CHONDROCYTE GROWTH FACTORS OF THE PITUITARY

by

ANNE-MARIE HAMEL

A thesis presented to the University of Manitoba in partial fulfillment of the requirements for the degree of Master of Science in the Faculty of Graduate Studies.

Winnipeg, Manitoba

ISBN 0-315-47913-2

CHONDROCYTE GROWTH FACTORS OF THE PITUITARY

BY

ANNE-MARIE HAMEL

A thesis submitted to the Faculty of Graduate Studies of
the University of Manitoba in partial fulfillment of the requirements
of the degree of

MASTER OF SCIENCE

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

I wish to thank Dr. H.G. Friesen for his supervision of my graduate research. Dr. R.P.C. Shiu and Dr. R.J. Matusik are also thanked for their advice in completing the research and for critically reviewing the thesis. The critical evaluation of the thesis by Dr. R. Bowness is also appreciated. For their guidance and the many discussions, I also thank France Croze, Paul Murphy, John Rowe, Cathy Too, Anne Walker, and Ian Worsley. The technical assistance of Agnes Fresnoza, Marg Klassen, Barbara Iwasiow, and Fred Pereira was much appreciated. Dr. K. Wrogeman is thanked for the use of western blotting equipment. I thank John Conroy and Sari Michaels for their computer and typing skills. Thank you also to my family and friends for their encouragement and support during my graduate work. And especially, I wish to thank my husband, Ralph, for his constant love and support and for his technical expertise in preparing the graphics and the references. This research has been supported by grants from the Medical Research Council of Canada.

ABSTRACT

Growth factors are involved in the regulation of normal and neoplastic growth proceses. A growth factor purified from human pituitary glands, which is mitogenic for rabbit fetal chondrocytes, has been purified in our laboratory.

Growth factors from a wide variety of tissues have been purified to homogeneity based on their strong affinity for heparin. These mitogens stimulate endothelial growth in vitro and angiogenesis in vivo. The similarity in the biological activity, estimated MWs and elution profiles of these proteins led to the term heparin-binding growth factors to denote this family of closely related or identical mitogens. The prototypes of this family are bovine brain acidic FGF (aFGF) and bovine pituitary basic FGF (bFGF).

Purification of this human chondrocyte growth factor (hCGF) was approached in three ways: Chromatofocused hCGF (CF-CGF) -- partially purified using sequential chromatography through Sephadex G-100, hydroxylapatite, DEAE-Affigel Blue and chromatofocusing; Heparin-Sepharose partially purified hCGF (hS-hCGF) -- further purification of chromatofocused mitogen using heparin-Sepharose chromatography; and Human Pituitary Growth Factor (hPGF) -- purified to homogeneity using heparin and copper affinity chromatography followed by CM cellulose 52. The

chromatofocused mitogen was reported to have an estimated MW of 40 kD and pI of 7.9. The hPGF had a pI of 7.5 and MW of 18 kD. Since the first eleven amino acids of hPGF are identical to bovine pituitary bFGF, our preparations probably represent the human pituitary equivalent of the bovine mitogen. To examine the relationship of the hCGF preparations to each other and to bovine bFGF, comparisons of the biological and immunological activities of these growth factors were completed. Mitogenic activity was examined in vitro using several cell types of mesodermal origin. Human CGF showed immunologic cross-reactivity with polyclonal antibodies raised to different regions of synthetic bovine bFGF. Western blot analysis of hS-hCGF and hPGF visualized bands at MW of 18-20 kD, similar to that reported for bovine bFGF.

Since the mechanism of release of FGF is unclear, a second component of the present research was to examine the release of FGF-like activity from pituitary tissue. Mitogenic activity was present in the rat pituitary organ culture medium. The pituitary organ cultures were capable of stimulating proliferation of three sequential chondrocyte assays, suggesting de novo synthesis and release of the mitogen by the rat pituitary.

The secretion of hCGF from organ cultures of pituitary adenomas has been reported previously. The presence of factor(s) in human pituitary tumor conditioned medium with FGF-like heparin-binding activity, as well as the

presence of similar activity in serum removed from the site of adenomectomy or peripheral serum from several patients was observed.

A final aspect of this research was to examine the in vivo effects of hCGF on the growth of the hormone-responsive transplantable Swarm rat chondrosarcoma. Preliminary results indicate that injection of hCGF may promote overall body growth as well as specifically stimulate testicular and tumor growth. In normal animals, an increase in the weight of the chondrosarcoma in animals injected with hCGF was observed.

The influence of the human and rat pituitary derived activity described in the present research is discussed in relation to the proposed role(s) of FGF in normal and neoplastic growth processes and in neovascularization.

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Hormones and Growth Factors

ACTH - adrenocorticotropic hormone
CF-hCGF - chromatofocused hCGF
EGF - epidermal growth factor
FGF - fibroblast growth factor
FSH - follicle stimulating hormone
GH - growth hormone
hCGF - human chondrocyte growth factor
hPGF - human pituitary growth factor
hS-hCGF - heparin-Sepharose purified hCGF
IGF - insulin-like growth factor
IL-1 - interleukin 1
LH - luteinizing hormone
LHRH - luteinizing hormone releasing hormone
PDGF - platelet-derived growth factor
PRL - prolactin
TGF - transforming growth factor
TNF - tumor necrosis factor

Cell Lines

BAEC - bovine aortic endothlial cells
Balb/c3T3 - mouse embryonic fibroblasts
BCEC - bovine capillary endothelial cells
BCE - bovine corneal endothelial cells
HUVEC - human umbilical vein endothelial cells
MCH6 - human foreskin fibroblasts
Nb_2 - human lymphoma cells
RFC - rabbit fetal chondrocytes
T-47D - human breast cancer cell line

Miscellaneous

FCS - fetal bovine serum
fg - femtogram
g - gram
h - hour(s)
HPX - hypophysectomy
HS - horse serum
kD - kilodalton
Kd - dissociation constant
L - liter(s)
min - minute(s)
mls - milliliters
MW - molecular weight
ng - nanogram
pg - picogram
pI - isoelectric point
RIA - radioimmunoassay
sc - subcutaneous
SDS - sodium dodecyl sulfate
[35S] - sulphur, isotope 35
[3H] - tritium
ug - microgram
WHO - world health organization

1. INTRODUCTION

The term **growth factor** defines a group of "growth-stimulating substances that are not nutrients.... and are active at low concentrations" (Gospodarowicz and Moran, 1976). Cell growth is manifested in two ways: hypertrophy (increase in cell size) and hyperplasia (increase in cell number). By these mechanisms, normal processes such as the growth and development to an adult state, replacement of aged cells, and repair of damaged tissues, may occur. Unfortunately, the growth of neoplastic tissues may also result from over-stimulation of the same processes.

The chemical messengers involved in the regulation of cell growth are primarily polypeptide in nature. Classical hormones such as insulin and growth hormone are cell growth promoters. A number of growth factors, including Platelet-Derived Growth Factor (PDGF), Epidermal Growth Factor (EGF), Nerve Growth Factor (NGF), Fibroblast Growth Factor (FGF), the Insulin-Like Growth Factors (IGF-I and IGF-II), and the Transforming Growth Factors (TGF-alpha and TGF-B) are also potent stimulators of hyperplastic/hypertrophic activity.

The classical mechanism of hormonal control of cell-to-cell communication and resultant cell growth, requires one of two possible modes of action:

i) the hormone may either be synthesized and

released at one site to affect target cells at a distant site (**Endocrine Mode**); or

ii) it may be released from one cell and bind to and elicit actions in an adjacent cell (**Paracrine Mode**). In the case of growth factors, a third mechanism has been proposed, that of **Autocrine** Regulation (Sporn and Todaro, 1980).

Autocrine control requires that a cell which is already responsive to a particular growth factor (i.e. possesses specific growth factor receptors), to begin producing (and secreting) this growth factor. The growth factor then binds to receptors on the cell from which it was secreted, resulting in a rapid, self-stimulated response. Such a response is speculated to be responsible for the rapid mitogenic activity observed in tumor growth (Sporn and Todaro, 1980; Stiles, 1984).

Growth factors are involved in the regulation of the cell cycle. Pledger and coworkers (1977) have defined two main processes or events mediated by growth factors which enable cells to leave G0 and enter the cell cycle:

i) **competence** -- under the influence of growth factors such as FGF and PDGF, called competence factors, the cells exit the quiescent state (G0) and become "competent" to divide (enter G1);

ii) **progression** -- under the influence of a second set of growth (progression) factors, such as EGF and IGF-I, competent cells will either continue through the cell

cycle, undergoing DNA synthesis and ultimate cell division, or alternatively, return to quiescence. The progression stage, thought to involve a specific labile protein, occurs at a restriction (R) point (located in G1) and is required for the cell to enter S phase. After this point, the cells are no longer influenced by growth factors (Stiles et al., 1979; Scher et al., 1979a; O'Keefe and Pledger, 1983; Pardee, 1987).

Growth factor regulation of the cell cycle and cell growth utilizing an autocrine mechanism was first suggested to explain the actions of PDGF in various cell cultures. Treatment of parent cells with PDGF during S phase prevented the daughter cells from becoming arrested in G0 (Vogel et al., 1978; Scher et al., 1979a); instead the cells continued to divide rapidly. The transforming protein ($p28^{sis}$), expressed by the simian sarcoma virus oncogene (a protein which shares 96% sequence homology with the B-chain of human PDGF) mimicked PDGF action, causing rapid stimulation of DNA synthesis in previously quiescent 3T3 cultures (Deuel et al., 1983). These transformed cells have a much lower PDGF requirement for growth than normal cells (Scher et al., 1979b). This information lead to the suggestion that an unregulated autocrine growth mechanism in which cells synthesize, release and respond to PDGF (or a PDGF-like oncogene product) (Seifert et al., 1984) could be responsible for the rapid and continuous cell growth found in neoplasia.

Knowledge that (almost) all of the cell types shown to be responsive to FGF have now been reported to also contain this mitogen (Gospodarowicz, 1987), provides strong evidence in support of an autocrine mode of regulation of normal and neoplastic cell growth by FGF as well. The following literature review will focus primarily on FGF, the growth factor to which our mitogen, human Chondrocyte Growth Factor (hCGF), is very similar if not identical. Reference to several other growth factors and their functional relationship to FGF will also be included.

1.1 Literature Review: Fibroblast Growth Factors

1.1.1 Early History of FGF

Almost 50 years have passed since crude brain homogenates were reported to contain mitogenic substances which stimulated cell division in primary cultures of fibroblasts (Trowell, et al., 1939; Hoffman, 1940). In the 1960's and 1970's, research relating to the presence of mitogenic activity in pituitary extracts and partially purified hormone preparations intensified interest in growth promoting substances (Holley and Kieran, 1968; Clark et al., 1972; Corvol et al., 1972; Armelin, 1973). Gospodarowicz subsequently reported the isolation of a fibroblast growth factor (FGF) from bovine pituitary

(Gospodarowicz, 1974) and brain extracts (Gospodarowicz et al. 1978, 1982), which stimulated the proliferation and phenotypic transformation of Balb/c3T3 fibroblasts. Gospodarowicz (1975) characterized pituitary FGF as a cationic polypeptide (pI>9.6) with a molecular weight of ~13,000; brain FGF (Gospodarowicz et al., 1978a, Westall et al., 1978) consisted of two basic peptides derived from a common precursor, with a similar size as pituitary FGF but bearing no resemblance to the pituitary mitogen. Further analyses demonstrated both FGFs to be potent mitogens for a wide variety of cells of mesodermal origin, including chondrocytes, adrenocortical cells, vascular smooth muscle cells and vascular endothelial cells (reviewed by Gospodarowicz and Moran, 1976; Gospodarowicz et al., 1978b and Gospodarowicz, 1979).

In 1978, Westall and coworkers proposed that brain FGF was produced by limited proteolysis of myelin basic protein and released in response to myelinated peripheral nerve injury. The intact protein was considered to be inactive at its basic isoelectric point since no activity was found in the myelin basic protein fragments following isoelectric focusing, but could only be generated as a result of proteolysis of myelin basic protein.

This hypothesis was immediately questioned by several researchers. First, Thomas et al. (1980) identified an active bovine brain FGF with an acidic

isoelectric point, unrelated to any fragment of the contaminating myelin basic protein. Lemmon et al. (1982) subsequently reported the presence in pituitary preparations of a basic mitogen, also distinct from this contaminant.

Although Westall's hypothesis was eventually disproved (Gospodarowicz et al., 1984), the confusion resulting from the association of presumably homogeneous FGF preparations with a contaminating protein intensified the efforts to purify FGF to homogeneity. Due to this purification difficulty, the relationship between the pituitary and brain FGFs, previously considered to be unrelated molecules, was also questioned (Thomas et al., 1980).

