characterise this activity and to possibly identify it either as belonging to a class of growth factors already studied or as a completely novel factor

Preliminary stability studies on a concentrated batch of conditioned medium that exhibited linearity with respect to concentration, indicated that the autocrine activity was partially heat-stable, with on average 26% activity remaining following boiling for 20 minutes The activity had only limited pH stability (pH 7 2-8 5) with residual activity remaining at the extremes of pH The activity was partially lost on treatment with trypsin indicating that a protein or peptide was involved in the response

The detrimental effect of low pH on the CM is particularly significant since the pH of collected CM often drops to below pH 7, which would result in reduced activity levels This undoubtedly contributes to the variation between different batches of CM collected The fact that all three batches of CM collected from the bioreactor runs which were maintained at pH 7 2-7 3, exhibited high levels of activity, supports this hypothesis

The presence of residual activity following acid, base and heat treatments indicated that there is more than one autocrine factor produced by the cells.

Subsequent stability work showed that the activity remaining following heat treatment was completely sensitive to acid and base treatments Also boiling of the pH treated samples indicated that the acid-stable activity was very sensitive to heat and the base treated activity was also sensitive but not to the same extent. Thus all the activity could be eliminated by successive heating and pH treatments

Initial size determination experiments using different molecular weight cut-off ultrafiltration membranes indicated that dilutable activity could be isolated in fractions greater than 30 kDa (R30) and in fractions between 1 and 30 kDa, with no activity presenting in the filtrates (F30 and F1) (although this is not surprising since no activity is usually detected in 1x samples)

The stability profiles of each of these fractions were shown to be quite different from each other indicating that two separate molecules or forms of molecules were isolated in each fraction

The smaller form (R1-30) is sensitive to heat, acid and base treatment and may account for the reduction in activity that is seen in the bulk R1 CM The larger form (R30) appears to be quite stable to extremes of pH and temperature and is sometimes capable of being activated under certain conditions (extended boiling for 20 minutes and base treatment) The presence of this molecule may account for the residual activity in the R1 fraction following pH and heat treatments

Candidates for the R1-30 fraction.

Because of its unstable nature the R1-30 fraction is not likely to be one of the more common transforming growth factors (TGF-α or TGF-ß), the insulin-like growth factors nor PDGF, all of which are acid- and heat-stable The members of the heparin binding growth factor family are, however in general unstable molecules with molecular weights ranging between 15-19 kDa which would fit the activity profile of the R1-30 fraction

Very little work has been published on heat-labile transforming growth factors. This is probably due to the definition of a TGF which describes TGFs as 'heat- and acid-stable peptides' and so their purification protocols were designed with this in mind The widespread occurrence of acid-stable growth factors and cytokines in cell systems may, in fact, point to some actions in acidified intracellular compartments (e g lysosomes) which remain to be elucidated. Lysosomes of animal cells contain a large array of different enzymes including proteases which could activate latent precursor forms under acidic conditions

A heat- and acid-labile transforming growth factor was identified in 77N1 cell (avian sarcoma virus transformed rat cell line) CM with an apparent molecular weight of 11 kDa (Hirai et al , 1983) that could stimulate the soft agar growth of BALB 3T3 cells This TGF, when purified, (Yamaoka et al , 1984) was was found not to bind to EGF receptors Subsequently it was suggested that this factor may be basic FGF (Rizzino and Ruff, 1986) Murine embryonal carcinoma cells produce a heat- and acid-labile growth factor that stimulates the soft agar growth of NR6-R cells (Rizzino, 1986) which has also been implicated as being bFGF (Rizzino and Ruff, 1986)

Platelet derived endothelial cell growth factor (Miyazonoet al , 1987) is heat and acid sensitive but its size precludes it from being the heat sensitive fraction (45 kDa)

The interleukins and colony stimulating factors

While the interleukins and colony stimulating factors (CSFs) have been generally identified for their action on cells of the immune system, it is becoming clear that many of them also act on other cell types, for example liver (Arai et al., 1990) and keratinocytes (Sauder et al , 1988)

Interleukin 1 (IL-1), originally identified as a product of activated macrophages, has since been reported to be produced by fibroblasts, endothelial cells, keratinocytes and smooth muscle

cells (Mizel, 1988). The purification of IL-1 (or hemopoietin 1) was described by Jubinsky and Stanley (1985) Conditioned media from a human urinary bladder carcinoma cell line (5637) was purified in a four step procedure employing batch DEAE-cellulose chromatography, chromatofocusing, gel filtration followed by hydrophobic chromatography Active fractions were eluted from the chromatofocusing column with 11% Polybuffer 74 (pH 3 9) It is unlikely that the R1-30 fraction is IL-1 since its activity would be lost at this pH

Interleukin 2 has been purified to homogeneity from T cells (Stern et al , 1984) The procedure employed ammonium sulfate precipitation, ion exchange chromatography followed by reverse phase HPLC The buffers used in the last two steps of the purification used were 0 05M NaCl/Hepes (25mM), pH 5 5 and acetic acid (9M)/pyridine (0 2M) buffer, pH 4 0 The acidity of these two buffers would destroy the R1-30 activity, and so it rules out IL-2 as the factor involved

Interleukin 3 (also called Multi-CSF) has been partially purified from the myelomonocytic leukaemia cell line, WEHI-3 (Bazill et al., 1983) and to apparent homogeneity from a cell line of similar origin (Clark-Lewis et al , 1984) Exposure of the purified IL-3 to pH 2 for 30 minutes did not cause any loss in activity This eliminates IL-3 as a possible candidate for the R1-30 activity

Interleukin 4 (or B cell stimulating factor (BSF)-1) is also discounted on the basis of acid stability IL-4 partially purified from the CM of EL-4 thymoma cell line that had been induced with 4ß-phorbol-12ß-myristate-12α-acetate (PMA) by a procedure which involved adsorption to and elution from TMS-CpG (trimethylsilyl-controlled pore glass beads) followed by reverse phase HPCL The CM was acidified to pH 4 0 prior to the adsorption step (Ohara et al , 1985)

Chromatofocusing, with acidic buffering systems, has been used in protocols for the purification of both interleukin 5 and 6. Interleukin 5 (previously called T cell replacing factor (TRF), B-cell growth factor II (BCGF II), and others) was purified from T cell hybridoma CM by ammonium sulphate precipitation, DE-52

chromatography, Mono P chromatography followed by gel permeation using a Superose 12 column and RP-HPLC (Takatsu et al , 1987) Fractions were eluted from the Mono P column with 10% Polybuffer 74 (pH 6 3-4 0)

Human interleukin 6 (or B Cell Differentiation Factor, BCDF) was purified from a T cell leukemia virus-transformed T cell line by sequential gel filtration and chromatofocusing, again using a Mono P column (Hirano et al , 1985). Fractions were eluted with Polybuffer 74, pH 4 5, which is outside the pH range at which the R1-30 remains stable

Lymphopoietin 1 (LP-1, now designated IL-7) was purified by a strategy which revealed that its activity was stable to a wide spectrum of conditions that included extremes of pH (2 1-8 0) and exposure to high temperatures (Namen et al , 1988) The procedure included DEAE-Sephacel chromatography, SP-Trisacyl chromatography, Blue B Chromatography and finally three phases of RP-HPLC

Interleukin 8 (Monocyte derived neutrophil activating peptide, MONAP, Neutrophil activating factor, NAF) was acidified to pH 3 0 prior to ultrafiltration (Schroder et al., 1988) The retentate was further processed by gel filtration followed by RP-HPLC Examination of the physiochemical properties of NAF showed that the factor was stable to pH 2 and 10 and was not sensitive to trypsin Exposing the factor to 95°C for 5 minutes only partially reduced its activity (Peveri et al , 1988) These results suggest that it is highly unlikely that the R1-30 fraction is IL-8

Interleukin 9 (Leukemia inhibitory factor, LIF) has also been shown to be acid and base stable A four step purification strategy described by Hilton et al (1988) incorporated anion-exchange chromatography (DEAE Sepharose), lentil lectin-sepharose chromatography, cation exchange-exchange chromatography (CM-Sepharose) followed by RP-HPLC The fractions were eluted from the cation-exchange column with a sodium acetate buffer, pH 5 0.

Interleukin 10 (originally known as cytokine synthesis inhibitory factor, CISF) is an acid-labile homodimer that is produced by T

helper cells. IL-10 has an approximate molecular weight of 35-40 kDa, however which may preclude it from being the autocrine factor (Mosmann and Moore, 1991)

Biologically active interleukin 11 (IL-11) was distinguished from IL-1 by a strategy employing chromatofocusing (Nordan and Potter, 1986) with Polybuffer 74, pH 4 0, suggesting its acid stability.

The colony stimulating factors (CSFs) have also been shown to be acid-stable.

Murine granulocyte-macrophage CSF (GM-CSF) was purified from IL-3 (Multi-CSF) by sequential fractionation using salt precipitation, gel filtration, anion and cation exchange chromatographies followed by HPLC (Prestidge et al., 1984) This protocol involved acidifying the partially purified fractions to pH 3 5 before the cation exchange chromatography step

A human lymphokine derived from a bladder carcinoma was purified to homogeneity by Watson et al , (1986), using a procedure which employed 0 9M Acetic acid and 0 2M pyridine, pH 4 0 as a buffer during the HPLC step This factor was assigned the name granulocyte CSF (G-CSF).