While the controversy surrounding the exact identity of FGF continued, several laboratories were reporting the isolation of mitogenic activity from various tissue sources. Using a number of assay techniques, mitogenic activity isolated from brain or pituitary sources was variously termed ovarian growth factor (OGF) (Gospodarowicz et al., 1974); chondrocyte growth factor (CGF) (Kasper et al., 1982); endothelial cell growth factor (ECGF) (Maciag et al., 1979); acidic FGF (Thomas et al., 1980, 1984; Lemmon et al., 1982; Gambarini et al., 1982); brain-derived growth factor (Barritault et al., 1982); adipocyte growth factor, AGF (Lau et al., 1983); glial growth factor (Brockes et al.,1980,1984;

Lemke and Brockes,1983) or otherwise (Kellet et al., 1981).

Activity similar to FGF was also described in other tissues including corpus luteum (Jakob et al., 1977, Gospodarowicz and Thakral, 1978), ovary (Koos and Le Maire, 1983; Makris et al., 1983, 1984), kidney (Preminger et al., 1980; Ekblom, 1981), adrenal (Kiss, 1975), retina (Glaser et al., 1980; Barritault et al., 1981), macrophages (Leibovich and Ross, 1976), cartilage (Klagsbrun and Smith, 1980) and tumor sources (Folkman, 1975) as well as in human milk (Klagsbrun, 1978). These mitogens caused similar in vitro effects on mesodermally derived cells and had certain biochemical similarities (eg. molecular weights). However, a continued lack of homogeneous preparations of these mitogens prevented clarification of any structural similarities. It was during this period, when intensive efforts by several laboratories to purify FGF to homogeneity were underway, that Shing and co-workers noted the enhancing effect of the mast cell product heparin, on the activity of a crude angiogenesis factor, and subsequently published the purification to homogeneity of an angiogenic peptide from chondrosarcoma (chondrosarcoma-derived growth factor; ChDGF) using heparin-Sepharose affinity chromatography. Using this extremely selective method of purification, a highly purified (500,000-fold) ChDGF preparation could be obtained from crude chondrosarcoma extracellular matrix

with only a two-step purification procedure. This angiogenic factor was a cationic polypeptide (pI 9.8) of about 18 kD which exhibited a strong affinity for heparin, eluting with 1.5 M NaCl. Like FGF, ChDGF stimulated DNA synthesis in Balb/c3T3 cells and capillary endothelial cell proliferation. (Shing et al., 1984).

1.1.2 Recent Advances in FGF purification: heparin-Sepharose Chromatography

The use of heparin-Sepharose as a rapid, highly efficient purification tool is readily apparent from the plethora of mitogens purified using this technique (see table 1) Klagsbrun and Shing (1985) suggested that an affinity for heparin was a property shared by growth factors that are mitogenic for capillary cells in vitro and induce angiogenesis in vivo. Lobb et al. (1985) established the presence of two distinct classes of endothelial mitogens, which they termed heparin-binding growth factor (HBGFs).

Class 1 HBGFs, such as brain-derived acidic FGF (Thomas et al., 1984), isolated from neural tissues are anionic peptides which elute from heparin-Sepharose with about 1 M NaCl. Class 2 HBGFs, isolated from a wide variety of tissues, are cationic and require about 1.5 M NaCl for elution. The prototype for this group is basic pituitary FGF (Gospodarowicz et al., 1984).

1.1.3 Characterization of Acidic and Basic FGFs

Basic FGF has been purified to homogeneity from a variety of tissue and cellular sources, primarily bovine and human in origin (Tables 1 and 2). Acidic FGF was initially thought to be only of neural origin, having only been isolated from brain, pituitary and retina (Thomas et al., 1984; Lobb and Fett, 1984). However, more recent discovery of aFGF in normal and tumor sources including uterus (Brigstock et al., 1986), kidney (Gautschi-Sova et al., 1987), benign prostate hyperplasia (Story et al., 1987) and rat Dunning tumor (a prostatic adenocarcinoma, Matuo et al., 1987), as well as in cultured smooth muscle cells and fibroblasts (Winkles et al., 1987), demonstrates a much wider distribution of this factor.

Biochemical characterization of homogeneous aFGF and bFGF preparations has been completed. Basic FGF preparations generally contain proteins of two sizes with apparent MW of 16 and 15 kD and pI of 9.6 (Gospodarowicz et al., 1984; Gospodarowicz et al., 1985a). Based on amino acid sequence analysis, the 16 kD form of bFGF is a single chain polypeptide containing 146 amino acids. A truncated form of bFGF, lacking the amino-terminal 15 amino acids (des. 1-15 or 16-146 bFGF), corresponds to the smaller protein (Esch et al., 1985b; Gospodarowicz et

al., 1985b). A larger amino-terminally extended molecule also exists (Klagsbrun et al., 1986; Ueno et al., 1986, Schweigerer et al., 1987b; Story et al., 1987). In the pituitary, brain, and retina, the intact (1-146) is the primary FGF molecule. The truncated form of bFGF is found in the kidney, adrenal, corpus luteum, retina, placenta and testes (Table 2). High molecular weight forms of immunoreactive bFGF have also been reported (Mormede et al., 1985) but have not been characterized. Figure 1 illustrates the molecular forms of bFGF.

Acidic FGF is a single chain polypeptide found in two microheterogeneous forms with MW in the range of 15-16 kD (Bohlen et al., 1984). The larger molecule is composed of 140 amino acids and has a blocked amino terminus (Esch et al., 1985a; Burgess et al., 1985) while the smaller form (des. 1-6) is missing an amino-terminal hexapeptide (Thomas et al., 1985; Esch et al, 1985a).

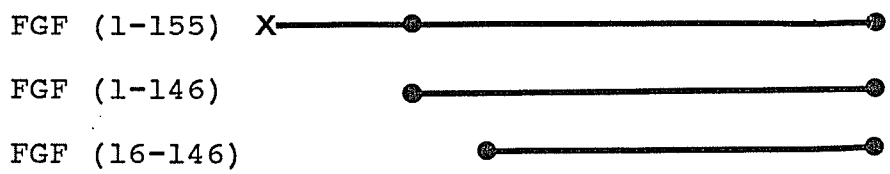


FIGURE 1. Molecular Forms of the bFGFs.
(modified from Ueno et al., 1986 and Baird et al., 1987c).

TABLE 1: FGF - like Heparin - Binding Growth Factors (HBGFs)

A. Acidic FGF - like (Class 1 HBGF)

	Source(s)	Bioassay(s)*	Characterization	Reference
Endothelial Cell Growth Factor (ECGF)	Bovine Hypothalamus	Swiss Albino Mouse 3T3 Fibroblasts HUVEC	MW 75 kD, 15 kD	Maciag et al., 1979; 1982
Brain - Derived Acidic FGF	Bovine Brain	Balb/c3T3	MW 16.6, 16.8 kD	Thomas et al., 1984
Pituitary - Derived Acidic FGF	Bovine Pituitary	3T3 Fibroblasts, Adrenal Cells, Glial Cells	MW 12 kD pI 4.7	Gambarini and Armelin, 1982
Eye - Derived Growth Factor II (EDGF - II)	Bovine Retina	HUVEC BAEC	MW 18 kD pI 5	Schreiber et al. 1985a
Retina - Derived Growth Factor (RDGF - alpha)	Bovine Retina	BCEC	MW 15 kD pI 5	D'Amore & Klagsbrun 1984; Baird et al., 1985b
Anionic Hypothalamus - Derived Growth Factor (aHDGF)	Bovine Hypothalamus	BCEC	MW 16-20 kD pI 5	Klagsbrun & Shing 1985

* HUVEC; human umbilical endothelial cells
BAEC; bovine aortic endothelial cells
BCEC; bovine capillary endothelial cells
RFC; rabbit fetal chondrocytes
BCE; bovine corneal endothelial cells
T-47D; human breast cancer cell line

A. Acidic FGF - like (Class 1 HBGF) (Cont'd)

	Source(s)	Bioassay(s)*	Characterization	Reference
Heparin - Binding Growth Factor (HGF - alpha)	Bovine Brain Bovine Hypothalamus Human Brain	BCEC Balb/c3T3	MW 16 kD	Lobb & Fett, 1984 Lobb et al., 1985
Glioma - Derived ECGF	Human glioma cell line	Human glioma cells mouse lung endothelial cells	MW 17 kD, 20 kD	Libermann et al. 1987
Rat Prostatic Growth Factor (RPGF)	Dunning Tumor (Rat Prostatic Adeno carcinoma)	Balb/c3T3	MW 19 kD pI 3.8	Matuo et al., 1987
Prostatropin (Prostate Epithelial Cell Growth Factor)	Bovine brain	Rat normal & tumor prostate epithelial cells	MW 18 kD 16 kD	McKeehan and Adams, 1988, Crabb et al., 1986
Prostatic Growth Factor (PrGF)	Human benign prostatic hyperplasia (BPH)	Human Foreskin Fibroblasts	-	Story et al., 1987
Bone - Derived Growth Factor (BDGF)	Bovine mineralized bone matrix	Balb/c3T3 BCEC Osteoblasts	MW 16.6 kD pI 5.2	Hause et al, 1986
Embryonic Brain - Derived Angiogenesis Factor (EBAF)	Embryonic Chicken Brain	BCEC Balb/c3T3	MW 16-18 kD	Risau et al., 1986

B. Basic FGF - like (Class 2 HBGF)

	Source(s)	Bioassay(s)*	Characterization	Reference
Chondrosarcoma - Derived Growth Factor (ChDGF)	Rat Chondrosarcoma	BCEC	MW 18 kD, pI 9.8	Shing et al. 1984
Cartilage - Derived Growth Factor (CDGF)	Bovine, Human and Chicken Cartilage	Balb/c3T3	MW 19 kD pI 9.8	Sullivan and Klagsbrun, 1985
basic FGF (bFGF)	Bovine Brain Bovine Pituitary	BAEC	MW 16 kD pI 9.6	Gospodarowicz et al., 1984
Heparin - Binding Growth Factor (HGF-B)	Bovine Brain Bovine Hypothalamus Human Brain	BCEC Balb/c3T3	MW 18 kD	Lobb and Fett, 1984 Lobb et al., 1985
Cationic Hypothalamus - Derived Growth Factor (CHDGF)	Bovine Hypothalamus	BCEC Balb/c3T3	MW 16-20 kD pI 8	Klagsbrun and Shing, 1985
Hepatoma - Derived Growth Factor (HDGF)	Human Hepatoma Cell Line	BCEC Balb/c3T3	MW 18.5 - 19 kD	Klagsbrun et al. 1986
Chicken Muscle Growth Factor (CMGF)	Chicken Skeletal Muscle	Chicken Myogenic Cells	MW 14-17 kD	Kardami et al. 1985
Mammary Tumor - Derived Growth Factor (hMTGF)	Human Mammary Tumor Tissue	RFC, BCE, T-47D, Human Foreskin Fibroblasts	MW 16 kD, pI 8	Rowe et al., 1986a

B. Basic FGF - like (Class 2 HBGF) (Cont'd)

	Source(s)	Bioassay(s)*	Characterization	Reference
Pituitary Growth Factor	Human Pituitaries	RFC, BCE	MW 18 kD pI 7.5	Rowe et al., 1986b
Heparin Binding Growth Factor	Mouse Bladder tumors, urine from tumor bearing mice	Balb/c3T3 BCE	MW 16, 26 kD	Chodak et al., 1986
Human Prostatic Growth Factor (hPGF)	Human benign hypertrophic prostates (cytosol)	Balb/c3T3	MW 11-13 kD pI 10.5	Nishi et al., 1985
Prostatic Growth Factor (PrGF)	Human benign prostatic hyperplasia	Human Foreskin Fibroblasts	MW 17.6 kD pI 10.2	Story et al., 1987
Embryonal Carcinoma - Derived Growth Factor (ECDGF)	Mouse PC13 Embryonal Carcinoma Conditioned Serum-Free Medium	3T3 Fibroblasts P19 Embryonal Carcinoma Cells	MW 17.5 kD	van Veggel et al., 1987
Retina - Derived Growth Factor (RDGF-B)	Bovine Retinas	BCEC BAEC	MW 16.5 kD	Baird et al., 1985
Eye - Derived Growth Factor (EDGF I)	Adult human retina	Bovine Lens Epithelial Cells BAEC	MW 16 kD	Courty et al., 1986

B. Basic FGF - like (Class 2 HBGF) (Cont'd)

	Source(s)	Bioassay(s)*	Characterization	Reference
Astroglial Growth Factor (AGF)	Bovine Brain	Rat Astroblasts	pI 9.5	Pettman et al., 1985
Bone - Derived Growth Factor (BDGF)	Bovine mineralized bone matrix	Balb/c3T3 BCEC Osteoblasts	MW 18-19 kD pI 9.5	Hauschka et al., 1986

TABLE 2: Distribution of Different Molecular Forms of Basic FGF.

A. Tissue Sources

Tissue	Species	Molecular Form	Reference
Brain	Bovine	1-146 (a)	Gospodarowicz et al., 1984
	Human	1-146	Bohlen et al., 1985
	Chicken	N.D.	Risau et al., 1988
	Guinea Pig	X-146 (b)	Moscatelli et al., 1987
Pituitary	Bovine	1-146, X-146	Bohlen et al., 1984; Ueno et al., 1986
	Human	1-146	Rowe et al., 1986b
Retina	Bovine	1-146	Baird et al., 1985b
Kidney	Bovine	16-146 (c)	Baird et al., 1985a
Adrenal Gland	Bovine	1-146, 16-146	Gospodarowicz et al., 1986a
Corpus Luteum	Bovine	16-146	Gospodarowicz et al., 1985b
Placenta	Human	1-146, 16-146, and X-146	Gospodarowicz et al., 1985c Moscatelli et al., 1986; Sommer et al., 1987
Testes	Bovine	16-146	Ueno et al., 1987
Thymus	Bovine	1-146, 16-146	Gospodarowicz et al., 1986b
Macrophages	Murine	N.D.	Baird et al., 1985c

(a) 1-146, the intact bFGF molecule
(b) X-146, amino - terminal extended bFGF molecule
(c) 16-146, truncated bFGF molecule

A. Tissue Sources (Cont'd)

Tissue	Species	Molecular Form	Reference
Benign Prostatic Hyperplasia	Human	X-146	Story et al., 1987
Chondro-sarcoma	Rodent	X-146	Shing et al., 1984; Klagsbrun et al., 1986
Bladder Tumors	Human	N.D.	Chodak et al., 1988
Kidney Tumors	Human	N.D.	ibid.
Mammary Tumors	Human	N.D.	Rowe et al., 1986a

TABLE 2: Distribution of Different Molecular Forms of Basic FGF.

B. Cellular Sources

Cell Type	Cell Assoc.	Cond. Media	Molecular Form	Reference
Normal Diploid Cells:				
Bovine Adrenal Cortex - or	+	+	N.D.	
Brain - derived Capillary	+	+	N.D.	Schweigerer et al., 1987
Endothelial Cells (EC)				
Bovine Aortic EC and	+	-	1-146	Vlodavsky et al., 1987
Extracellular Matrix		+	N.D.	Baird and Ling, 1987
Bovine Capillary EC	+	-	N.D.	Moscatelli et al., 1986a
and Extracellular Matrix		+	N.D.	Baird and Ling, 1987
Chicken Embryo Fibroblasts	+	-	N.D.	Moscatelli et al., 1986a
Human Embryo				
Lung Fibroblasts	+	-	N.D.	ibid.
Bovine Embryo				
Skin Fibroblasts	+	-	N.D.	ibid.
Bovine Adrenal Cortex Cells	+	-	N.D.	Schweigerer et al., 1987
Bovine Granulosa Cells	+	N.D.	N.D.	Neufeld et al., 1987
Bovine Retinal Pigment				
Epithelial Cells	+	N.D.	X-146	Schweigerer et al., 1987
Human Astrocytoma Cells	+	±	N.D.	Murphy et al., 1988b

B. Cellular Sources (Cont'd)

Cell Type	Cell Assoc.	Cond. Media	Molecular Form	Reference
Tumor Cell Lines:				
Human Hepatoma (SK-HEP-1)	+	-	X-146	Klagsbrun et al., 1986; Moscatelli et al., 1986b
Human Rhabdomyosarcoma (A-204, RD, A-673)	+	N.D.	1-146, X-146	Schweigerer et al., 1987
Human Melanoma (RPMI 7272)	+	-	N.D.	Moscatelli et al., 1986b
Human Cervical Carcinoma (HeLa)	+	-	N.D.	ibid.
Human Leukemia	+	-	N.D.	ibid.
Chicken Leukemic Marrow Cells	N.D.	+	N.D.	Dodge, 1985
Transformed Cells:				
Vaccinia - Infected CV-1 Cells	+	-	X-146	Abraham et al., 1986c
Monkey COS 7 Cells	+	+	1-146	Kurokawa et al., 1987
NIH 3T3 Fibroblasts	+	-	X-146	Rogelj et al., 1988
Balb/c3T3 Fibroblasts	+	+	N.D.	Sasada et al., 1988
BHK 21 Fibroblasts	+	+	1-146, X-146	Neufeld et al., 1988

TABLE 2: Distribution of Different Molecular Forms of bFGF

C. Other Sources

Tissue	Species	Molecular Form	Reference
Serum	rat	N.D.	Mormede et al., 1985
	human	N.D.	Baird et al., 1986
Urine (patients with bladder or kidney cancer)	human	N.D.	Chodak et al., 1988
	mouse	N.D.	Chodak et al., 1986
Cerebro-spinal Fluid (patients with brain tumors)	human	N.D.	Lopez-Pousa et al., 1981
Aqueous Humor (patients with eye tumors)	human	N.D.	Tapper et al., 1979

Nucleotide sequence analysis of bFGF cDNA showed that both aFGF and bFGF are probably synthesized initially as 155 amino acid (MW~18,000) proteins containing a 9 residue amino-terminal extension (Ueno et al., 1986; Abraham et al., 1986; Klagsbrun et al., 1986; Klagsbrun et al., 1987; Story et al., 1987). This larger precursor bFGF has been isolated from several human tumor sources including hepatoma, rhabdomyosarcoma, and benign prostatic hyperplastic tissue, as well as from a rat transplantable chondrosarcoma and bovine pituitaries (Table 2). A larger aFGF (prostatropin) has been described in bovine brain. In the extended aFGF and bFGF forms, where sequence analysis has been attempted, the amino-terminus is usually blocked. Several researchers have suggested that the particular FGF molecule isolated may be a function of the extraction conditions used (i.e. acid or neutral pH) as well as the tissue source (Klagsbrun et al., 1987; Story et al., 1987).

With the publication of the complete amino acid sequences of bovine acidic and basic FGF (Gimenez-Gallego et al., 1985, Esch et al., 1985a, b, respectively) which was quickly followed by the human acidic and basic FGF sequences (Gimenez-Gallego et al., 1986a, b; Abraham et al., 1986b, respectively), comparative structural analysis of these biologically similar mitogens was completed.

Bovine pituitary bFGF and bovine brain acidic FGF

share a 55% sequence identity in overlapping regions (Esch et al., 1985b; Gimenez-Gallego et al., 1986a). In the remaining nucleotide, only a single base change is required to cause an amino acid replacement and resultant sequence homology (Esch et al., 1985a). Human and bovine bFGF exhibit complete sequence identity over the first 40 amino-terminal residues (Gimenez-Gallego et al., 1986a) and are 98.7% homologous over their entire sequences, with only 2 different amino acids (Abraham et al., 1986b). Human and bovine aFGF are also extremely homologous molecules; they share 92% sequence identity with only 11 different amino acids. Conservative changes with 8 of these amino acids would bring sequence similarity up to 98% (Harper et al., 1986; Gimenez-Gallego et al., 1986a, b).

Two functional domains for the FGFs, each containing heparin and receptor recognition sites, have been described (reviewed in Baird et al., 1987c). Heparin-binding sites, previously described in fibronectin, antithrombin 3 and platelet factor 4, are characterized by clusters of basic residues or pairs of basic and aromatic residues (Schwarzbauer et al., 1983). Both FGFs possess two such clusters of basic residues found in equivalent structural loci: basic FGF [18-22] and acidic FGF [9-12]; basic [107-110] and acidic FGF [100-102] (Esch et al., 1985a; Strydom et al., 1986). Basic FGF also contains two sequences (residues 37-40 and 78-

81) for the cell surface recognition site (also found in proteins such as fibronectin, fibrinogen, collagen and thrombin) although positioned in an inverted orientation to that found in fibronectin (Baird et al., 1986; Yoshida et al., 1987). These residues, found on the exterior of FGF, may allow the protein to attach to cell surface recognition sites. A receptor activation site (residues 25-68) is also present in the bFGF molecule. The locations of two cysteine residues are highly conserved in the two FGFs: positions 16 and 83 in aFGF and at residues 25 and 92, the corresponding positions in bFGF (Harper et al., 1986). The presence of a disulfide bond between these residues in the FGFs would bring the two heparin-binding sites into close proximity (Strydom et al., 1986; Harper et al., 1986). Finally, the amino-terminal truncated forms of aFGF (7-140) and bFGF (16-146) are both cleaved in exactly homologous positions; a specific protease is responsible for degradation or processing of both molecules from larger precursors (Esch et al., 1985a; Klagsbrun et al., 1987). Since the regions of the bFGF molecule involved in heparin and receptor binding and receptor activation are all contained in the homologous core sequence, this explains why all three amino-terminal bFGF variants are equipotent mitogens.

Weak sequence homologies have been noted between the FGFs, the interleukins (IL-1 alpha and IL-1 B) and

several neuropeptides. The carboxyl-terminal regions of human IL-1 alpha and IL-1 B show structural homology to aFGF (19% and 27%, respectively); IL-1 B is 25% homologous to bFGF (Esch et al., 1985a) -- all significant levels of similarity since the two interleukins only share 26% identity themselves (Gimenez-Gallego et al., 1985). Since IL-1 B and the FGFs are mitogenic for primary fibroblasts, these growth promoting proteins may all be members of a family of homologous growth factors (Gimenez-Gallego et al., 1985).

Distant sequence homology also exists between a decapeptide in aFGF (residues 102-111), an octapeptide in bFGF (residues 111-118) and several neuropeptides (Gimenez-Gallego et al., 1985). The FGF neuropeptide-like amino acid sequences resemble those of neuromedin C, bombesin, neuromedin K, substance K, substance P, physalaemin and eledoisin. The sequences are especially similar in the carboxyl-terminal halves, a highly conserved region thought to participate in receptor binding and biological actions of these neuropeptides Several of these neuropeptides are mitogens, acting on a diverse array of cell types including chondrocytes, bronchial epithelial cells, smooth muscle cells, fibroblasts, and lymphocytes (reviewed in Sporn and Roberts, 1988). Situated on either side of these FGF sequences are basic (Lys-Lys) dipeptides, considered to be proteolytic signals for cleavage of many active

polypeptides from their precursor proteins (Gimenez-Gallego et al., 1985; Thomas and Gimenez-Gallego, 1986).

The FGFs have been isolated from mammalian avian and piscean sources (Risau et al., 1988; Lagente et al., 1986). Basic FGF has been implicated in amphibian embryonic mesoderm induction (Slack et al., 1987). The extremely high degree of sequence conservation between bFGF and aFGF (98% versus 92% for human and bovine forms), combined with their wide distribution throughout evolution implies stringently controlled significant physiological role(s) for the FGFs.

1.1.4 The Genes for aFGF and bFGF

The strong homology between aFGF and bFGF, their common tissue sources, and their similar biological activities both in vitro and in vivo, all suggest that these mitogens are derived from a single ancestral gene (Esch et al., 1985a; Thomas and Gimenez-Gallego, 1986). The weaker homology found between the FGFs and IL-1s suggests that these proteins " ... long ago diverged from a common ancestral protein, followed by a split between the two interleukins, and finally by the emergence of the two FGFs" (Thomas and Gimenez-Gallego, 1986).

The genes for aFGF and bFGF as well as complementary DNA (cDNA) sequences for these mitogens have been

isolated from bovine and human libraries (Abraham et al., 1986a, b; Jaye et al., 1986) Bovine and human nucleotide sequence analysis revealed a common initiator codon (encoding the initiating methionine) for the FGFs which would in both cases, produce an apparent primary translation product composed of 155 amino acids (Abraham et al., 1986a). Proteolytic cleavage of the first 9 (bFGF, 1-146), or 15 (aFGF, 1-140) residues from this FGF precursor would result in the commonly purified aFGF and bFGF proteins. Further cleavage, known to occur in homologous positions (Ueno et al., 1986) would produce the amino-terminal truncated bFGF (des. 1-16; 16-146) or aFGF (des. 1-6; 7-140) mitogens (Abraham et al., 1986a; Gospodarowicz et al., 1986a, b).