Macrophage CSF (M-CSF) has been reported to lose activity when stored for one week at pH 2 1 in the presence of acetonitrile or isopropanol These conditions apparently cause subunit dissociation and so inactivation The purification strategy however includes RP-HPLC from which proteins were eluted in a gradient of aqueous sodium chloride (adjusted to pH 2 1 with HCl) and acetonitrile When eluted the pH of the fractions was adjusted to pH 6.5 with phosphate buffer/Tween (Burgess et al , 1985) The exposure of the factor to pH 2 1 would undoubtedly cause loss of activity in the R1-30 fraction

A purification strategy for erythropoietin (EPO) was described (Sasaki et al , 1987) in which biologically active EPO was eluted from immunoaffinity columns at pH 2 5 This again suggests that EPO is not the R1-30 fraction

It therefore appears that the autocrine factor present in the R1-30 fraction may be a novel growth factor, although any definitive conclusion on this will depend on purification to homogeneity and sequencing

Candidates for the R30 fraction.

The R30 fraction on the other hand may belong to any one of the families of growth factors mentioned above The IGF molecules are about 7 kDa although they are normally found bound to various larger plasma carrier proteins which would result in a higher molecular forms (Hossonlop et al., 1990, Baxter and Martin, 1989; Kiefer et al , 1991) It may be possible that the larger molecule may be acidic or basic FGF in a complex form with either heparin, α_2-macroglobulin or some other binding protein which would by its presence both increase the molecular weight of the molecule and protect it from acid, pH and trypsin treatments

The activation of this fraction following boiling and base treatment is interesting and was examined further Out of 4 different batches of CM, 2 lots were found to be activated on extensive boiling The assays on these samples were carried out with the same serum, cell pretreatment, etc each time Eventhough each CM batch was collected according to the same procedure, however, the pH varied from one batch to the next While this may not be a direct cause of the differences in activity in the CM, it may be an indicator of the how the producer cells were growing during CM collection

Extensive boiling results in the trypsin stable R30 moiety becoming trypsin sensitive, again supporting the theory of a binding moiety protecting the R30 fraction On boiling this moiety is released making the R30 fraction more labile to physical treatments (e g pH or proteases) Boiling at low pH resulted in loss of activity.

An alternative theory suggests that the R30 fraction has a particular conformation that allows it to be very stable but on boiling, this conformation is altered in some way making the molecule more susceptible. The results in Table 3 6 7 corroborates

the second theory, since the R30 fraction does not appear to be broken down into smaller moieties on boiling, although the possibility of a very large complex being broken down into subunits greater that 30,000 MW must not be discounted.

The data presented in this thesis does not allow for any conclusion whether or not the R30 autocrine activity (after boiling or pH treatment) becomes identical to the R1-30 factor, or whether a totally different molecular species is involved. The treated R30 is pH and trypsin labile and loses activity following boiling at low pH, but it cannot be concentrated in the R1-30 fraction (Section 3 6 6)

Molecules have been identified which reversibly bind and with low affinity to their ligands and seem to act as carrier molecules These binding molecules in some cases have been shown to protect the growth factors from proteolytic attack and exposure to extremes of pH and temperature

Heparin has been shown to potentiate aFGF-induced proliferation of human endothelial cells (Thorton et al , 1983, Schreiber et al , 1985, Mueller et al , 1989) and rat pheochromocytoma cells (Damon et al , 1989) In contrast it exerts positive or negative effects on the bioactivity of bFGF depending on the cell type and on experimental conditions (Neufeld et al , 1987, Barzu et al , 1989, Shipely et al , 1989, Gospodarowicz et al , 1990) Heparin has been shown to protect both acidic and basic FGF from heat and acid inactivation (Gospodarowicz and Cheng, 1986), bFGF from plasmin (Saksela et al , 1988), trypsin and partially from chymotrypsin treatments (Sommer and Rifkin, 1989)

An interesting paper was published on the trypsin sensitivity of bFGF by Doble and his co-workers (1990). High molecular weight (23 7 and 27 kD), immunoreactive bFGFs from bovine pituitary and heart extracts were found to be susceptible to proteases which break it down to its standard 18 8 kD form This form, which has a high affinity for heparin, is apparently stablilised to subsequent protease treatment by binding to heparin.

Heparın ıs a mucopolysaccharıde wıth molecular weıght ın the range 6-20 kDa dependıng on source (Merck Index). When heparın bınds the 18.8 kDa form of bFGF ıt forms a complex ın the regıon of 24 - 38 kDa. Such a complex may be concentrated ın the RPMI 2650 R30 fractıon On boılıng the R30 fractıon, the bındıng component ıs removed (heparın), releasıng free bFGF, whıch should be concentrated ın the R1-30 fractıon Thıs ıs not the case however sınce Table 3 6 7 shows that the actıvıty of the R30 fractıon does not shıft ınto the R1-30 fractıon followıng boılıng Therefore a heparın-bFGF complex does not seem to be an explanatıon for thıs effect.

Endothelıal cells have been shown to secrete hıgh molecular mass heparan sulfate proteoglycans and glycosamınoglycans whıch can bınd bFGF ın the same manner as heparın, elıcıtıng sımılar protectıve effects (Saksela et al , 1988) A sımılar secretıon from RPMI 2650 may account for the presence of a bındıng protein ın the R30 fractıon, whıch ıs partıally removed on boılıng

Bındıng proteıns for a number of growth factors have been ıdentıfıed These proteıns ın general ınactıvated theır lıgands and so regulate actıvıty by reducıng concentratıon of the free lıgand

α_2-Macroglobulın (Harpel PC, 1973) a serum protease ınhıbıtor (MW 280 kDa) has been found to act as a bındıng protein for PDGF (Huang et al., 1984), TGF-ß (O'Connor-McCourt, et al , 1987, Huang et al , 1988) and bFGF (Dennıs et al , 1989) Because of ıts tıght bındıng, thıs protein does not seem to be a carrıer protein for these factors, rather a means of elımınatıng ıt from the cırculatıon

α_2-Macroglobulın cannot be removed from PDGF by pretreatment wıth eıther 8M urea, 1M acetıc acıd, 0 1M NaOH or 1% $NaDodSO_4$ at 100°C for 10 mınutes, whıch suggests that covalent bındıng ıs ınvolved Dıssocıatıon does occur however followıng treatment wıth 2 mercaptoethanol

It is important to note that α_2-macroglobulin attachment to bFGF results in its lack of affinity with heparin (Dennis et al , 1989) This may be significant to the R30 factor which as shown in Table 3 8.3 does not bind heparin sepharose.

α_2-Macroglobulin complexes with either TGF-ß or bFGF are unlikely to be the R30 factor, since dissociation would result in autocrine activity concentrating in the R1-30 fraction and not remaining in the R30 fraction PDGF is a dimer with a molecular weight of approximately 30 kDa It is possible that dissociation of this complex from α_2-macroglobulin would result in the activity remaining in a form that could be concentrated in the R30 fraction PDGF is however acid-stable, so removal of the α_2-macroglobulin should result in an acid-stable moiety which is not the case with the R30 fraction

α_2-Macroglobulin binding results in loss of biological activity, which is not the case with the R30 fraction which is active prior to boiling Therefore, for a number of reasons it is unlikely that α_2-macroglobulin is involved in complex formation with the autocrine activity in the RPMI 2650 R30 fraction

The binding proteins for IGFs appears to play a similar role to α_2-macroglobulin Various forms of IGF-carrier protein complexes have been described, with 5 different IGF binding proteins (IGF-BP) been reported to date Their native molecular weight on SDS-PAGE under non-reducing conditions is shown in the table below

TABLE 4.2 IGF binding proteins

	Native MW	Reference
IGFBP-1	28-30	Hossenlopp et al., 1990,
IGFBP-2	33-35	ibid
IGFBP-3	38 5, 41.5	Baxter and Martin , 1989,
IGFBP-4	22 6	Kiefer et al , 1991,
IGFBP-5	26	ibid

In serum, IGF is found in two main fractions of about 50 and 150 kDa (Zapf et al , 1975) The smaller complex is made up of IGF and the binding proteins 1, 2, 4 or 5. The 150 kDa complex is composed of three subunits: an acid labile 80-85 kDa subunit, IGFBP-3 and either IGF I or IGF II.

It is unlikely that IGFBP is responsible for the stability (and possible stimulation) of the RPMI 2650 R30 activity for a number of reasons IGFBPs block binding of IGF to its receptor and so inhibit its action, while activity is present in the untreated R30 sample Also, acid treatment of the IGFBP-IGF complex releases free IGF (heat-stable) (Smith et al , 1987), whereas acid treatment results in the R30 fraction becoming heat-labile (see Table 3 6 5)

TGF-ß has also been shown to bind reversibly to the extracellular matrix proteoglycan, decorin, via its core protein (Yamaguchi et al , 1990) Association of the complex results in loss of TGF-ß activity, indicating again that this type of complex formation is not a factor in the autocrine activity present in the R30 fraction

TGF-ß is secreted in latent forms consisting of the active molecule, a N-terminal peptide (LAP) (Gentry et al , 1989) and a high molecular weight binding protein (Miyazono et al , 1988) This latent form can be activated by acidification (Pircher et al , 1984) and/or trypsin treatment (Lyons, 1988) which is not the case with the R30 fraction where acid treatment and treatment with trypsin left the autocrine activity unchanged. Other reports

suggest that brief heat treatment may activate latent TGF-ß (Brown et al , 1990), although again short heat treated did not activate the R30 fraction, it required extensive boiling.