Southern blot analysis of human genomic DNA for the FGFs shows that they are both single copy genes (Abraham et al., 1986b; Gospodarowicz et al., 1987). Abraham's group has mapped the bFGF and aFGF genes to two separate chromosomes, chromosomes 4 and 5, respectively (Mergia et al., 1986; Jaye et al., 1986). Citing the fact that genes for other related growth factors exist on different chromosomes -- human insulin and IGF-II reside on chromosome 11, whereas the related IGF-I gene is located on chromosome 12; the human PDGF A and B chains are located on chromosome 7 and 22, respectively. These researchers maintain that the structural and functional homologies shared by aFGF and bFGF still implies their

common ancestral origin (Mergia et al., 1986). Thus, the heparin-binding growth factors so far described and/or characterized (see Table 1) are probably encoded by one of two genes -- the aFGF gene or the bFGF gene. Their slight structural differences (such as MW) are probably the result of posttranslational processing or modification in vivo as well as due to differing purification procedures (Abraham et al., 1986c).

The human bFGF gene encodes two major polyadenylated RNA species of approximately 3.7 and 7.0 kilobases (kb) (Abraham et al., 1986b, c). The gene for aFGF encodes only a single 4.8 kb RNA species (Jaye et al., 1986). Based on restriction enzyme mapping data, both aFGF and bFGF genes are greater than 34 kb in size (Abraham et al., 1986c; Gospodarowicz et al., 1987). The human bFGF coding region is interrupted by two introns -- one is contained within codon 60, the second intron is located between codons 94 and 95 (Abraham et al., 1986c). Alignment of the amino acid sequences of the two FGFs to provide the greatest sequence homology locates aFGF introns at positions identical to codons 60 and 95 of bFGF (Abraham et al., 1986c). Therefore aFGF and bFGF share not only amino acid sequence homology, but gene structural homology as well.

In several tissue sources from which bFGF has been purified, the levels of bFGF mRNA are very low or undetectable; the cDNA are often unspliced, still

containing intron sequences (Abraham et al., 1986b). Using a bovine bFGF cDNA clone as a hybridization probe, Abraham's group did not detect bFGF mRNA (by Northern blot analysis) in kidney, term placenta, breast carcinoma, fetal heart and fetal liver human libraries (Abraham et al., 1986b). To explain their results these researchers proposed the following: normal synthesis of bFGF mRNA occurs at low levels; and/or cytoplasmic bFGF mRNA is quite unstable or is normally under tight regulatory control.

Preliminary studies of the mechanisms regulating bFGF mRNA expression have been conducted by Paul Murphy and Yuji and Reiko Sato in our laboratory. Using a human astrocytoma cell line which expresses high levels of bFGF mRNA, they studied the effects of serum and cell density on bFGF mRNA expression line (Murphy et al., 1988a). Either the addition of serum to serum-deprived cultures, or the replating of density arrested cells to lower cell density resulted in a rapid increase in the levels of both the 7.0 and 3.7 kb bFGF mRNAs. The mRNA levels then declined sharply as the cells entered log phase growth. Their findings suggest that bFGF expression is a tightly regulated phenomena which is closely associated with the transition of density arrested (quiescent) cells from G0-G1 of the cell cycle. These results substantiate previous reports that bFGF acts as a competence factor stimulating transition from G0 to G1 of the cell cycle

(Lathrop et al., 1985). Therefore, a possible explanation for the low levels of bFGF mRNA experienced by Abraham's group could be the result of normal regulation of cell-to-cell contact in normal adult tissues such as those examined by Abraham and coworkers (Murphy et al., (1988a). Low levels of bFGF gene expression have been detected in several tissue and cultured cell sources. Tissue sources include bovine pituitary and hypothalamus (Abraham et al., 1986a, b), and human primary intracranial tumors, including schwannomas, craniopharyngiomas, and astrocytomas (Murphy et al., 1988b). Cellular bFGF mRNA sources include: human hepatoma cell line (Abraham et al., 1986a); human rhabdomyosarcoma cells (Schweigerer et al., 1987b); human astrocytoma cell line (Murphy et al., 1988a); human breast cell line (HBL-100, Murphy et al., 1988a); human foreskin fibroblasts (Kurokawa et al., 1987); bovine granular cells (Neufeld et al., 1987); bovine adrenal cortex cells (Schweigerer et al., 1987a); bovine capillary endothelial cells (Schweigerer et al., 1987c); and retinal pigment epithelial cells (Schweigerer et al., 1987d). In the above cells and tissues, aFGF gene transcripts were not detected. At present, human aFGF has only been detected in human brain stem (Jaye et al., 1986) and human gliomas (Libermann et al., 1987).

Both aFGF and bFGF lack a classical signal peptide sequence (Abraham et al., 1986a), a feature normally

associated with secretory proteins. The amino-terminal (residues 1-13) of the bFGF precursor does contain many hydrophobic residues, possibly providing an atypical signal peptide core sequence; the corresponding region of aFGF does not contain this hydrophobic domain (Abraham et al., 1986a).

The FGFs are extracellular growth factors which are known to bind to specific, high affinity membrane-associated cell-surface receptors (Neufeld and Gospodarowicz, 1985, 1986). As such, the lack of a secretory signal peptide places the mechanism of release of these potent mitogenic agents in question. In cultured cells, bFGF is generally considered to be cell associated and not released into the conditioned media (see Table 2). In cases where bFGF has been localized in conditioned media, its release has usually been attributed to cell lysis or leakage as a result of long term culture conditions (Schweigerer et al., 1987c; Jaye et al., 1986, Folkman and Klagsbrun, 1987). This mechanism of release has also been suggested for the structurally related IL-Is which also lack proper signal peptides (Auron et al., 1985). Prevention of bFGF secretion and receptor binding, thereby preventing uncontrolled autostimulation of cell growth, may be a consequence of this lack of a proper secretory signal (Abraham et al., 1986a).

Several laboratories have recently tested this

speculation. Klagsbrun and coworkers fused bFGF cDNA to sequences encoding an amino-terminal immuno-globulin (Ig) secretory-signal peptide (which mediates passage of proteins into endoplasmic reticulum). Transfection of NIH 3T3 cells with the plasmids thus formed (pIgbFGF) caused cellular transformation and were highly tumorigenic in NIH/NSF mice. Plasmids containing bFGF only were unable to induce transformation; bFGF was not, however, present in conditioned medium of the transfected cells. (Rogelj et al., 1988).

Transfection of baby hamster kidney-derived (BHK-21) cells, mouse Balb/c3T3 cells, monkey COS7 cells or vaccinia-infected CV-1 cells with plasmids containing bFGF cDNA ligated to and under the control of promoter regions, also induced transformation, anchorage-independent growth in soft agar and autocrine growth stimulation (Kurokawa et al., 1987; Sasada et al., 1988; Abraham et al., 1986c; Neufeld et al., 1988). The conditioned media of all but the vaccinia-infected CV-1 transformed cells contained biologically active bFGF, which could be inhibited by anti-bFGF antibodies. Addition of bFGF antibodies to transformed Balb/c3T3 cells caused reversion to the normal phenotype (Sasada et al., 1988). Transfection of 3T3 NR6 fibroblasts with aFGF plasmid also resulted in phenotypic transformation and tumor development (Jaye et al., 1988).

Based on the above information, two possible

mechanisms to explain the autocrine growth transformation in these transfection studies were postulated:

i) bFGF stored intracellularly is released by cell lysis or specific transport (Abraham et al., 1986c; Sasada et al., 1988) and immediately sequestered at the cell surface or in the extracellular matrix (ECM) to heparin sulfate glycosaminoglycans, where its activation could result in extracellular autocrine stimulation of growth and transformation (Rogelj et al., 1988; Vlodavsky et al., 1987; Baird and Ling, 1987). Specific transport of FGF (in association with ECM components) to the cell surface where it could bind to specific receptors, has been suggested (Baird et al., 1987c).

ii) the signal peptide directs bFGF into the same intracellular locations utilized by the FGF receptors, intracellular receptor-ligand association could occur, resulting in intracellular autocrine stimulation (Rogelj et al., 1988). The latter process has been previously suggested to explain transformation by the PDGF related sis oncogene (Betsholtz et al., 1984; Robbins et al., 1985).

The observation that addition of anti-bFGF antibodies causes reversion from the malignant morphology to the normal phenotype, supports the first proposed mechanism -- i.e. that bFGF exerts its effects through receptors at the cell surface (Sasada et al., 1988). In contrast, anti-bFGF antibodies do not inhibit the growth

of transfected BHK-21 cells, supporting the latter possibility of intracellular activation (Neufeld et al., 1988). In these same cells, however, protamine sulfate, an inhibitor of angiogenesis in vivo (Taylor and Folkman, 1982), known to prevent binding of bFGF to its receptor, inhibited proliferation (Neufeld et al., 1988). This latter evidence would suggest a receptor mediated bFGF action. These contrasting investigations only serve to emphasize the necessity for further research to determine the mechanism(s) by which FGF stimulates cell growth. The transfection studies demonstrate the potential oncogenic effects of the bFGF and aFGF genes -- the induction of transformation, anchorage-independent growth and tumorigenesis are all characteristics typical of malignancy. In fact, three oncogenes with homology to the gene encoding the FGFs have recently been described in the literature.

1.1.5 FGF Oncogenes

A proto-oncogene (c-onc) is a gene which functions in normal regulation of cell growth and/or differentiation. Oncogenes are derived from normal cellular genes that undergo some change that either causes them to produce an abnormal product or to disrupt their control so that they are expressed inappropriately -- making their products in excessive amounts or at the

wrong time, and thereby allowing them to bypass the normal cellular controls that regulate cell division and differentiation (Nicolson, 1987).

Oncogene stimulated growth factor secretion may occur by several mechanisms:

i) cellular transformation by a number of oncogenes (such as ras,src, mos, fes, abl, fps, erbB, yes and mil/raf) stimulates the induction of genes which code for growth factors, thus indirectly stimulating their production and secretion. Several growth factors, such as the TGFs, PDGF, EGF, IGF-II and FGF are, under appropriate conditions, capable of inducing phenotypic transformation of fibroblasts (Roberts and Sporn, 1986). These growth factors may then activate the proto-oncogenes c-myc and c- fos which are involved in cell differentiation and cell proliferation (Muller and Wagner, 1984; Armelin et al., 1984).

ii) other oncogenes directly encode growth factors -- the cellular oncogene c-sis gene product is similar to PDGF (Doolittle et al., 1983; Waterfield et al., 1983) and the oncogenes int-2 and hst/KS encode an FGF-like product (Peters et al., 1983; Sakamota et al., 1986; Delli Bovi and Basilico, 1987). Release of the growth factors could rapidly stimulate cell growth by an autocrine mechanism.

iii) oncogenes that encode receptors for growth factors -- the oncogene v-erb-B specifies a truncated EGF

receptor (Downward et al., 1984), its homologue, neu has an EGF receptor-like gene product (Schecter et al., 1984). Another oncogene, v-fems is related to the receptor for the macrophage specific colony stimulating factor (CSF-1) (Sherr et al., 1985). The oncogene c-erb-A gene product is related to steroid hormone receptors (Wienberger et al., 1985; Green et al., 1986a, b). Oncogene alteration of a growth factor receptor could allow the receptor to continually release growth stimulatory signals even in the absence of growth factor.

iv) oncogene alteration of signal transduction-- for several growth factor receptors such as PDGF, EGF, FGF, insulin and certain lymphokines, receptor occupancy leads to activation of tyrosine kinase activity. For example, the EGF receptor stimulates the nucleotide binding of the ras protein (Kamata and Feramisco, 1984). Altered forms of the transducing proteins that carry the signals (which lead to growth stimulation) from the cell surface receptors to intracellular targets could continuously send out signals even in the absence of growth factors. (reviewed in Weinberg, 1985; Breakefeld and Stern, 1986; Weinstein, 1987; and Rollins and Stiles, 1988).

All of these mechanism allow for rapid stimulation of cell growth in the presence of low levels or even in the complete absence of growth factors. The finding that transformed cells have a lower requirement for serum and

exogenous growth factors (Scher *et al.*, 1979) is consistent with the above scenarios of oncogenic-stimulated cell growth. Three recently identified oncogenes encode proteins that are structurally similar to fibroblast growth factors. These oncogenes have been identified from cancerous as well as normal tissues.

The *int-2* gene, located on mouse chromosome 7, was originally identified as a frequent integration site for mouse mammary tumor virus (MMTV), and has subsequently been implicated in murine mammary carcinogenesis (Peters *et al.*, 1983; Dickson *et al.*, 1984; Moore *et al.*, 1986). Following publication of the complete nucleotide sequence and predicted amino acid sequence of mouse *int-2* (Moore *et al.*, 1986), homology to bovine aFGF and bFGF was established (Dickson and Peters, 1987). *Int-2* shows greater similarity to bFGF than to aFGF. The *int-2* protein has 245 amino acids with an estimated MW of 27,000 making it almost twice as large as the FGFs (Moore *et al.*, 1986; Smith *et al.*, 1988).

Multiple species of *int-2* RNA (2.9, 2.7, 1.8, and 1.6 kb) reported by a number of researchers, are suggested to result from initiation at variable capsites within two distinct promoter regions (P1 and P2), and termination at two alternative polyadenylation signals (A1 and A2) (Smith *et al.*, 1988; Wilkinson *et al.*, 1988). The *int-2* gene is therefore highly complex allowing for the production of mRNA transcripts comprising either

three or four exons. The four int-2 RNA species all encode the same protein product (Smith et al., 1988).

Expression of int-2 appears to be highly restricted in normal cells. Although int-2 gene expression has been detected in normal mouse embryogenesis, it has not been found in normal adult tissue (Dickson et al., 1984; Jakobovits et al., 1986).

Using an int-2 probe, Jacobovits and coworkers (1986) detected four species of RNA (3.2, 2.9, 1.8 and 1.4 kb) prior to day 7.5 of gestation in peri-implantation mouse embryos. Transcripts were not detectable during mid-gestation (days 8-13). The int-2 RNA was most abundant in cells of primitive endodermal lineage. Wilkinson et al. (1988) studied the expression of four RNA transcripts (2.9, 2.7, 1.8, and 1.6 kb) during early gastrulation and neurulation (days 7.5 to 9.5). Using in situ hybridization, correlations between int-2 gene expression and two embryonic events -- the migration of mesodermal cells, and the induction of otocyst (embryonic inner ear) development, were discovered.

The above results led Dickinson and Peters' group to postulate a dual role for the int-2 gene:

i) as a normal growth regulatory gene which encodes an autocrine growth modulator with a highly specialized role in embryogenesis; and

ii) as an oncogene involved in the genesis of

MMTV-induced tumors (Wilkinson et al., 1988; Smith et al., 1988).

The second oncogene, hst, is a transforming gene initially identified in transformed NIH 3T3 cells that had been transfected with DNA of a human stomach cancer (Sakamoto et al., 1986). These transformed cells were also tumororigenic in nude mice. Other human sources of hst DNA include hepatomas, colon cancer, peripheral leukocytes of a patient suffering from chronic myelogenous leukemia, as well as non-cancerous stomach and colon mucosae and normal leukocytes. The variety of sources makes the hst gene one of the most frequently occurring transforming genes, second only to the ras gene (Yoshida et al,, 1987; Sakamoto et al., 1988). These transformed cells were also tumorigenic in nude mice.

Simultaneous but independent research by Delli Bovi and Basilico (1987) had isolated an oncogene from Kaposi's sarcoma (KS) also capable of transforming NIH 3T3 cells transfected with KS DNA. Kaposi's sarcoma is an angiosarcoma of endothelial/mesenchymal origin which frequently occurs in patients with AIDS (acquired immune deficiency syndrome) (Snover and Rosai, 1985).

Publication of the complete nucleotide sequences of the hst and KS oncogenes revealed their exact identity. The predicted amino acid sequence of the hst/KS protein is homologous to selected regions of human bFGF (43%), human aFGF (38%) and to the mouse int-2

protein (40%) (Yoshida et al., 1987). The oncogene product contains 206 amino acids and has an ~MW 22,000 (Taira et al., 1987; Delli Bovi et al., 1987).

Structural comparisons between the four proteins --the FGFs, hst/KS and int-2 have been reported. Two functional domains of bFGF (Esch et al., 1985b), the cell recognition site, and the heparin-binding site, are conserved in the hst/KS protein (Yoshida et al., 1987). Two cell recognition site sequences occur in human bFGF; one is also found in the hst protein, but these sites are not present in either human aFGF or mouse int-2. Acidic FGF and bFGF each contain two heparin-binding sites; the hst protein also has a potential heparin-binding sequence. Two cysteine residues, Cys-88 and Cys-155, are present in homologous regions of the FGFs and the hst/KS and int-2 proteins. These four proteins all contain a central exon of exactly 104 nucleotides, implying their common ancestry (Smith et al., 1988). The amino-terminus of int-2, like bFGF, has a short stretch of non-charged residues that may function as an atypical signal sequence (Abraham et al., 1986; Dickson and Peters, 1987). In contrast to the FGFs, the hst protein has a classical signal-peptide sequence and internal hydrophobic domains.

To date, a third oncogene found to encode an FGF-like protein has been isolated from human bladder cancer cells by Xi Zhan and Martin Goldfarb (reported by J.L. Marx, 1987a).

The isolation of several oncogenes which code for proteins that are potential oncogene products, are members of the FGF gene family, and have the potential to be secreted proteins able to act on a variety of target cells, indicates a mechanism by which the FGF's could regulate angiogenesis and tumor growth.

1.1.6 FGF Receptors

Specific cell surface receptors for aFGF and bFGF have been reported on a variety of cell types known to respond to these mitogens (Table 3). FGF receptors have been characterized using a baby hamster kidney cell line (BHK-21), chosen for its high density of FGF receptors/ cell (Neufeld and Gospodarowicz, 1985). The density of FGF cell surface receptors in most diploid cells is low (10^3-10^4) compared to the density in BHK-21 cells (10^5). The process of receptor binding is time and concentration dependent, saturable and reversible. Receptor down-regulation occurs as a result of receptor occupancy or increasing cell density (Neufeld et al., 1985; Schreiber et al., 1985a, b). Binding of the FGFs to their receptors is highly specific; insulin, IGF-I, EGF, PDGF, NGF and transferrin do not compete for these receptors (Neufeld and Gospodarowicz, 1985; Olwin and Hauschka, 1986).

Neufeld and Gospodarowicz (1986) reported that

TABLE 3: Characterization of FGF Receptors on Various Cell Types

Cell Types	MW	Kd	Receptors/ Cell	Reference
Skeletal Muscle Myoblasts	165 (a)	11 pM	2×10^4	Olwin and Hauscka, 1986
Swiss 3T3 Cells	165 (a)	45 pM	6×10^4	ibid.
Murine Lung Capillary Endothelial Cells	150 & 130 (b)	40 pM	4×10^4	Schreiber et al., 1985 a Friesel et al., 1986
Human Glioma Cell Lines	150 & 130 (b)	N.D.	$0.3\text{-}8 \times 10^3$	Libermann et al., 1987
BHK-21 Cells*	145 & 125 (a)	270 pM	1×10^5	Neufeld and Gospodarowicz, 1985
Rhabdomysarcoma Cells	145 & 125 (a)	N.D.	N.D.	Schweigerer et al., 1987c
PC12 Pheochromocytoma	145 (a)	20 pM	3.6×10^3	Neufeld et al., 1987
Human Umbilical Vein Endothelial Cells	130 (b)	20 pM	4×10^4	Schreiber et al., 1985a
Bovine Lens Epithelial Cells	130 (c)	53 pM	2×10^4	Moenner et al., 1986

(a) Determined with aFGF and bFGF.
(b) Determined with aFGF only.
(c) Determined with bFGF only.

* Used for Initial Receptor Characterization due to High Receptor Concentration Per Cell.

TABLE 3: Characterization of FGF Receptors on Various Cell Types (Cont'd)

Cell Types	MW	Kd	Receptors/ Cell	Reference
Balb/c3T3 Fibroblasts	N.D. (b)	40 pM	3×10^4	Schreiber et al., 1985a
Human Foreskin Fibroblasts	N.D. (b)	70 pM	2×10^4	ibid.
Rabbit Aortic Endothelial Cells	N.D. (b)	80 pM	2×10^4	ibid.
Bovine Aortic Endothelial Cells	N.D. (b)	60 pM	2×10^4	ibid.
Human Hepatoma Cell Line	N.D. (c)	38.4 pM	2.5×10^3	Moscatelli, 1987**
Chicken Embryo Fibroblasts	N.D. (c)	7.3 pM	1.3×10^4	ibid.
Human Melanoma Cells	N.D. (c)	16.6 pM	1.6×10^4	ibid.
Bovine Embryo Skin Fibroblasts	N.D. (c)	12.9 pM	3.2×10^4	ibid.
Bovine Capillary Endothelial Cells	N.D. (c)	20 pM	8×10^4	ibid.
Mouse Sarcoma	N.D. (c)	47 pM	6×10^4	ibid.
Bovine Outer Rod Segment	N.D. (b)	N.D.	N.D.	Plouett et al., 1988

** High Affinity Binding Sites only.

bFGF and aFGF interact with the same cell surface receptors -- a single class of high affinity binding sites con-sisting of two species, MW 145,000 and 125,000 kD. They observed that qualitatively, aFGF and bFGF could interact with either membrane component. However, quantitatively, bFGF displayed a higher affinity for the 145,000 dalton receptor whereas the affinity of aFGF was greater for the 125,000 dalton species. The differences in affinity of the two mitogens towards their common receptors was suggested as a possible explanation for their differing biological potencies (aFGF is less mitogenic that bFGF). A single class of binding sites for aFGF and bFGF has also been identified in Swiss 3T3 cells and MM14 myoblasts (Olwin and Hauscka, 1986) and in murine and human endothelial cells and fibroblasts (Friesel et al., 1986; Schreiber et al., 1985a).

Moscatelli (1987) reported the presence of high and low affinity binding sites in several cell lines, including BHK cells, human hepatoma, and bovine corneal endothelial cells. High affinity sites were pH sensitive and thought to represent binding to the bFGF receptor. Binding to low affinity sites could be competed and decreased with heparinase or by heparin treatments, suggesting that these sites represent binding to cell-associated heparin or heparin sulfate.

The possibility that aFGF and bFGF bind to different receptors has been suggested by Baird and

coworkers based on their discovery that bFGF but not aFGF stimulated the growth of cultured human melanocytes (Halaban et al., 1987). As well, bFGF did not compete for binding to an aFGF receptor on bovine aortic endothelial cells (Schreiber et al., 1986). The different MWs reported for the FGF receptor may be the result of different degrees of receptor glycosylation (Gospodarowicz et al., 1987).

Only partial receptor occupancy is required for FGF mitogenicity (Neufeld and Gospodarowicz, 1985; Schreiber et al., 1985a, b; Friesel et al., 1986). So called "spare" receptors have been described for other polypeptide growth factors such as the TGF-B receptor (Massague and Like, 1985) and the EGF receptor (Carpenter and Cohen, 1979).

Interaction of aFGF or bFGF with their cell surface receptors can be inhibited by several factors. Mono-clonal antibodies to aFGF prevent receptor occupancy by this ligand (Schreiber et al. 1985a), thereby inhibiting its mitogenic activity. The glycosaminoglycan heparin, can modulate binding of aFGF or bFGF to receptors. Addition of heparin significantly increases the binding of aFGF to endothelial cells (Schreiber et al., 1985a); whereas heparin has been reported to inhibit the binding of bFGF to BHK 21 cells (Neufeld and Gospodarowicz, 1985) and PC 12 neuronal cells (Schubert et al., 1987). The possibility that heparin may alter

the actual structure of aFGF resulting in an increased affinity for its receptor, or that the receptor may in fact require a heparin-aFGF complex for an ideal fit has been considered (Schreiber et al., 1985a). Conversely, protamine sulfate prevents the interaction of bFGF with its receptor (Neufeld and Gospodarowicz, 1987). (The effects of heparin and protamine sulfate on FGF activity in vitro and in vivo are discussed in sections 1.17 and 1.18).

The rate of aFGF internalization and degradation following binding to endothelial cell receptors has recently been reported (Neufeld and Gospodarowicz, 1985; Friesel and Maciag, 1988). ^{125}I-aFGF was found to be rapidly internalized, apparently as a result of receptor down regulation. Internalized ^{125}I-aFGF was found to have a long half-life, with subsequent degradation in a lysosomal cellular compartment occurring quite slowly. IL-I, which is structurally homologous to aFGF shares this low rate of degradation (Mizel et al., 1987).

Early studies suggested that FGF did not stimulate tyrosine phosphorylation of the FGF receptor in vivo or in vitro (Gospodarowicz et al., 1986; Neufeld and Gospodarowicz, 1985). This was in sharp contrast to PDGF (Ek and Heldin, 1982), EGF (Cohen et al., 1980, 1982), insulin (Kahn, 1985) and IGF-I (Jacobs et al., 1983) all of which stimulate tyrosine kinase activity by their receptors. In 1986, Huang and Huang reported that brain-

derived aFGF stimulated tyrosine phosphorylation of a 135 kd protein *in vitro*. More recently, Coughlin and colleagues (1988), demonstrated the ability of both aFGF and bFGF to stimulate tyrosine phosphorylation of a 90 kd protein *in vivo*. In PDGF, EGF, IGF-I and insulin, the tyrosine kinase activity is intrinsic to the growth factor receptor with the receptor being a major substrate of the kinase (Coughlin *et al.*, 1988). In contrast, the 90 kD protein appears to be a specific substrate of FGF-stimulated tyrosine kinase rather that a component of the FGF receptor (Coughlin *et al.*, 1988).