Lyons et al (1988) found a large (62 kD) inactive form of TGF-ß in the CM of NRK-49F and HT-1080 cells which could be activated by treatment with plasmin but not by acid or urea, indicating that there could be different latent forms of TGF-ß, one of which may account for the R30 activity

The large molecular weight factor may also be due to covalently linked carbohydrate moieties, which on their removal reduces the molecular weight of the molecule without loss in activity, such is the case with the human colony-stimulating factors (CSFs) Human CSFs (GM-CSF, G-CSF, M-CSF and IL-3) are glycosylated polypeptide chains with native molecular weights of 18-30, 20, 45-90 and 15-30 respectively (Metcalf, 1991) Cloning of these factors has shown that removal of the carbohydrate moieties does not impair their biological function, while reducing their respective molecular weights to 14.7; 18 6; 21 and 18, and 15.4 kD. Although it has not been reported in this case, the loss of the carbohydrate moieties may result in decreased stability and increased susceptibility to proteolytic attack Working on the basis of size, none of these homeopoetic growth factors is likely to be the R30 fraction, although a similar effect could result from other glycoprotein growth factors

Also, a 17 kDa protein has been identified which binds both acidic and basic FGF (Wu et al , 1991) in a non-covalent, reversible way. Binding causes inhibition of the biological activities of both acidic and basic FGF But both the binding and inhibition are abolished by the presence of heparin

From these stability results a number of hypotheses are suggested.

(a) There are (at least) two distinct components in the R30 fraction Component I is inactive in the assay and cannot be activated until after extensive boiling/base treatment. Component II is particularly heat-stable and is able to withstand prolonged boiling In some batches of CM component I is not present or at very low concentrations, which would account for CM batches which could not be activated on boiling Component I may be a latent form of growth factor (e g TGF-ß) or associated with a blocking binding molecule (e.g. α_2-macroglobulin).

(b) There are (at least) two distinct components in the R30 fraction Component I is only partially active and its activity is enhanced by boiling and so it contributes to the overall R30 (unboiled) activity Component II is partially heat-stable, and its loss in activity on boiling is overshadowed by the activation of component I The actual ratio of component I·component II is CM batch dependent

(c) There is one major component in the R30 fraction but in 2 different conformational forms, one of which is heat-stable and a second that is capable of activation Again the concentration of the inactive or subactive form is a function of the CM

(d) There is one stimulatory and one inhibitory component in the R30 fraction. Boiling results in the distruction of the heat-labile inhibitor, but does not effect the stimulator which continues to elicit a response in the conditioned medium

4 3 PURIFICATION

Before further characterisation of the autocrine response could be undertaken, a fraction with higher purity was required A number of strategies were examined to try to purify the factor.

Ion exchange and hydrophobic interaction chromatographies were not particularly successful due to a combination of the effect of pH extremes and dialysis (in the case of ion-exchange chromatography) and dialysis alone (in the case of hydrophobic interaction). The autocrine growth activity had been shown previously to be dialysible (Dooley, 1987, Section 3.14.1), but in the experiments described in this thesis, activity was lost in the controls compared with undialysed CM retentates It is possible that this was due to the particular batch of dialysis tubing used for these experiments

Interestingly, Bazill et al (1983) found that dialysis led to variable loss of IL-3 activity, while it was possible to ultrafiltrate the activity using YM ultrafiltration membranes Similar findings were reported by Burgess et al (1980) during the purification of GM-CSF from T19 1 cells

Originally, Dooley (1987, thesis) carried out a purification strategy which incorporated dialysis against 1M acetic acid and Bio-gel P60 and P30 gel chromatographies using 1M acetic acid as a running buffer. Some activity was isolated although this was not compared to the original CM activity so the yield of the eluted fractions was difficult to determine The activity presenting in these experiments was most likely due to the acid-stable R30 fraction and not to the bulk activity in the bulk conditioned medium

Because of the stimulatory effects of acidic and basic fibroblastic growth factors in the autocrine assay, heparin sepharose chromatography was examined as an alternative to ion-exchange, gel filtration and hydrophobic interaction chromatographies.

Heparin sepharose chromatogrpahy is a well established procedure for purifying members of the fibroblast growth factor family. Heparin is a natural mucopolysaccharide with anticoagulant properties It consists of D-glucosamine and D-glucuronic acid residues and a large number of amino groups combined with sulphate groups As a result of its composition, heparin is a general ligand and can combine with a number of proteins and polycationic organic compounds, by either specific or ionic mechanisms

For every run through the heparin sepharose column, two distinct fractions were isolated, a fraction that bound weakly to heparin which could be eluted with 0 5M NaCl and a fraction that appeared to have no affinity for heparin

In the initial run activity was eluted following addition of 0 5 M NaCl with some activity being eluted at 0 25 M NaCl Above this concentration of NaCl no further activity was eluted Initially this was attributed to growth inhibition by high salt concentrations In these experiments the control medium samples supported low colony formation and so the assay was unable to pick up inhibition But subsequent diafiltration of pooled fractions showed that while the high salt concentration did in fact cause some inhibition, no autocrine activity was eluted above 0 5M NaCl

The broad specificity of heparin sepharose chromatography makes identification of the heparin binding moiety difficult Many proteins have a stronger affinity for heparin than the RPMI 2650 factor

Heparin-binding growth factors are isolated primarily by their strong affinity for heparin which requires salt concentrations of higher than 1M NaCl for elution Acidic and basic FGFs elute from heparin sepharose at 1 0M and 1 5 M NaCl respectively (Shing et al., 1984) FGF4 (*hst*/K-fgf) and FGF 5 have similar affinities (Klasgsburn, 1989) FGF3 (*int*-2) appears to be non-heparin binding KGF, the seventh member of the FGF family has been reported to elute from heparin sepharose columns at 0 6M NaCl (Rubin et al., 1989).

A heparın-bındıng EGF was also descrıbed (Hıgashıyama et al , 1990, Hıgashıyama et al., 1992), thıs elutes from heparın sepharose at 1 0 to 1 2 M NaCl.

Two heparın-bındıng growth factors have been ısolated from rat braın. Pleıotrophın, an 18 kDa neurıte-promotıng factor (also called HB-GAM and FGF-8) (Lı et al , 1990, Merenmıes and Rauvala, 1990) bınds heparın tıghtly, beıng eluted at salt concentratıons of 1 5M NaCl (Rauvala and Pıhlaskarı, 1987). The sequence shows, however, that ıt ıs not a member of the heparın-bındıng growth factor famıly and cannot be desıgnated FGF 8 as previously assıgned (Merenmıes and Rauvala, 1990). A 30 kDa component (p30) was ısolated ın these studıes as a major neuron-bındıng protein, and was suggested to have a regulatory role ın neuronal growth (Rauvala and Phlaskarı, 1987) p30 ıs detached from heparın sepharose at 0 75-1 0M NaCl ın salt gradıents

The protein encoded by the MK gene has been ıdentıfıed as a heparın bındıng protein (Tomomura et al , 1990) Thıs gene was expressed temporarıly durıng the early stages of retınoıc acıd-ınduced dıfferentıatıon of embryonal carcınoma cells as well as durıng the mıdgestatıon perıod of mouse embryogenesıs (Kadomatsu et al , 1988). Thıs study led to the ısolatıon of a 19 kDa heparın-bındıng protein secreted by chıcken embryos (Vıgny et al , 1989) whıch can be ınduced by retınoıc acıd, called retınoıc acıd ınduced-heparın bındıng (RI-HB) protein Thıs protein can be eluted from heparın sepharose columns at 1-1 1 M NaCl and ıt has the same apparent bıologıcal actıvıtıes as HB-GAM (neurıte outgrowth and prolıferatıon of PC12 cells) (Raulaıs et al , 1991). RI-HB and HB-GAM have very sımılar N-termınal sequences It ıs not known ıf the MK protein ıs the mammalıan counterpart of RI-HB or whether they are two dıstınct members of the same famıly

Other growth factors bınd heparın sepharose less tıghtly and can be eluted at lower salt concentratıon, sımılar to that of the bound RPMI 2650 actıvıty.

Vascular endothelial cell growth factor (VEGF), a mitogen for endothelial cells, has been isolated from a variety of sources including bovine pituitary folliculo-stellate cells (Ferrara et al , 1989, Gospodarowicz et al , 1989); rat glioma cells (Conn et al., 1990) and a human epidermoid carcinoma line (Myoken et al , 1991). This 40-46 kDa protein elutes from heparin sepharose columns at 0 3 M NaCl

Epithelial transforming growth factor (TGFe) (Halper and Moses, 1983) was originally isolated in a 59 kDa form that did not bind heparin Subsequently a lower molecular weight form of this molecule was isolated from bovine kidney (Dunnington et al , 1990, Parnell et al , 1990) which could be eluted from heparin sepharose at 0 5 M NaCl.

Also, an IGF-II-like peptide isolated from fetal bovine serum was shown to have a weak affinity for heparin and could be eluted from heparin sepharose columns at about 0 5 M NaCl (Li et al., 1990) In this same study IGF I was also applied to a heparin sepharose column and was found to bind, being eluted at salt concentrations less than 0 5M.

PDGF also has been shown to bind heparin and can be eluted with 0 5 M NaCl (Shing et al., 1984, Klagsbrun et al , 1985).