Further investigation is required to firmly establish whether aFGF and bFGF interact with the same, similar or distinct receptors and to determine the exact role of tyrosine phosphorylation in the receptor-ligand coupling and the subsequent biological actions of these growth factors.

1.1.7 In Vitro Biological Effects of FGF

The *in vitro* biological actions of FGF include effects on the proliferation, morphology, transformation, differentiation and senescence of various cell types.

Although the functions of aFGF and bFGF are very similar, bFGF is 30- to 100-times more active (Esch *et al.*, 1985a; Bohlen *et al.*, 1985). In the presence of heparin, however, the activity of aFGF and bFGF appear

equivalent (Schreiber et al., 1985a, b). Heparin appears to interact structurally with aFGF to enhance and stabilize the activity of this mitogen, to increase the affinity of aFGF for its receptor, and to modify monoclonal antibody recognition of aFGF. Passage of biologically inactive aFGF over heparin-Sepharose restores its biological activity. Heparin also protects both aFGF and bFGF from acid and heat inactivation (Schreiber et al., 1985a, b). The stabilizing function of heparin may explain the high levels of FGF that appear to be stored in the ECM for long periods. Protamine, a protein which binds avidly to heparin, inhibits the heparin-induced enhancement of FGF actions both in vitro and in vivo.

Table 4 provides a list of the wide spectrum of FGF responsive cells. The FGFs are potent mitogens for a variety of normal and tumor cells, primarily of mesodermal and neuroectodermal origin, as well as for epithelial cells. As such, the term Fibroblast Growth Factor, originally chosen to describe the effects of an impure mitogen on 3T3 fibroblast cultures, could be considered obsolete as well as misleading in its scope.

Several morphological changes associated with FGF addition have been observed in fibroblasts, endothelial cells and smooth muscle cells. The changes are apparently the result of increased locomotor activity and cellular migration (Gospodarowicz and Moran, 1975;

TABLE 4: Cell Types for which bFGF or aFGF is Mitogenic.*

	bFGF	aFGF
Normal Diploid Cells:		
Glial and astroglial cells	+	+
Oligodendrocytes	+	+
Trabecular meshwork cells	+	N.D.
Endothelial cells from capillary large vessel, and endocardium	+	+
Corneal endothelial cells	+	+
Fibroblasts	+	+
Myoblasts	+	+
Vascular smooth muscle	+	+
Chondrocytes	+	+
Osteoblasts	+	+
Blastema cells	+	N.D.
Adrenal cortex cells	+	+
Granulosa cells	+	+
Prostatic epithelial cells	+	+
Lens epithelial cells	+	+
Mesothelial cells	+	+
Neuronal cells	+	N.D.
Keratinocytes	+	N.D.
Established Cell Lines:		
Rat fibroblast - 1	+	+
Balb/c3T3 Fibroblasts	+	+
Swiss 3T3 Fibroblasts	+	+
BHK - 21 Fibroblasts	+	+
A-204 Rhabdomyosarcoma	+	N.D.
PC12 Pheochromocytoma	+	N.D.
Melanoma cell line	+	N.D.

* - Modified from Gospodarowicz et al., 1987a.
\+ - Positive effect on cell proliferation.
N.D. - Effect on proliferation not determined.

Gospodarowicz et al., 1985a, b).

In the presence of FGF, the regular cobblestone appearance of resting Balb/c3T3 cells changes dramatically. As the cells lose their contact inhibition and begin to proliferate, they appear spindle-shaped and exhibit an irregular criss-crossed pattern and membrane ruffling (Gospodarowicz and Moran 1974; Gospodarowicz, 1979). These features (i.e. loss of "contact inhibition", reduced adhesion to the cell substratum, growth in a criss-cross pattern, and membrane ruffling) are typical of transformed cells (Gospodarowicz, 1986, 1987). This FGF-induced transformed phenotype is reversible. Both aFGF and bFGF induce anchorage-independent soft agar growth of non-transformed cells (aFGF effects are potentiated by heparin); FGF potentiated TGF-B activity in this assay (Rizzino and Ruff, 1986; Rizzino et al., 1986). Since anchorage-independent growth in vitro is an indication of tumorigenic potential in vivo, these authors contemplate the involvement of the FGFs in tumor vascularization and subsequent tumor growth.

Basic FGF alone and in combination with EGF, dramatically improves the lifespan of several cultured cells, by stabilizing the normal phenotypic expression of the cells (Gospodarowicz et al., 1986, 1987), thus allowing for their long-term culture.

In low density cultures of vascular or corneal

endothelial cells (Gospodarowicz et al., 1976, 1977), smooth muscle cells (Gospodarowicz et al., 1981) and neuronal cells (Walicke et al., 1986), FGF is considered a survival agent required for their continued normal morphology and function. This ability of FGF may be due to its regulation of the synthesis and deposition of essential components of the ECM which affect the normal polarity and gene expression of cells such as endothelial cells (reviewed in Gospodarowicz et al., 1986,1987). The ECM components affected by FGF include collagen, fibronectin and proteoglycans (Gospodarowicz, 1983).

Endothelial cells are bipolar, having a luminal surface and a basal (basement membrane) surface which is involved in ECM production (Robinson and Gospodarowicz, 1983; Folkman and Klagsbrun, 1987). In the absence of FGF, confluent endothelial cultures do not acquire this polarity; upon addition of FGF, the cells revert to the normal phenotype which is characteristic of the vascular endothelium in vivo (Greenburg et al., 1980; Vlodavsky et al., 1979; Vlodavsky and Gospodarowicz 1979; Tseng et al., 1982). Both aFGF and bFGF induce plasminogen activation (PA) and collagenase production in endothelial cells (Moscatelli et al., 1986; Saksela et al., 1987). Secretion of these enzymes aids in the proteolytic breakdown of the basement membrane and the ECM; structures which otherwise limit endothelial cell migration (Gross et al., 1982; Baird and Durkin, 1986;

Frater-Schroder et al, 1986).

In cultured chondrocytes, bFGF is not only mitogenic but also delays cellular differentiation (Gospodarowicz and Mescher, 1977). Addition of FGF to low density chondrocyte cultures stabilizes the chondrocyte phenotype once the cultures become confluent, allowing for the production of the essential ECM components (i.e. chondroitin sulfate, proteoglycans and collagen type II) found in vivo; whereas, chondrocytes grown in the absence of FGF assumed a fibroblastic appearance (Kato and Gospodarowicz, 1985). Although FGF suppresses [35S]-sulfate incorporation into proteoglycans during logarithmic growth, once the cultures achieve confluency, this mitogen stimulates the synthesis of proteoglycans, associated with the cartilage matrix. The differentiating chondrocytes surround themselves in a thick ECM as is typical of cartilage in vivo (Gospodarowicz, 1985).

Hiraki et al. (1988) recently reported the effects of TGF-B on chondrocyte proliferation and glycosaminoglycan synthesis. This factor inhibited thymidine uptake and stimulated glycosaminoglycan synthesis in rabbit growth plate (costal) chondrocytes. However, TGF-B potentiated DNA synthesis induced by serum, FGF, PDGF, and EGF, and also inhibited IGF-I activity. TGF-B alone was twice as potent as IGF-I, considered to be the main serum factor involved in

glycosaminoglycan synthesis, in stimulating this differentiated function. TGF-B also potentiated the effects of IGF-I and PDGF on glycosaminoglycan synthesis. Like FGF, TGF-B appears to be involved in chondrocyte growth and differentiation. Since both FGF and TGF-B have been isolated from cartilage and bone, the role(s) of these factors in cartilage and endochondral bone formation via paracrine and/or autocrine mechanisms has been suggested (Seyedin et al., 1982; Hiraki et al., 1988).

Basic FGF also induces the differentiation of preadipocyte fibroblasts into adipocytes (Broad and Itam, 1983; Serrero and Khoo, 1982).

By stimulating myoblast proliferation, bFGF delays the terminal differentiation (i.e. myogenic cell fusion to form myotubes) of both smooth muscle (Gospodarowicz et al., 1976) and skeletal muscle (Kardami et al., 1985; Clegg et al., 1987) in vitro. Addition of FGF to myogenic cultures represses the synthesis of muscle specific proteins (such as creatine phosphokinase) associated with differentiation and causes the muscle cells to exit from G0 into G1 of the cell cycle (Lathrop et al., 1985; Spizz et al., 1986). Simply by controlling the level of bFGF in these cultures, one can control the phenotypic expression of the muscle cells: in the absence of FGF, the muscle cells arrest in G0 and express a differentiated phenotype; upon FGF addition, the cells

enter G1 and a relatively undifferentiated state (Lathrop et al., 1985; Clegg et al., 1987). In contrast, TGF-B inhibits terminal muscle differentiation (Massague et al., 1986; Olson et al., 1986).

As mentioned previously, aFGF and bFGF promote growth and survival of cultured neuronal cells. FGFs also affects neuronal differentiation. Addition of bFGF to astroglial cells stimulates the synthesis of glial fibrillary acidic protein (Pettman et al., 1985; Morrison et al., 1985). The mitogen also enhances neurite extension (critical for the development of the nervous system) in hippocampal neurons (Walicke et al., 1986), cerebral cortical neurons (Morrison et al., 1986), cerebellar granule neurons (Hatten et al., 1988) and pheochromocytoma (PC 12) cells (Togari et al., 1983, 1985; Schubert et al., 1987). In addition to improved neuronal survival and neurite outgrowth, Schubert and coworkers (1987) showed that PC 12 cells cultured in the presence of bFGF exhibited an increased cell-substratum adhesion as well as morphological changes. Heparin modulated the effects of bFGF in the PC 12 assays. The above results have led to the speculation that bFGF may function as a neurotrophic factor in the central nervous system (Morrison, 1987).

Interactions between FGF and several hormones have been reported. In cultured adrenal cortical cells, ACTH inhibits FGF mitogenicity (Gospodarowicz et al., 1977).

Glucocorticoids enhance FGF effects of fibroblast growth (Gospodarowicz, 1974; Gospodarowicz and Moran, 1974a, b). Basic FGF can increase the release of PRL and thyrotropin and decrease the release of GH from primary cultures of rat anterior pituitary cells (Baird et al., 1985d; Gospodarowicz et al., 1986). The in vitro actions of FGF and TGF-B oppose each other. FGF and estradiol act synergistically to modulate the release of PRL in vitro (Mormede et al., 1985). The latter results suggest that in the pituitary, FGF may act as a differentiating factor, possibly involved in the development of estrogen-dependent prolactin-secreting tumors and lactation (Baird et al., 1986).

Several biochemical events are induced by FGF. The addition of FGF to quiescent fibroblasts causes rapid induction of diacylglycerol formation, protein kinase C activation and Ca++ mobilization -- events associated with c-myc activation and tumor development (Tsuda et al., 1985; Kaibuchi et al., 1986).

Doctrow and Folkman (1987) recently reported an attenuation of the mitogenic response of bovine capillary endothelial cells to hepatoma-derived FGF in the presence of protein kinase C activators. These researchers speculate that the role of protein kinase C activation may be to act as a regulatory intracellular signal involved in angiogenesis, perhaps by inducing proliferating capillary endothelial cells to

differentiate and form non-growing capillary tubes.

Other in vitro responses induced by FGF include an increased uptake of uridine, thymidine and total amino acids (Rudland et al., 1974), increased polysome formation, and the synthesis of many secretory proteins (Nilsen-Hamilton et al., 1981) including a thiol-dependent cathepsin (Denhardt et al., 1986) and the mitogen proliferin, a member of the GH-PRL family (Linzer and Nathans, 1984). TGF-B inhibits both the synthesis and release of these proteins in response to bFGF (Chiang and Nilsen-Hamilton, 1986).

1.1.8 In Vivo Biological Effects of FGF

1.1.8.1 Embryonic Development

Acidic and basic FGF mimic the in vivo effect of the ventrovegetal signal in early amphibian embyros (Slack et al., 1987). This signal induces cells normally destined to form ectoderm to differentiate into mesoderm instead. The inducing effect of FGF is highly specific, since no effect was observed with TGF-alpha, TGF-B, tumor necrosis factor, interferons alpha and B, insulin, IL-1 alpha, IL-1 beta and the colony stimulating factors, G-CSF and GM-CSF. Slack and colleagues (1987) suggest an embryonic role of FGF as a morphogen, controlling the developmental pathway selected by cells.

Two recent publications have further

established a role for FGF in early developmental processes. Risau et al. (1988) reported FGF-like endothelial cell mitogens in embryonic chick brain and suggested a specific role for this mitogen in embryonic angiogenesis. The regulated expression of int-2 mRNA observed in early mouse embryogenesis (during gastrulation and neurulation) also suggests a role for FGFs in embryonic development (Wilkinson et al., 1988).

1.1.8.2 Limb and Lens Regeneration

Basic FGF has been implicated in the regeneration of certain tissues. It promotes the regeneration of amphibian (newt) forelimb blastemas in vivo (Mescher and Gospodarowicz, 1979; Mescher and Loh, 1980). The blastemas are composed of FGF-responsive dedifferentiated myoblasts or chondrocytes. Increased forelimb regeneration also occurs in adult frogs (Rana pipiens) in the presence of bFGF (Gospodarowicz and Mescher, 1981). Thus, FGF may be involved in the recruitment of primitive cells required for blastema formation and limb regeneration (Gospodarowicz et al., 1986).

Retina- and pituitary-derived bFGF and aFGF may be involved in lens regeneration in vivo (Yamada et al., 1982). Evidence in support of this suggestion includes the requirement of these tissues for lens regeneration to occur in organ culture and the

potent stimulation of cultured lens epithelial and iris cells by FGF from these sources (Courty et al., 1985).

1.1.8.3 Angiogenesis

Angiogenesis is a term used to describe the formation of new blood vessels. Research associating capillary growth with tumor development prompted Folkman and his colleagues to propose the following mechanism of tumor growth: "solid tumors are angiogenesis dependent ... once tumor take has occurred, every increase in tumor cell population must be preceded by an increase in new capillaries that converge upon the tumor" (Folkman, 1986). Using several in vivo assays for angiogenesis activity, including the rabbit cornea, the chick chorioallantoic membrane and the hamster cheek pouch systems, a potent endothelial cell growth factor was isolated (Maciag et al., 1979). This mitogen was capable of stimulating a variety of processes thought essential to the neovascularization required for tumor development.

In normal circumstances, angiogenesis activity is tightly regulated. Blood vessel growth is extremely active only during embryonic development, and essentially absent in adults, with the exception of ovulation and the formation of the corpus luteum and placenta in women. In pathologic conditions such as wounds, chronic inflammation, and certain immune reactions, limited angiogenesis also occurs. Neoplastic

angiogenesis differs from the other circumstances in that the capillary growth is both persistent and uncontrolled.

The normal processes of ovulation, corpus luteum formation, and placenta formation all exhibit rapid neovascularization. FGF has been isolated from granulosa cells as well as corpus luteum and placenta tissue (Gospodarowicz et al., 1985b, c). Granulosa and luteal cells respond to FGF. Granulosa cells are normally separated from the outer layer of the follicle by a basement membrane. As such, they are in an avascular environment where their proliferation is limited. Following ovulation, the basement membrane breaks down and the rapid vascularization associated with luteinization occurs. The granulosa cells and capillaries proliferate, and the corpus luteum is formed (reviewed in Gospodarowicz, Mescher and Birdwell, 1976). FGF is also a potent inhibitor of FSH-stimulated aromatase activity thereby inhibiting the conversion of androstenedione to estradiol (Baird et al., 1986). Since estradiol is required for proliferation of the endothelium, by decreasing the level of this hormone, FGF effectively mediates follicular atresia. How FGF can mediate these two apparently opposed actions -- corpus luteum formation and follicular atresia is not understood.

Folkman and colleagues have described four processes which are associated with the formation of

new capillaries:

i) local degradation of the vascular basement membrane of the parent vessel;

ii) outward migration of endothelial cells to form a capillary sprout;

iii) proliferation of endothelial cells within the sprout; and

iv) formation of a lumen within the sprout, followed by an anastomoses of several sprouts to form a capillary loop through which blood will flow (reviewed in Folkman, 1986; Folkman and Klagsbrun, 1987a, 1987b).

These authors describe a family of unrelated proteins which share the functional ability to promote angiogenesis *in vivo*. Included in this group is aFGF and bFGF, TGF-alpha and TGF-B, angiogenin, and TNF-alpha.

The identity of the endothelial cell growth factor (ECGF) isolated by Maciag *et al*. (1979) with aFGF came about due to the observation that one of the most frequent cell types attracted to a tumor site is the mast cell. Since these cells produce heparin, this substance was tested in their assays and found to promote the effects of a crude tumor angiogenesis factor on endothelial cell migration *in vitro* and capillary growth *in vivo* (Taylor and Folkman, 1982; Folkman *et al*., 1983). Biochemically, their mitogen bound avidly to a heparin-Sepharose column, a feature which led to the ultimate

purification of the aFGF-like and bFGF-like mitogens listed in Table 1. The factor stimulating the induction of angiogenesis was found to be (heparin-Sepharose purified) aFGF (Lobb et al., 1985; Thomas et al., 1985).

The functional significance of heparin is not limited to the endothelial cell since heparin also potentiates the mitogenicity of aFGF and bFGF for hamster fibroblast and bovine lens epithelial cells (Uhlrich et al., 1986) and acts synergistically with the bFGF-like human pituitary growth factor (Rowe et al., 1986b). Heparin sulfate on the surface of dorsal root ganglion neurons is required to elicit Schwann cell proliferation (Ratner et al., 1985). Conversely, heparin inhibits the growth of smooth muscle cells (Clowes and Karnovsky, 1977; Castellot et al., 1981), an effect which can be reversed by EGF (Reilly et al., 1987). Glomerular epithelial cells secrete heparin-like molecules that prevent mesangial-cell proliferation (Castellot et al., 1985).

Angiogenin is a single chain polypeptide (MW 14,400; pI 9.5) initially isolated from the conditioned medium of a human adenocarcinoma cell line (Strydom et al., 1985). Structurally, angiogenin is 35% homologous to a family of pancreatic ribonucleases, and shares their ribonucleolytic activity (Kurachi et al., 1985; Fett et al., 1985; Shapiro et al., 1986). Whether the action of angiogenin on RNA is a component of its

angiogenic activity is unknown (Harper et al., 1988). Angiogenin is not an endothelial cell mitogen, does not bind to heparin and is a secretory protein with a signal peptide (reviewed in Folkman and Klagsbrun, 1987a). The above features suggest that the FGFs and angiogenin regulate angiogenesis by different mechanisms.

The transforming growth factors (TGF-alpha and TGF-beta) are structurally unrelated polypeptides, originally isolated from the conditioned medium of virally transformed cells. They were named according to their ability to alter the phenotype of normal fibroblasts to that of transformed cells, resulting in anchorage-independent growth (DeLarco and Todaro, 1980; Roberts et al., 1983; Moses et al., 1981).

Transforming growth factor-alpha contains 50 amino acids and shares 35% sequence homology with EGF. Both mitogens bind competitively to the EGF receptor. Both TGF-alpha and EGF stimulate microvascular endothelial cell proliferation in vitro and angiogenesis in vivo (Schreiber et al., 1986). The proteins EGF, TGF-alpha and another homologous protein called vaccinia virus growth factor (VGF) accelerate epithelial regeneration in vivo, thus aiding in the healing of damaged epidermal tissue (Schultz et al., 1987).

The second transforming factor, TGF-B is a homodimer (112 amino acids/chain), MW 25,000 (Dernyck et al., 1985) which binds to a specific receptor.

Sources of TGF-beta include platelets, placenta, kidney and tumors (Assoian et al., 1986; Sporn and Roberts, 1985, Frolik et al., 1983). In vitro, TGF-B acts primarily as a growth inhibitor although a growth-stimulating effect on fibroblasts has been reported (Keski-Oja et al., 1987). Most of the stimulatory effects reported for this factor appear to be indirect, by mediating the expression of other direct acting growth factors. For example, in fibroblasts, TGF-B induces expression of c-sis mRNA and the production of the PDGF-like sis-protein; PDGF acts directly on the cell, activating the cellular oncogenes c-fos and c-myc, and causing autocrine stimulation of cell growth (Leof et al., 1986). Addition of EGF to the cultures inhibits the activity of TGF-B (Roberts et al., 1985). In vivo, TGF-B causes an increase in macrophage, fibroblast and collagen production (Roberts et al., 1986). Although TGF-B and FGF have similar in vivo angiogenic activity, the in vitro actions of TGF-B directly oppose those FGF: TGF-B inhibits endothelial cell proliferation and the synthesis of plasminogen activator (required for ECM dissolution) (Frater-Schroder et al., 1986; Baird and Durkin, 1986; Saksela et al., 1987).

Tumor Necrosis Factor (TNF-alpha) is a polypeptide originally identified in the serum of bacillus-infected mice (Carswell et al., 1975). It is identical to cachectin and is one of the main products

secreted from activated macrophages. TNF-alpha is an established anti-cancer agent, known for its ability to cause haemorrhagic necrosis and regression of tumor growth and metastasis (reviewed in Frater-Schroder et al., 1987). The potent angiogenic activity of TNF-alpha is, therefore, in sharp contrast to its tumor necrosing actions. TNF-alpha inhibits endothelial proliferation in vitro, and acts as a non-competitive antagonist of FGF (Frater-Schroder et al., 1987; Leibovich et al., 1987).

The angiogenic potential of this factor appears dependent on its route of administration to the bipolar endothelium. Exposure of the basal cell surface to TNF-alpha stimulates capillary outgrowth from parent vessels, whereas injection of TNF-alpha into the vessel lumen stimulates the procoagulant activity associated with thrombosis and haemorrhage but not capillary growth (Frater-Schroder et al., 1987; Leibovich et al., 1987). The contrasting effects of TNF-alpha, that is, its ability to stimulate angiogenesis, an event considered essential to tumor development, as well as its tumor necrosing ability, may relate to the responses of the bipolar endothelium.

Folkman and Klagsbrun (1987a, b) have suggested several mechanisms to explain the interaction of these multiple angiogenic factors. The following scenario explains the interactions of these factors in the process of wound healing.

Upon injury, neovascularization may result from direct stimulation of capillary endothelial cells to follow the steps (outlined above) as required for new capillary growth. Direct angiogenic factors would include aFGF, bFGF and TGF-alpha, since they all promote cell motility and proliferation. Angiogenesis may also be mediated indirectly, with the involvement of macrophages. Since TGF-B is a strong chemoattractant for macrophages, this factor might stimulate these cells to release direct angiogenic factors, such as FGF and TNF-alpha. Macrophages are strongly attracted to wounds and would therefore be present to indirectly aid the healing process by promoting the regeneration of capillaries. TNF-alpha, in turn, is a powerful chemoattractant for the endothelial cells needed to repair the wound (Leibovich et al., 1987). As blood begins to flow through the new capillaries, the increased tissue oxygen content shuts off the macrophage angiogenic capacity (Folkman and Klagsbrun, 1987a).

Since the FGFs do not have secretory signals, the mechanism of release of FGF from intra- or extracellular stores is generally thought to be the result of cell lysis or leakage such as would occur during wounding. Indirect angiogenic factors may mediate the release of inactive endothelial mitogens such as FGF-heparin complexes stored either intracellularly or in the extracellular matrix. Mast cells located at the wound

site as well as the endothelial cells themselves can produce the heparin molecules. Heparinase, an enzyme released by platelets or activated macrophages, could degrade heparin sulfate in the ECM, and cause solubilization of biologically active heparin-FGF complexes (Gospodarowicz et al., 1987). Once released from intracellular stores, stimulation of collagenase and plasminogen activator proteolytic activity could aid in the dissolution of the basement membrane and the ECM releasing further extracellular stores of FGF. Endothelial proliferation and migration to form the new capillaries required to heal the wound could progress. These effects could be opposed by the TGF-B released from local platelets. Since platelet-endothelial interactions are involved in homeostasis and wound healing, the opposing effects of these factors could regulate these processes (Saksela et al., 1987)..

In the case of uncontrolled tumor angiogenesis, growth factor interactions are similar to that already explained for wound healing. Two recent reports have been critical to the understanding of growth factor induced tumorigenesis. The first is the finding that capillary endothelial cells express the bFGF gene, as well as produce and apparently release bFGF which is capable of stimulating capillary endothelial cell proliferation by an autocrine mechanism (Schweigerer et al., 1987c). Supporting this finding is the detection of

bFGF in the culture medium of BCE cells using a sensitive 2-site immunoradiometric assay, work completed by Yuji and Reiko Sato (1988) of our laboratory. Release of FGF from such a ubiquitous cell type, would allow the mitogen to stimulate *in vivo* the proliferation of other cell types found in close association with the endothelium--chondrocytes, smooth muscle cells, fibroblasts, glial cells and many others. Tumors themselves are sources of this mitogen and could release FGF to initiate rapid angiogenesis. Mast cells and macrophages, which also frequent tumor sites, could release heparin and FGFs to further stimulate FGF activity. The capillary network established, tumor growth, stimulated by tumor-derived and capillary-derived FGF, could progress rapidly.