Mouse embryonal carcinoma (EC) cells have been shown to produce two heparin-binding factors one, thought to be related to PDGF, which is eluted at low salt (0 3-0 5M NaCl) and a second, related to bFGF, which is eluted at high salt concentrations (Tiesman et al , 1988)

Granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin 3 (IL-3) (Roberts et al., 1988), as well as melanoma growth stimulatory activity (MGSA)/gro, Interleukin 8 (IL-8) (Derynck et al., 1990) and connective tissue activating peptide (CTAP III) (Castor et al , 1991) are all eluted from heparin at low salt concentrations

Secreted Int-1 protein from a cloned fibroblast cell line (MXI-5/R2) was found to bind heparin agarose and to be eluted with a salt concentration of about 0 6M NaCl (Bradley and Brown, 1990)

Many purified growth factors are presumed not to be heparin-binding, but in a lot of cases these studies are carried out using a running buffer of 0 6M NaCl, which would preclude any factors weakly binding heparin sepharose (such as those secreted by RPMI 2650 cells) and would not isolate factors that might bind to heparin sepharose under physiological salt concentrations Examples of such running systems are presented in the table below

TABLE 4 3 Examples of heparin sepharose systems using running buffers containing NaCl

Source of growth factor	NaCl (M) of running buffer	Reference
Human malignant gliomas	0 6	Rutka et al , 1989
Chick basement membrane	0 65	Vigny et al , 1989
Mouse mammary tumour	0 6	Kasayama et al , 1991

Other growth factors have no affinity for heparin sepharose These include EGF, TGFα, TGFß, Tumour necrosis factor (TNF)-α and IL-1 (Klagsburn, 1989)

To get a better idea of what factors could be present in the fraction bound and unbound to the heparin sepharose, stability studies similar to those carried out on the bulk conditioned media and on the samples fractionated by ultrafiltration were done These results indicated that the unbound fraction exhibited characteristics similar to that seen in the R30 retentates (i e acid- and base-stable, and stable to boiling for 6 minutes, with an

activation after 20 minutes), while the fraction bound to the heparin was acid- and base-labile and sensitive to temperature (similar to the R1-30 fraction)

The similarity of the bound activity to the R1-30 activity and the unbound to the R30 warranted further investigation Samples fractionated by ultrafiltration into R1, R1-30 and R30 fractions were applied to heparin sepharose in the same manner as before. The R1 fraction gave similar results to the straight CM with activity distributed between the bound and the unbound fractions. The R1-30 fraction was found to be predominantly heparin-binding while the R30 fraction was predominantly non-heparin binding This result gives an indication of the sizes of the factors involved and may be used along with stability information to narrow down the number of characterised growth factors that may be acting in the RPMI 2650 system

The number of pH and temperature sensitive growth factors that weakly bind heparin is limited

Basic and acidic FGF are both heat-labile. While acidic FGF can withstand acidic conditions, basic FGF, when exposed to a pH lower than 4 0, losses 95-97% of its potency (Gospodarowicz et al , 1985) However, aFGF and bFGF have higher affinities for heparin than the bound RPMI 2650 fraction, being eluted from heparin sepharose columns at salt concentrations of above 1M NaCl KGF is also heat and acid labile (Rubin et al , 1989) but has been found to be eluted at salt concentrations of 0 6M NaCl and so is unlikely to be the RPMI 2650 factor Other members of the FGF family are not well characterised as regards acid and heat stability but can be discounted as a possible candidate for the R1-30 factor since, besides FGF-3, they all have higher affinities for heparin

TABLE 4 4 **The Fibroblast Growth Factor Family**

Name	Elution from heparin sepharose (M NaCl)	Size (kDa)	Stability Heat	acid
aFGF	1-1 2	15-18	L	S
bFGF	1 5-2	16-18 5	L	L
int-2/FGF3	0	28 5		
hst-1/FGF4	1-1 2	17.5		
FGF5	1 0			
hst2/FGF6	1 0			
KGF	0 6	28	L	L

S=stable L=labile

The bound fraction is not likely to be VEGF which is eluted from heparin sepharose at 0 3M NaCl This factor has been reported to be stable to boiling for 5 minutes and to acidification to pH 2 5 for 5 hours (Myoken et al , 1991) Also glioma derived and pituitary folliculo-stellate cell derived VEFGs are discounted as being the heparin-binding fractions since their molecular weights were each determined to be 46 kDa (Conn et al , 1990, Gospodarowicz et al , 1989).

TGFe has been shown to be stable to boiling for 5 minutes following a purification procedure which employed acidic running buffers (Halper and Moses, 1987), and so could not be the bound factor

IGF I and II are bioassayed following acidification to remove binding proteins (Tamura et al , 1989, Greenstein et al., 1987), indicating their acid stability

MGSA has been designated a member of a family of acid- and heat-stable polypeptides, which includes CTAP III, that act in an autocrine fashion to stimulate the growth of malignant melanoma cells in serum free medium, (Richmond et al., 1986) so it is unlikely that the bound factor in the RPMI 2650 system belongs to this family

PDGF has been well characterised as a heat stable growth factor (Antoniades, 1981) and purified preparations are routinely stored in 1 M acetic acid (-20°C) without loss of activity for several months (Heldin et al , 1987).

As described previously GM-CSF, IL-3 and IL-8 are also acid stable.

The R1-30 factor is unlikely to be int-1 since this protein is eluted at 0 6M salt The size restriction also rules out the possibility of the secreted int-1 protein being the factor involved Bradley and Brown (1990) have shown that the int-1 protein has a molecular weight of 44 kDa under reducing conditions

TABLE 4 5 Non-FGF Heparın-bındıng Growth Factors

Name	Elutıon from heparın sepharose (M NaCl)	Sıze (kDa)	Stabılıty Heat	acıd
HB-EGF	1-1 2	19-23		
VEGF	0 3	46	S	S
TGFe	0 5	11	S	S
IGF II	0.5	16,8,7	S	S
IGF I	<0.5	7	S	S
PDGF	0 5	28-36	S	S
GM-CSF	0 5	22	S	S
IL-3	0.5	25	S	S
MGSA	0 5	8	S	S
IL-8	0 5	8	S	S
ınt-1	0 6	44		

A range of adhesıve molecules bınd weakly to heparın sepharose These ınclude mouse lamının, whıch ıs eluted at salt concentratıons of 0 2-0 4M NaCl (Engvall et al , 1983) Fıbronectın has also a weak affınity for heparın and ıs eluted from heparın-agarose columns wıth 0 5M NaCl (Bober Barkalow and Schwarzbauer, 1991) These factors are not lıkely to be the heparın bındıng entıty ın the RPMI 2650 CM sınce these factors normally have a much larger molecular weıght than would be concentrated ın the R1-30 fractıon

Affınity for heparın reflects bındıng to heparın-lıke molecules on cell surfaces, ın extracellular matrıces and basement membranes In general the affınity of a protein for heparın sepharose ıs hıgher than ıts affınity wıth heparan sulfate Such ıs the case wıth the 19 kDa protein ısolated from chıck embryos (Vıgny et al , 1989, ıbıd) and bFGF (Vıgny et al , 1988) In the case of the 19 kDa protein, elutıon from heparın sepharose occured at 1-1.1 M NaCl whıch ıt could be dısplaced from the basement membranes at only 0.5M NaCl Thus the weak affınity of the RPMI 2650 factor for

heparin does not necessarily imply that the factor is bound under normal physiological conditions to heparan sulfate chains of proteoglycans in the extracellular matrix

It has been known for some time that heparin inhibits growth of non-transformed BHK/21/13 cells in agarose (Sanders and Smith, 1970) Whether this may be simply a matter of heparin taking growth factors out of the autocrine circulation system or whether heparin is a true inhibitor is not clear. Heparin however does not affect the growth of RPMI 2650 colonies in soft agar.

An interesting study was undertaken by Seno et al (1990), where they constructed terminally truncated bFGF in *E. coli* The first group of truncated proteins lacked the basic region present at the carboxyl-terminus of bFGF (including one of the heparin binding sites Arg^{107} Ser^{108} Arg^{109} Lys^{110}) and was eluted from heparin sepharose in a single peak at 0 3M NaCl, and retained weak mitogenic activity on BALB/c 3T3 cells The second group had the heparin binding site but lacked Phe^{139}, Leu^{140} and Pro^{141} This group is present in cell membranes and is also eluted in a peak of 0 3M NaCl along with an equal sized peak at 1-1 3M NaCl As the number of amino acids truncated was reduced, the affinity of the protein for heparin appeared to shift to the 1 3M NaCl eluted form and high mitogenic activity

Any of the characterised growth factors that have no affinity for heparin listed above (EGF, TGFα, TGFß, TNF, IL-1) could account for the non-heparin binding fraction of conditioned medium, since each are stable to acid and heat treatments Alternatively, the unbound fraction may be a result of heparin- or heparan sulfate-bound FGF Heparin protects FGF from heat, pH and protease treatments and also blocks binding to heparin sepharose columns (as described before)

An interesting result was presented in Table 3 8 4, where TGF-ß activity in the R1 and R30 samples was predominantly heparin binding with considerably lower levels of activity in the unbound fractions This is unexpected since TGF-ß has not been previously reported as being heparin binding (Klagburn, 1989) It has been reported, however that IGF I and II have a weak affinity for

heparın sepharose Thıs together wıth the results presented previously that IGF I and II caused a posıtıve response ın the NRK-49F assay system, would ındıcate that possıbly RPMI 2650 cells produce an IGF-lıke molecule whıch bınds heparın sepharose and can be detected by the NRK-49F soft agar bıoassay. Thıs hypothesıs could easıly be dıscounted ıf thıs TGF-ß-lıke actıvity was heat sensıtve sınce both IGF I and II are heat-stable molecules

4 4 SCALE-UP OF THE GROWTH AND AUTOCRINE FACTOR PRODUCTION FROM RPMI 2650 CELLS

Routinely conditioned media from RPMI 2650 cells were collected from roller bottle cultures This involved seeding the cells from one large flask into a roller bottle and growing the culture for one week, after which collection of CM is possible Up to 600 mls of CM could be collected from each roller bottle over one week (100 mls daily, discarding the first collection) The collection procedure is long (2 weeks from innoculation to final collection), quite labour intensive and the contamination risk is high

The large-scale aspect of this project was designed to improve on this procedure by producing more (active) conditioned medium in a shorter time, with less risk of contamination This was to be achieved in a computer controlled bioreactor (1.8 litre capacity) Before this capacity could be attempted, a number of preliminary questions had to be addressed Can microcarriers be used, and what type? Can the cells grow in suspension? What is the most appropriate medium and sera to use? Does agitation rate effect growth factor production? Does oxygen levels effect growth factor production?

Microcarrier cultures

RPMI 2650 cells grow in monolayer on plastic (in tissue culture flasks) and on glass (in roller bottles) In the first set of experiments different types of microcarrier beads were examined for their suitability as regards growth and autocrine activity production.

Microcarriers (van Wezel, 1967) are small spherical beads suspended in culture medium which provide a surface for cell attachment and allow monolayer cell growth to occur The beads must have a continuous even surface for the cells to attach and be

large enough that the curvature of the surface will not affect growth (ideally about 200-300μm) It is important that the distribution in size of the beads is narrow This ensures that the cells on each bead become confluent at the same time The beads are slightly more dense than water, so they settle quickly, but can be kept in suspension with little agitation.

The first type of bead examined were glass, plastic and collagen coated Biospheres (Whatman) (plastic matrix, density 1 02 g/ml, diameter 150-210μm). Although they allowed attachment and growth of cells, they were found to be unsuitable since, because the beads were not translucent, it was difficult to make routine observation of the cells The dextran Cytodex beads (Pharmacia) had excellent optical properties and therefore had more potential as a scale-up tool for the RPMI 2650 cells

The surface coating of the bead is very important for attachment and growth Plastic coatings (sulphonated polystyrenes) mimic the surface of culture flasks, and so the cells should grow and secrete product in the same manner as culturing them in flasks The main disadvantge of this type of bead is its optical properties. Glass beads provide a negatively-charged surface and this allows rapid cell attachment and detachment Because of its high density, glass is usually coated on less dense materials (e.g plastic), so it can be kept in suspension more easily The growth of cells on these beads is similar to growth in roller bottles. In practice, however, the growth rate from both these bead types is also affected in spinner flasks by shear effects (discussed later) and so it cannot be considered identical to conventional monolayer systems.

The connective tissue protein, collagen, has been widely used as a cell culture substrate Cytodex 3 microcarrier beads are coated with a surface layer of denatured collagen covalently bound to a matrix of cross-linked dextran The denatured collagen (MW 60,000-200,000) is derived from pig skin type I collagen which has been extracted and denatured by acid treatment, concentrated and purified by ion-exchange before coupling to dextran Collagen is

present in basement membranes on which cells adhere in vivo and has a high affinity for fibronectin which promotes cell attachment and spreading (Kleinman et al , 1981)

Cytodex 1 and 2 consist of dextran beads cross-linked with positively-charged molecules N,N-diethylaminoethyl (DEAE) groups are distributed evenly through the dextran matrix in Cytodex 1 beads. Whereas Cytodex 2 beads have an outer layer of positive charges (N,N,N-trimethyl-2hydroxy-aminopropyl groups) (See Appendix E). Because Cytodex 2 beads do not have charged groups present inside their matrix, negligible binding of medium components and cell products occurs

The exchange capacity of microcarrier beads has been considered a critical factor for attachment and growth of animal cells. This parameter is based on the total protonation of the molecules attached to the supporting backbone polymers Complete protonation occurs at very low pH levels (below pH 2 0) At pH 7 4, only a portion of the molecules are protonated Thus the charge density under physiological conditions is the actual critical parameter (rather than exchange capacity) for cell attachment (Himes and Hu, 1987).

Cell attachment to Cytodex 1 microcarrier beads has been found to be primarily an electrostatic effect. Attachment rates can be reduced in the presence of serum (Himes and Hu, 1987) However, the cells remain rounded and do not spread until after serum has been added Because of this some researchers suggest that the initial attachment of cells onto the beads should be done in PBS rather than in medium supplemented with serum.

The overall growth kinetics of RPMI 2650 cells on each of the Cytodex beads tested were similar However, the production of autocrine activity was variable depending on the bead type When the activity was calculated on a 'per cell' basis, the results indicated that the cells growing on the Cytodex 3 microcarrier beads were not producing autocrine activity or else the autocrine activity was being adsorbed onto the bead Either way, the level of activity collected in the CM was negligible compared with the CM collected from cultures of cells grown on Cytodex 1 or 2

The studies on attachment rates described in the literature are based on how quickly the cells attach to the beads, in terms of minutes. In the work presented in this thesis the percentage of attached cells is calculated on a daily basis This gives a better indication of the performance of the beads during a culture run (with agitation), and is probably more appropriate for deciding the suitability of the bead type.

The fact that quite a large number of cells could grow well in suspension implied that the cells were capable of anchorage independent growth Microcarrier culture is an excellent way of propagating large numbers of anchorage-dependent cell lines But if the cells could grow well and produce CM containing reasonable levels of autocrine activity without a surface to attach to, there would be no need to use them

Suspension cultures

The remainder of the scale-up work in this thesis was carried out on cells grown in suspension The cells did not grow as single cells (like hybridoma cells) but rather in small clumps or flocs This type of system is termed a "cell aggregate suspension" culture This system has been optimised for other anchorage dependent cell lines for example HepG2 and 293 (Hu and Peshwa, 1991) Some transformed cell lines have also been cultivated as aggregrates (Tolbert et al , 1980)

In microcarrier cultures the ratio of the settled bead volume to culture volume is low This is a major disadvantage as it difficult to maintain a homogeneous suspension while keeping shear on the cells low (Hu and Wang, 1986) Compared with a microcarrier culture, the settled volume of the solid phase in an aggregate culture is much lower at the same cell concentration

Different methods have been used to induce aggregate formation for cells which normally grow on or prefer to grow on surfaces This usually involves the use of medium containing a low concentration of calcium in conjunction with a moderately high agitation rate

This method of cell cultivation allows for an easier cell retention in perfusion systems due to the large size of the particles, and thus high cell concentrations can be acheived

There has been some studies on lectin-induced agglomeration of cells after trypsinisation (Jones and Perry, 1980). But this approach has not been developed any further.

Aggregate cultures can also be induced by adding microspheres to the cells (Goetghebeur et al , 1990, Goetghebeur and Hu, 1991/92). Microspheres are similar to conventional microcarriers but are much smaller in size (10-60 μm) The method of action is similar to the seeding of crystals in a crystallization process to induce crystal growth The cells attach preferentially to the microsphere surface rather than to each other An aggregrate is formed by outgrowth of cells and/or by agglomeration of a number of beads

The RPMI 2650 cells were 'encouraged' to grow in suspension by siliconising the culture vessel before autoclaving This made the glass surfaces hydrophobic and so prevented the cells sticking and attaching The cells then apparently used each other as attachment substrates and formed tight, discrete clumps

The initial suspension work done with RPMI 2650 cells used standard MEM media (supplemented with foetal calf serum) (Gibco) containing 200 mg/litre calcium chloride The cells grown in suspension with this medium formed tight aggregates which were very difficult to trypsinise and count

MEMS (Gibco) (see Appendix G for composition) performed much better in the RPMI 2650 system The cells grew well and formed loose flocs This type of floc formation has a number of other advantages Oxygen and nutrients can pass easily through the floc to the cells growing in its core. Waste products can pass out into the surrounding medium Therefore loose aggregates provide a more homogeneous system Also, CM collected from the MEMS culture system exhibited higher levels of autocrine activity than that from the MEM culture after a three day period

Unlike in monolayer cultures, the batch of serum used does not appear to have a dramatic affect on the rate of growth of the suspension cultures A number of different batches of donor horse sera were examined and compared with the batch of FCS that had been used for routine culture of RPMI 2650 cells Comparable growth curves were generated This may be a reflection of the variable presence of attachment factors in the sera which do not hold the same importance in suspension cultures as they would have in anchorage dependent systems

Following these experiments a growth and production protocol was set up for the RPMI 2650 cells in suspension. This involved seeding the cells at approximately 10^5 cells per ml into MEMS supplemented with the chosen batch of DHS The cells were grown for 4 days The media was then removed from the cells and replaced with fresh MEMS without serum The cells conditioned the medium for three days after which time the CM was collected and processed (ultra-filtration or heparin sepharose chromatography) This reduced the length of time from flask to last collection of CM from 2 weeks, for roller bottles, to one week and the process could be easily scaled up using the bioreactor with the potential of collecting 1 8 litres in one week.

Agitation Effects

The agitation rate had a dramatic effect on RPMI 2650 growth in serum-free medium At high agitator speeds the cell count was reduced from 5 x 10^5 cells/ml to about 2 x 10^4 cells/ml, after two days in medium without serum At lower rates the drop was not as great (down to 3 x 10^5 cells/ml)

Historically, it has been believed that suspended animal cells are extremely fragile relative to other microorganisms However, when the effects of mechanical agitation alone were properly examined experimentally, it was found that cells of both insect and mammalian origin were generally far more robust than expected (Oh et al , 1989)

Estimating shear effects is a complex procedure Hybridoma cells may be grown successfully in slowly stirred laboratory vessels, but are increasingly difficult to handle when the systems are scaled up This difficulty may be associated with the damaging effects of turbulent shear which are more predominant on a larger scale While the average bulk shear stress in small and large reactors may be comparable, the modes of operation in the two may differ. There a tendency at very large scale towards turbulent flow, rather than the less damaging laminar flow, with the possibility of both macroturbulence (large eddy formation due to impeller action) and microturbulence (smaller eddies, at a cellular level, formed by dissipation of larger eddies) (Bliem and Katinger, 1988) Presumably, therefore, scaling this 100 ml culture to production scale could increase the detrimental effects of shear on the cell number and viability

Initial work by de St Groth (1983), reported that the growth rates of ten different hybridoma cell lines were 15% lower in a stirred vessel, with minimal agitation, than in a stationary culture This work was subsequently discounted by Dodge and Hu (1986) as lacking proper controls to account for the possibility of localised concentrations of conditioning factors in the stationary culture that might improve cell growth In their study they found detrimental effects on hybridoma growth only at agitation rates which were four times that required to keep the cells in suspension Lee et al (1988) looked at the effects of shear caused by agitation in the stationary and decline phases of batch growth of hybridoma cells (when a significant portion of monoclonal antibody is secreted (Velez et al , 1986) It was found that cells in stationary and decline phases of growth were sensitive to shear forces, but cells in the growth phase seemed less sensitive The shear sensitivity of hybridoma cells has been shown to be increased by ammonia accumulation and pH changes (Petersen et al , 1988), decreases in culture medium viscosity (Croughan et al , 1989), decreases in concentration of sera (Kunas and Papoutsakis, 1990) and the absence of surface active agents (Michaels et al., 1991), at low temperature and on depletion of nutrients (Al-Rubeal et al., 1990)

The protecting effect of serum is interesting in relation to the work presented in this thesis It has been shown that progessively higher concentrations (up to 10%) of FCS reduces cell death and allows growth of cells at substantially higher agitation rates (Kunas and Papoutsakis, 1990, Lee et al , 1989) The possible mechanism of serum protection has not been fully elucidated and may be either fluid-mechanical (Kunas and Papoutsakis, 1990) or biological (Michaels et al , 1991)

The RPMI 2650 cells appear to be dramatically shear sensitive, with a huge reduction in cell numbers over a relatively small agitation range (20-60 rpm) Whether this is due to the absence of serum or due to the nature of the cells themselves, cannot be elucidated from the results presented here To study the hypothesis that serum protects the RPMI 2650 cells from increasing shear rates, additional controls would need to be included For example, flasks could be treated in the same manner as described here, but instead of being set up in serum free medium, the cultures could be grown at various levels of serum and exposed to different agitation rates.

The response to shear exposure are diverse at the cellular level, depending on both the type of cell and on the intensity and length of exposure to shear Schurch et al (1988) found that the hybridoma cells remaining viable after exposure to shear stress showed growth and monoclonal antibody production rates similar to unexposed cells

At shear rates below fatal levels, while DNA synthesis rates are inhibited, metabolic rates (measured by the MTT assay) may be increased with increasing shear (Al-Rubeai et al , 1990) Frangos et al (1988) followed the metabolism of arachidonic acid by primary human umbilical vein endothelial cells, by measuring the production rate of prostacyclin They found that maximising shear stress caused subsequent increases in metabolite production.

Cyclic adenosine monophosphate (cAMP) levels in osteoblasts <u>in vivo</u> were shown to increase by increasing shear (Reich et al., 1990).

Increasing shear stress in human embryonic kidney cells showed that above a critical shear level, the viability of the cells dropped but the surviving cells produced up to 2 5 times more urokinase than the untreated controls (Stathopoulos and Hellums, 1985).

Giard et al. (1979) previously found that human fibroblasts secrete up to 30-fold greater amounts of interferon when maintained on microcarriers in spinner flasks compared to cells in roller bottles They suggest that this may be attributed to the shear effects in the spinner flasks

In one report the levels of bFGF mRNA was monitored for increasing shear rates (Diamond et al , 1990) Primary cultures of umbilical vein endothelial cells were either maintained in stationary phase or exposed to arterial levels of shear stress (25 dynes/cm^2) for 24 hours. Total cellular RNA was isolated and levels of bFGF mRNA were determined using a coupled reverse transcriptase/polymerase chain reaction method The level was compared with similarly prepared glyceraldhyde 3-phosphate dehydrogenase (GAPDH) and tPA mRNAs It was found that the levels while the levels of tPA mRNA increased with shear, bFGF and GAPDH mRNAs remained unchanged. In a follow up study Nollert et al., (1991) found that these cells secreted decreased levels of endothelin peptide and mRNA in response to increasing shear rates.

Steady shear stress has also been shown to induce a transient increase in PDGF A and B mRNAs levels in human umbilical vein endothelial cells (Hsieh et al , 1991), which appears to be mediated by protein kinase C activation (Hsieh et al , 1992)

Neither autocrine nor TGF-ß activity levels varied with increasing agitation rate in the RPMI 2650 spinner flasks studied. TGF-α levels were higher at the higher rate indicating a possible correlation, although more work would have to be done at a range of agitation rates to verify this result. Presumably, also, scaling

up this process into production levels would result in even greater increases in TGF-α levels because of the associated increase in turbulent shear

Following this small scale experiment, It was decided not to change the agitation speed in the culture for the CM production phase, since it did not affect the autocrine activity

Oxygen Effects

The next step was to scale-up the 100 ml suspension cultures to 1.8 litres This was achieved with the Braun Biostat MC bioreactor, which provided temperature, agitation, pH and dissolved oxygen control pH was maintained at 7 3 by means of CO_2 gas and sodium bicarbonate (1M) addition The oxygen level was maintained by a balance of nitrogen and air. The gases (CO_2, nitrogen and air) were introduced into the system indirectly by a silicone tubing aeration basket and directly by surface aeration

In a number of preliminary runs, the oxygen levels were controlled at 40-60% for the growth phase, which lead to consumption of excessive amounts of gases. Because of this it was decided not to have an upper oxygen limit on the medium and to allow natural depletion of the dissolved oxygen levels (due to cellular metabolic activities) A lower limit of 40% DO was imposed below which air would be added This lower limit was never reached in the growth phases of the three runs carried out The profile of oxygen depletion was different for the runs described This, however, was not indicative of the growth of the cells from one run to the next

Based on the curves generated in Figure 3 9 9, the average oxygen uptake rate for RPMI 2650 cells is in the region of 0 0010 to 0 0018 mmol oxygen/hour for a cell population of about 10^5/ml in a culture of 1 8 litres Reported values for oxygen uptake rates range from 2-10 x 10^{-12} g/cell/hour (Speir and Griffiths, 1984) or 0 0531-0 59 mmol O_2/litre/hr for cultures of 10^6 cells per ml

(Glacken et al , 1983) or from 0 1 (Vero) to 0 05 (human intestinal cells) g O_2/litre/hr for cultures of $4x10^6$/ml (Freschaker and Sinskey, 1981, Wang et al , 1988) As shown in Appendix F RPMI 2650 cells may be considered to have a particularly low oxygen consumption rate

On average the oxygen was consumed at a rate of approximately 0.3-0 5 % per hour It would take this culture of RPMI 2650 cell 8-13 days approximately to deplete this level of oxygen Therefore, at a two-litre-scale oxygen limitation is not a problem over a four day batch run Although at higher cell densities it may become rate limiting (Pugh, 1988).

The effect of three different oxygen levels on serum-free growth and viability of RPMI 2650 cells were examined Cell viability remained high for the culture run at 40% DO (Mid oxygen) and the number of cells increased At the high and low oxygen levels, the total cell count dropped and the viabilities were lower Around 40% appears to be optimum for this system This correlates with figures for most cell lines Typical oxygen concentrations are estimated between 2-4 mg/litre (i.e. 32-64% of saturated levels) (MacMichael, 1989) In batch culture, oxygen levels between 8% and 100% were found to have no real effect on the cell growth of the hybridoma cell line, NB1 (Boraston et al , 1987) Reuveny et al (1985) found that the optimum dissolved oxygen level for growth of hybridoma cells was between 50% and 60%

At low levels of oxygen (less than 50%), Reuveny et al. found that glucose ultilisation was reduced, and the cells retained viability longer, although the maximum cell concentration was not as high As DO is decreased, lactic acid formation is increased (Miller et al , 1987) Lactic acid production rates decrease four fold (from 0 3 mg of lactic acid per 10^5 hybridoma cells per day) when the dissolved oxygen level is increased from 25% to 75% (Reuveny et al , 1986) High lactic acid levels at low oxygen concentration are due to incomplete glutamine oxidation resulting in the consumption of glucose as the primary energy source

At high levels of oxygen, cells die due to the cytotoxic effects of hyperoxia The oxygen-derived radicals formed can attack all cellular components, leading to membrane peroxidation, enzyme inactivation and nucleic acid alterations (Freeman and Crapo, 1982) To protect against these reactive species, cells contain low molecular weight molecules like ascorbic acid, α-tocopherol, glutathione and anti-oxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase and glutathione reductase (Fridovich, 1986)

Michiels et al (1990) carried out a comparative study of oxygen toxicity in human fibroblasts and endothelial cells They found that the endothelial cells were more sensitive to the increased oxygen tension Looking at levels of intracellular anti-oxidants present in each cell type after oxygen exposure, they found that levels of most of the antioxidants listed above are lower in the endothelial cells than in the fibroblasts, which may account for the differences in sensitivity.

Sullivan et al (1991) reported that replacement of media in cell cultures during exposure to hyperoxia was found to alter oxygen toxicity in Chinese hamster fibroblast cells Membrane peroxidation during hyperoxia gives rise to lipid breakdown products, such as hydroperoxides and aldehydes, which are also highly reactive and are capable of causing tissue injury (Spitz, et al., 1990) Sullivan et al (1991) suggest that replacing the medium removes these toxic aldehydes and replaces them with additional substrates for the synthesis of biomolecules required for the repair and/or detoxification reactions

The expresssion of PDGF, its receptor and related genes was measured in the lung tissue of rats exposed to air and 85% oxygen (Han et al , 1992) While the PDGF-A chain mRNA was unaffected by exposure to 85% O_2, transcription of the PDGF-B chain mRNA was increased

Low levels of oxygen can also effect product formation Erythropoietin (EPO) is a glycoprotein hormone that regulates erythrocyte production, and thus, blood oxygen-carrying capacity in

mammals Steady state EPO mRNA levels increase when Hep3B human hepatoma cells (Goldberg et al , 1987, Goldberg et al , 1988) are made hypoxic

Low levels of oxygen have also been implicated in a transient multiple drug resistance (MDR) in EMT6/Ro cells (Sakata et al , 1991), which does not seem to be associated with P-glycoprotein-associated MDR When the cells return to a normal oxygen environment, they lose their resistance. This oxygen effect has serious implications as regards control of tumour growth in vivo.

Hypoxia also induces TGF-ß1 mRNA levels in infant rat brains, where TGF-ß1 is speculated to be involved in post-asphyxial repair mechanisms (Klempt et al , 1992)

Autocrine activity did not vary with DO levels, but when this activity was separated into heparin binding and non-heparin binding fractions, differences were apparent At mid and low levels of oxygen, the activity was predominately non-heparin binding But at high oxygen levels the activities of bound and unbound fractions were comparable, with the level of unbound activity reduced at higher oxygen and the level of bound activity increased.

TGF-α activity also dramatically increased with oxygen levels (16 fold), while TGF-ß activity appears to drop slightly The limited results presented do not clearly demonstrate whether these results are indicative of the relatively low viability or represent a a direct effect of oxygen itself

5 0 CONCLUSIONS AND FUTURE DIRECTION OF PROJECT

5.0 CONCLUSIONS AND FUTURE DIRECTION OF THE PROJECT

The results in this thesis show that RPMI 2650 cells produce at least three different growth stimulatory activities, which can be collected in their conditioned medium, a TGF-α-like activity, a TGF-ß-like activity and an autocrine activity

While the TGF-α-like assay was specific for TGF-α and EGF, IGF-I and to a lesser extent IGF II could cause a positive response as well as TGF-ß in the NRK-49F assay

The autocrine activity could be fractionated into two distinct activities

Fraction 1 = Heparin-binding, acid-, base- and heat-labile activity concentrating in the range 1-30 kDa,

Fraction 2 = Non-heparin-binding, acid-, base- and heat-stable activity greater than 30 kDa
Boiling renders labile to acid and trypsin

The published information on the stability and fractionation properties of acid- and heat-labile growth factors is unlike the activity profile of Fraction 1, which is eluted from heparin sepharose gels at low concentrations of salt

The properties of Fraction 2, however, may correspond to those of previously characterised growth factor in association with either a specific binding protein (like the IGF-BP family), a non-specific binding protein (e.g. α_2-macroglobulin) or a mucopolysaccharide (e g heparin) Because the free growth factor is not concentrated in the R1-30 fraction (following extensive boiling), Fraction 2 is unlikely to be one of the common transforming growth factors (TGF-α, TGF-ß, PDGF, aFGF, bFGF, IGF I or II) Alternatively, the stable nature of the R30 fraction may be accounted for by a particularly stable protein conformation, which can be modified by boiling without loss in activity

The work presented in this thesis is by no means final. Many questions remain to be answered For example the heat stability profile of the R30 fraction is unusual Why did boiling for 3

minutes cause a drop in autocrine activity in the non-heparin-binding fraction, while extensive boiling appears to activate it? Other pertinent questions should be addressed

What determines the ratio of the activity in the R1-30 fraction to that of the R30 fraction?

Are the R1-30 and the R30 effects additive?

What other treatments cause a similar response to extensive boiling?

Would glycosidases have an effect on activity?

Is the effect of the autocrine factor reversible or irreversible (as in the case of murine epithelial cells, Wilder and Rizzino, 1991)?

For definitive conclusions on the identity of the two factors, N-terminal sequencing is an obvious step Before this could be attempted, it would be necessary to have a very pure fraction To achieve this, other purification steps would need to be investigated and most likely combined with heparin sepharose Hydrophobic interaction chromatography may be considered as a possible candidate, since preliminarly experiments did indicate some fractionation on the phenyl sepharose column Gel chromatography under non-acidic conditions may also be considered If the factors proved to have homology with previously reported sequences, neutralising antibodies against these factors could be used to ascertain their autocrine mechanism

If either of the fractions appears to be novel at this stage, polyclonal antibodies could be raised against them These could then be used in affinity columns to aid purification and, possibly more interestingly, could be used as probes to study if these autocrine growth factors are extensively expressed in different tumour cell lines or tissues, or whether they have a role in normal cell growth.

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8 0 APPENDICES

APPENDIX A

Interleukins and hemopoietic growth factors

	IL-1		IL-2	IL-3
	IL-1α	IL-1ß		
Proprotein	271 aa	269 aa	153 aa	152 aa
	30 6 kDa	30 7 kDa		
Mature protein	159 aa	153 aa	133 aa	133 aa
MW (kDa)	38,31,17, 15,4 2	38,31,17 15,4,2	19-22	28
Structure	Monomer or non colvalent polymer			Monomer
Chromosome	2	2	4	5

	IL-4	IL-5	IL-6
Proprotein	153	133	212
Mature protein	129	125	184
MW (kDa)	20	45, 60	21
Structure	Monomer	Dimer, trimer	Monomer
Chromosome	5	5	7

	IL-7	IL-8	IL-9
Proprotein	177	90	202
Mature protein	152	72	179
MW (kDa)	20-28	8	21-41
Structure	Monomer	Monomer	Monomer
Chromosome	*	*	7

	IL-10	IL-11	IL-12
Proprotein			
Mature protein			
MW (kDa)	24	23	
Structure			
Chromosome			

	EPO	GM-CSF	G-CSF	M-CSF
Proprotein	193 aa	144 aa	207 aa	224 aa
Mature protein	166 aa	127 aa	177 aa	127 aa
MW (kDa)	27-60	22	23	40-45
Structure	Monomer	Monomer	Monomer	Homodimer
Chromosome	7	5	17	5

APPENDIX B

Interleukin	Alternative names
IL-1	Endogenous pyrogen (EP), Lymphocyte-activating factor (LAF), B-cell activating factor (BAF), Leukocyte endogenous mediator (LEM), Catabolin, Mononuclear cell factor (MCF)
IL-2	T-Cell Derived Growth Factor (TCGF), T-cell Maturation/ Stimulating factor (TMF/TSF), Killer Helper Factor (KHF), T-Cell Replacing Factor (TRF)
IL-3	Mast cell growth factor (MCGF), P-cell stimulating factor (PSF), Burst promoting activity (BPA), Haematopoietic cell growth factor (HCGF), Erythroid colony stimulating factor (ECSF), Megakaryocyte colony stimulating factor (MEG-CSF), Eosinophil colony stimulating factor (Eo-CSF), Multiple colony stimulating factor (Multi-CSF)
IL-4	B Cell growth factor (BCGF), B cell stimulatory factor-1 (BSF1), B-cell stimulatory factor p1(BSFp1), Macrophage fusion factor (MFF), Macrophage Activating Factor (MAF)
IL-5	T-cell Replacing Factor (TRF), Eosinophil differentiating factor (EDF), B Cell growth factor II (BCGFII), Killer helper factor (KHF), IgA-enhancing factor (IgA-EF), Eosinophil colony stimulating factor (Eo-CSF), IL-4
IL-6	HP-1, Plasmacytoma growth factor (PCT-GF), Hybridoma growth factor (HGF), IFN-ß2, B-cell stimulatory factor-2 (BSF-2), hybridoma/plasma-cytoma growth factor (H/PGF), B-cell differentiation factor (BCDF), B cell stimulatory factor p2 (BSFp2)
IL-7	Lymphopoietin-1 (LP-1)
IL-8	Monocyte-derived neutrophil chemotatic factor (MDNCF), neutrophil activating factor (NAF), Monocyte-derived neutrophil activating peptide (MONAP), lymphoccyte-derived neutrophil activating peptide (LYNAP), granulocyte chemotatic peptide (GCP), macrophage-derived inflammatory protein 2 (MIP-2)
IL-9	Human interleukin for DA cells (HILDA), leukemia inhibitory factor (LIF), differentiation inhibiting activity (DIA)
IL-10	B cell derived T cell growth factor (B-TCGF) cytokine synthesis inhibitory factor (CISF)

(Oppenheim et al , 1986, Dinarello, 1985, Di Giovine and Duff, 1989, MacNeil et al , 1990)

APPENDIX C

Calculations of TGF-α-like activity

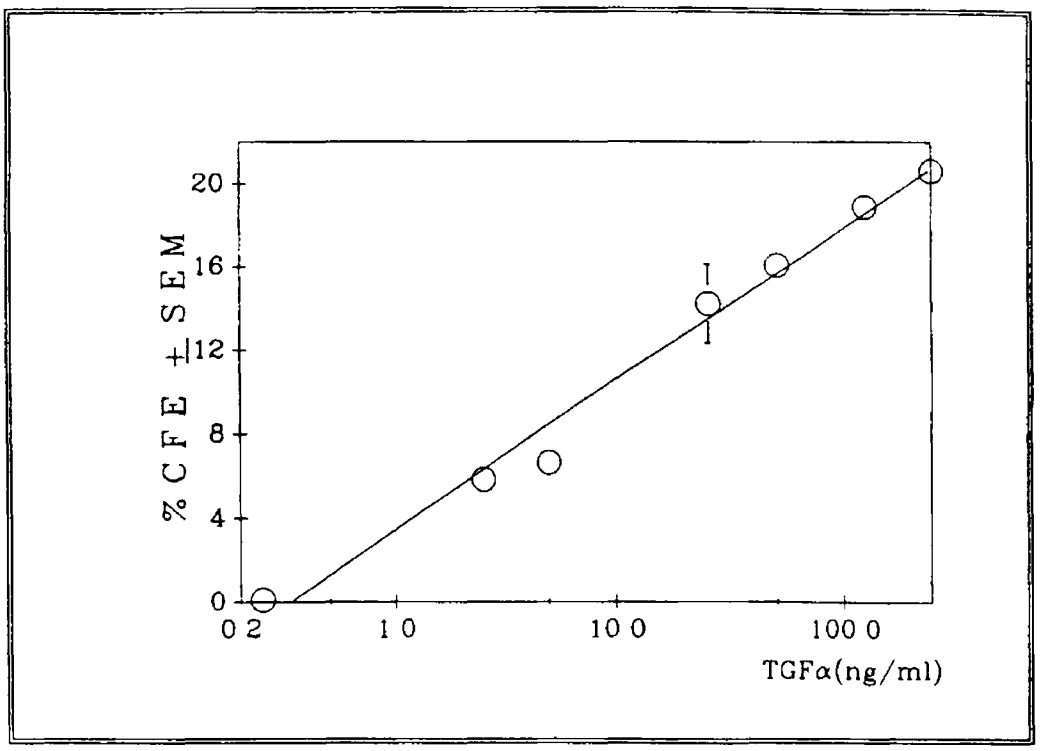


FIGURE C 1 Standard curve of TGF-α in the NRK agar assay

RPMI 7650 CM	CFE±SEM	CFE-C	Equilavent TGF-α (ng/ml)
R1 10x	1.18±0 19	1 14	0 50
5x	0 39±0 06	0 35	off scale
1x	0 00±0 00	-0 04	off scale
High O_2 10x	9 82±0 81	9.82	7 74
Low O_2 10x	1 45±0 65	1 45	0.47
Controls (C)			
MEM	0 04±0 05		
MEMS	0 00±0 00		

APPENDIX D

Calculations of TGF-ß-like activity

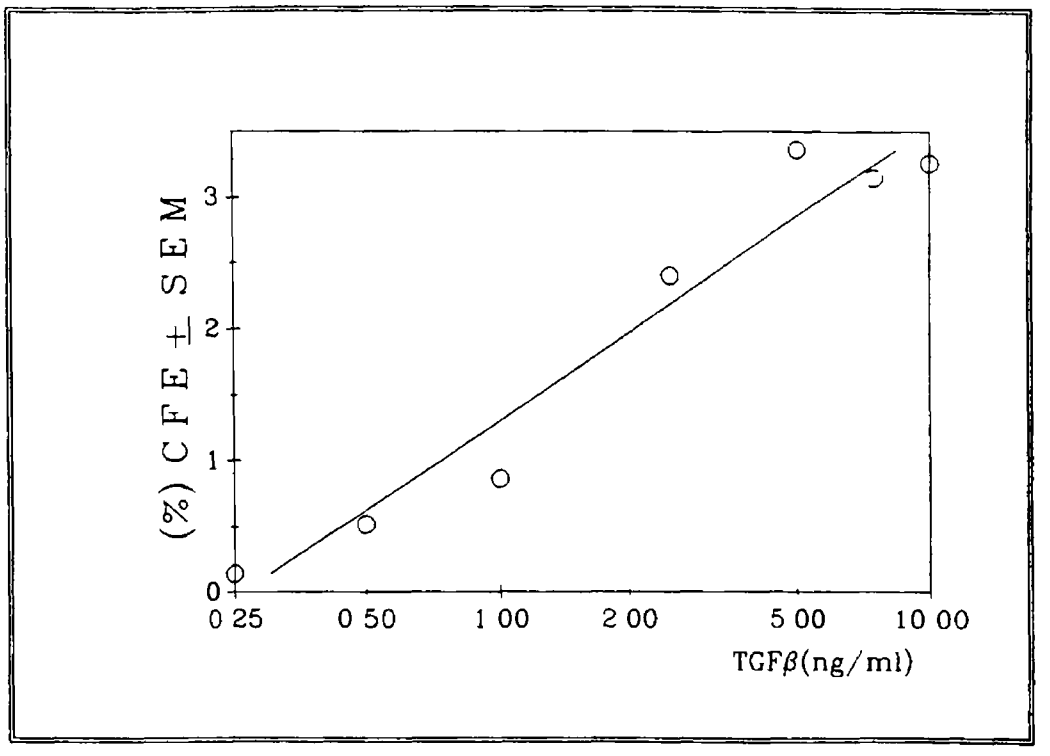


FIGURE D 1 Standard curve of TGF-ß in the NRK-49F agar assay

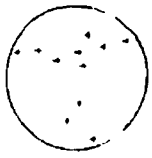
RPMI 2650 CM -	CFE±SEM	CFE-C	TGF-ß (ng/ml)
RPMI 2650 R30 10x	3 69±0 13	3 51	off scale
5x	2 47±0 03	2.29	3 25
1x	1 65±0 01	1 47	1 17
R5-30 10x	1 41±0 02	1 31	1 00
5x	0 94±0 01	0 76	0 56
1x	0 21±0 01	0 03	off scale
BSC-1 R10 10x	2 85±0.06	2 67	4 00
1x	0 43±0 01	0.25	0 34
MSV 3T3 R1 10x	0 04±0 01	-0 27	off scale
5x	0 32±0 01	0 01	0 00
1x	0 13±0 01	-0 18	off scale
Controls			
MEM	0 18±0 01		
DME	0.31±0 01		

APPENDIX E

Structures of Cytodex microcarrier bead side chains

Cytodex 1

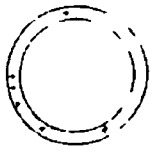
charges throughout matrix



cross linked dextran – O CH CH – N(CH_2CH_3)(CH_2CH_3) HCl

Cytodex 2

surface layer of charges



cross-linked dextran – O – CH_2 – CH(OH) – CH_2 – $N^{\oplus}$(CH_3)(CH_3) $Cl^{\ominus}$

Cytodex 3

collagen layer coupled to surface

cross-linked dextran – O – CH_2 – CH(OH) – CH_2 – NH – (ε LYS COLLAGEN)

APPENDIX F

Calculation of oxygen consumption rates

	Low Oxygen			Mid Oxygen			High Oxygen		
Hrs	DO	Hrs	Rate	DO	Hrs	Rate	DO	Hrs	Rate
0	97 0			98 2			92 0		
10	87 0	10	-9 4*	92 7	10	-5 5	87 2	10	-4 9
20	83 8	10	-3 8	88 5	10	-4 2	82 5	10	-4 7
29							79 3	9	-3 2
30	81 0	10	-2 8	84 3	10	-4 2			
31							63 5	2	-15 8*
40	78 8	10	-2 2	80 7	10	-3 6	73 3	9	-3 6
50	75 9	10	-2 9	77 0	10	-3 7	69 2	10	-4 1
60	74 8	10	-1 1	72 7	10	-4 3	63 9	10	-5 3
70	71 0	10	-3 8	67 8	10	-4 9	58 3	10	-5 6
80	68 5	10	-2 5	61 8	10	-6 0	52 1	10	-6 2
90	65 8	10	-2 7	54 9	10	-6 9	46 0	10	-6 1
Average Rate	0 273 %/hr			0 481 %/hr			0 494 %/hr		

Oxygen Solubility = 6 4 mg/litre (0 2mmol/litre)
(Oller et al, 1989)

100% DO in 1 8 litres = 6 4 x 1 8 = 11 5 mg of O_2
= 0 2 x 1 8 = 0 36 mmol O_2

Average Consumption Rate	0 031 mg/hr 0 0010 mmol/hr	0 055 mg/hr 0 0017 mmol/hr	0 057 mg/hr 0 0018 mmol/hr

Reported Consumption Rates

Speir and Griffiths (1984)

$2 - 10 \times 10^{-12}$ g/cell/hr

$= 2 - 10 \times 10^{-9}$ mg/cell/hr

RPMI 2650 culture = approximately 1×10^5 cells/ml in 1 8 litres

$= 1\ 8 \times 10^8$ cells

Rate = $(2 - 10 \times 10^{-9}) \times (1\ 8 \times 10^8)$ mg/hr

= 0 36 - 1 8 mg/hr

Glacken et al (1983)

0 0531-0 59 mmol $O_2/10^9$ cells/hr

= 0 0096-0 1062 mmol O_2/1 $8x10^8$ cells/hr

= 0 31 - 3 40 mg/hr

Fleischaker and Sinskey (1981)

0 1 - 0 5 g $O_2/4x10^9$ cells/hr

= 0 0045-0 0225 g/1 $8x10^8$ cells/hr

= 4 5 - 22 5 mg/hr

APPENDIX G

Composition of Minimum Essential Media (MEM and MEMS) (Gibco Brl)

	MEM (10x) with Earl's salts without L-glutamine, without sodium bicarbonate (Cat No 042-01430) (mg/litre)	MEMS (10x) Modified for suspension cultures with Earl's salts (modified) without L-glutamine, without sodium bicarbonate (Cat No 042-01650) (mg/litre)
Inorganic Salts		
$CaCl_2$ $2H_2O$	2640 00	-
KCl	4000 00	4000 00
$MgSO_4$ $7H_2O$	2000 00	2000 00
NaCl	68000 00	68000 00
NaH_2PO_4 $2H_2O$	1580 00	15800 00
Other Components		
D-Glucose	10000 00	10000 00
Phenol Red	100 00	100 00
Amino Acids		
L-Arginine HCl	1260 00	1260 00
L-Cystine	240 00	240 00
L-Histidine HCl H_2O	420 00	420 00
L-Isoleucine	520 00	520 00
L-Leucine	520 00	520 00
L-Lysine HCl	730 00	730 00
L-Methionine	150 00	150 00
L-Phenylalanine	320 00	320 00
L-Threoine	480 00	480 00
L-Tryptophan	100 00	100 00
L-Tyrosine	360 00	360.00
L-Valine	460 00	460 00
Vitamins		
D-Ca Pantothenate	10 00	10 00
Choline Chloride	10 00	10 00
Folic Acid	10 00	10 00
i-Inositol	20 00	20 00
Nicotinamide	10 00	10 00
Pyridoxal HCl	10 00	10 00
Riboflavin	1 00	1 00
Thiamine HCl	10 00	10 00