In addition to FGF, many tumors also contain TGFs, which could add to the angiogenic response. Dernyck and coworkers (1987) found mRNAs encoding TGF-alpha, TGF-B, and the EGF receptor in a variety of human tumors and tumor cell lines. Members of our laboratory recently reported the differential expression of bFGF and TGF-B in human primary intracranial tumors and glioma cell lines (Murphy *et al.*, 1988a, b). TGF-B could also function by attracting macrophages to the tumor site. Release of TNF-alpha from the macrophages, although previously thought beneficial to tumor regression, could further stimulate capillary proliferation.

To add to this already sinister

scenario, the FGF-induced destruction of the basement membrane, which is also a requirement for tumor metastasis, could increase the metastatic potential of certain tumors. In fact, transformation of NIH 3T3 cells with bFGF results in the production of the metastatic phenotype in nude mice (personal communication, S. Egan, PhD. student, Manitoba Institute of Cell Biology). Cassian Yee and Robert Shiu (1986) have demonstrated the ability of FGF-responsive human breast cancer cells to invade the basement membrane. Since mammary tumors are sources of bFGF (Rowe et al., 1986a), this mitogen may be responsible for the basement membrane degradation. The invasive activity of Kaposi's sarcoma cells in vitro is potentiated by aFGF (Albini et al., 1988).

Autocrine stimulation of tumor growth could actually be initiated by cellular oncogene activation (described in section 1.1.5) or due to the deletion of a normal repressor gene which prevents tumorigenesis. Gene deletion as a mechanism of tumorigenesis has been described for acoustic neuromas (Seizinger et al., 1986), retinoblastoma (Dryja et al., 1984), rhabdomyosarcoma (Koufos et al., 1985) and Wilms tumor (Fearon et al., 1984).

Physiologic inhibitors of angiogenesis, called angiostatic agents, also exist. The ability of mast cells and heparin to stimulate the migration of capillary endothelial cells is inhibited by protamine

(Taylor and Folkman, 1982). This protein also blocks capillary proliferation induced by embryogenesis, inflammation, immune reactions and the growth of solid tumors (Taylor and Folkman, 1982). Protamine also inhibits the synergistic effects of hPGF and heparin on RFC cell growth (Rowe et al., 1986b).

Pericytes and smooth muscle cells in direct contact with endothelial cells, suppress endothelial cell growth (Orlidge and D'Amore, 1987). Mature capillaries are composed of endothelial cells and pericytes. These researchers noted that prior to the rapid growth that causes diabetic retinopathy, the pericytes disappeared.

In the presence of heparin, certain corticosteroids found in the circulation inhibit angiogenesis and suppress tumor growth (Folkman et al., 1983; Crum et al. 1985). Such angiostatic steroids may act in concert with extracellular heparin-like molecules to restrain capillary growth in vivo.

1.1.9 Physiological Significance of the FGFs

The primary physiological role of the FGFs appears to be their involvement in angiogenesis. The rapid angiogenesis observed in embryogenesis, ovulation, and corpus luteum formation and wound healing are examples of normal tightly regulated capillary growth. The only difference between normal angiogenesis and tumor

angiogenesis may be the extreme intensity and duration of neoplastic growth. Tumors induce neovascularization continuously; the normal control mechanisms having been overridden.

Non-neoplastic persistent angiogenesis is the dominant pathology present in the variety of disorders discussed below (Folkman and Klagsbrun, 1987a; Marx, 1987b; Baird et al., 1987b). In diabetic retinopathy, the small blood vessels in the retina proliferate rapidly until they rupture, causing blindness. Blindness resulting from abnormal neovascularization is also found in neovascular glaucoma and retrolental fibroplasia and may be a factor in the case of premature infants exposed to high levels of oxygen. Aberrant capillary growth is also observed in rheumatoid arteries, where it destroys joint cartilage. Hemangiomas in newborns and angiofibromas in the nasopharynx of adolescents are the result of abnormal capillary proliferation. The excessive epidermal proliferation suffered by patients with psoriasis may be due to abnormal growth of dermal capillaries. Finally, the formation of dense capillary networks called vasa vasorum in the region of atherosclerotic plaques, are potential sites of haemorrhage which can occlude coronary arteries and initiate a heart attack.

Other roles for FGFs include that of a morphogen in early embryonic development, cartilage and

endochondral bone formation, and as a neurotrophic factor involved in neuronal survival and early CNS development. Conversely, the rapid capillary growth observed following injection of FGF into the brain may indicate a method of treatment for stroke victims by promoting new vascular tissue and therefore decreasing neuronal damage (Baird et al., 1987b). The presence of FGF activity in the rat outer segments of the retina suggests a potential role for FGF in phototransduction (Plouett et al., 1988).

The potent activity of the FGFs, observed in the diverse array of systems which have been tested may reflect the multiple mechanisms of release available to this factor. The presence of FGF in serum suggests a classical endocrine mode of action. The ability of capillary endothelial cells as well as tumors to produce and release FGF to affect neighboring cells reflects a paracrine mechanism. Finally, the presence of FGF receptors on the variety of cell types that contain FGF, including capillary endothelial cells and tumor cells, establishes an effective autocrine system of action.

1.2 The Swarm Rat Chondrosarcoma Tumor Model

The growth of a cartilagenous tumor, the Swarm rat chondrosarcoma, both in vitro and in vivo is hormone responsive (McCumbee and Lebovitz, 1980a,b). This non-metastatic tumor has been maintained in vivo by serial

subcutaneous transplantation. Morphologically, the chondrosarcoma forms a pink, somewhat translucent nodular mass and is poorly vascularized, the vessels being located primarily in the septae separating the nodules. Histological and biochemical analysis indicated that the cells comprising the tumor are immature chondrocytes (Breitkrutz et al., 1979). This chondrosarcoma is considered a good model for cartilage growth and development in vivo since the direct actions of the IGFs and insulin on chondrocytes are the same as in normal cartilage. These hormones stimulate amino acid transport, RNA synthesis, and glycosaminoglycan synthesis in chondrosarcoma cells in vitro in a manner similar to that described in normal chondrocytes (McCumbee and Lebovitz, 1980a).

The pituitary-dependence of the growth of this tumor is well established. Although tumors will grow rapidly in normal Sprague Dawley rats, tumor growth is almost completely abolished in hypophysectomized (HPX) animals (McCumbee and Lebovitz, 1980b). Injection of bGH, cortisone or a combination of these hormones only partially restores the rate of tumor growth. Redding and Schally (1983) were able to partially inhibit the growth of Swarm chondrosarcoma by chronic injection of analogs of the hypothalamic hormones LHRH (decreases circulating LH and FSH levels) and somatostatin (lowers serum PRL, GH and ACTH levels). The above studies demonstrate the

pituitary-dependence of this chondrosarcoma, and has implied a role for a variety of known pituitary hormones such as GH, in chondrosarcoma growth. However, the almost complete reduction in tumor growth observed following HPX could not be mimicked by hypothalamic analogs, nor could injection of hormones completely restore the rate of tumor growth to that observed in the normal animals. These findings suggest that other pituitary-derived factors, such as hCGF, may play a significant role in the development of cartilage tumors. The observation by Mormede et al. (1985) that HPX increases circulating FGF levels in rats further suggests pituitary involvement in the control of FGF pools. Redding and Schally (1983) speculated upon a use for their analogs in the formation of a new endocrine therapy for chondrosarcomas, osteosarcomas, and related hormone-dependent neoplasias.

In addition to the possible involvement of pituitary FGF-like growth factors in the growth of cartilage tumors, normal cartilage (Sullivan and Klagsbrun, 1985) and rat chondrosarcoma (Shing et al., 1984) are also sources of FGF. Baird et al. (1986) have shown that injection of antiserum to FGF substantially decreases the growth rate of a mouse chondrosarcoma, implicating the growth factor in the etiology of cartilage tumor development in vivo. Whether the effect of the antibodies was via an inhibition of neovascularization or

due to an inhibition of chondrocyte proliferation or both was not defined.

1.3 Objectives of the Present Research

The studies presented in this thesis fall into three basic categories:

i) comparison of the biological and immunological activities of three human pituitary-derived hCGF preparations with that of bovine pituitary FGF. The *in vitro* mitogenicity of these and several other growth factors were tested on several cell types of mesodermal origin;

ii) the ability of human and rat pituitary tissue to synthesize and/or release hCGF-like mitogenic activity *in vitro* and *in vivo*; and

iii) the effects of a relatively impure hCGF preparation on the *in vivo* growth of a rat chondrosarcoma.

2. MATERIALS

2.1 Protein Purification

Sephadex G-100, PBE Chromatofocusing gel, PB 96 polybuffer, heparin-Sepharose CL 6B and Spectropor dialysis tubing (MW cutoff 6000-8000) were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Hydroxylapatite and DEAE Affigel Blue were purchased from Bio-Rad (Richmond, CA).

2.2 Tissue Culture Supplies

Culture media, i.e. Ham's F-10, Dulbecco's Modified Eagles Media (DMEM), and Fischer's media; as well as fetal bovine serum (FBS; lot 19N2651), horse serum (HS), penicillin-streptomycin, and trypsin-EDTA were purchased from Gibco Canada (Burlington, Ont.). L-glutamine was obtained from Flow Laboratories (McLean, VA). Tissue culture dishes, flasks, and microwell plates were purchased from either Gibco or Flow.

2.3 Cells, Tissues and Animals

The Balb/c3T3 clone A31 mouse embryonic fibroblast cell line was obtained from American Type

Tissue Culture Collection (ATCC). The T-47D human breast cancer cells were the gift of Dr. R.P.C. Shiu. Bovine corneal endothelial (BCE) cells were provided by Dr. Y. Sato or Dr. R.P.C. Shiu. The human foreskin fibroblasts (MCH6) were generously supplied by Dr. K. Wrogemann. The Nb_2 rat lymphoma cells were provided by Dr. F. Croze or Dr. A. Walker.

The Swarm rat chondrosarcoma was obtained from Dr. Richard Swarm of Hoffman Laroche.

The normal Buffalo rats and the normal and hypopysectomized Sprague Dawley rats were purchased from Charles River Canada Inc. (St. Constant, Que.).

2.4 Hormones, Growth Factors, and Antisera

The human GH and human PRL as well as monoclonal antisera to human GH (3D5) and human PRL (A31) were prepared in our laboratory by H. Cosby.

The growth factors, FGF (10 ug/vial) and EGF (1 ug/vial) were purchased from Collaborative Research (Lexington, MA). TGF-alpha and IGF-II were generously provided by Dr. R.P.C. Shiu. Polyclonal antibodies to basic FGF were the gift of Dr. Andrew Baird, Salk Institute, La Jolla, CA. These antibodies were raised in rabbits against synthetic peptides to bovine basic FGF, conjugated to BSA. Dr. Baird also provided an aliquot of

basic pituitary FGF.

2.5 Patient Human Pituitary Tumor Tissue and Serum Samples

The patient samples of pituitary tumor tissue and serum (from patients with pituitary tumors) were supplied by Dr. Fewer and Dr. M. West.

2.6 Electrophoresis and Blotting Supplies

The reagents for SDS-polyacrylamide gel electrophoresis were purchased from Bio-Rad. Western blot supplies, including nitrocellulose, immuno-blot (GAR-HRP) assay kit, and dot-blot apparatus were also purchased from Bio-Rad. Electrophoretic transfers were completed in a Hoeffer Transfer Apparatus.

2.7 Miscellaneous Reagents

Scintillation fluid (Biofluor) and [3H]-thymidine were purchased from New England Nuclear (Boston, MA). Various other reagents, including BSA (fraction V) and BSA (fatty acid and globulin-free) were obtained from either Sigma (St. Louis, MO) or Fischer Scientific (Edmonton, Alta.).

3. METHODS

3.1 Purification of Human Pituitary-Derived Chondrocyte Growth Factor (hCGF)

3.1.1 Chromatofocused hCGF (CF-hCGF)

Human CGF was partially purified using a protocol established by S. Kasper (Phd. thesis, 1984), with modifications. Human pituitary glands, removed at autopsy, were stored at -70°C until extraction. All steps of the purification were performed at 4°C.

One thousand human pituitaries were homogenized in 0.15 M $(NH_4)_2SO_4$, pH 5.5, as described in Too et al., 1987. The homogenate was stirred for 2 h, centrifuged (50 min at 10,000 x g), and the supernatant filtered through cheesecloth. Following a pH adjustment to 3.5 (using 0.5 M H_3PO_4), the supernatant was recentrifuged (10,000 x g for 50 min), and the pH immediately readjusted to 6.5-7.2 with 1 M NaOH. The supernatant was concentrated 3 fold using a 21 Amicon diaflo cell and YM-10 membrane filter (MW cutoff 10,000). The concentrated material was applied to a Sephadex G-100 column and eluted in 0.01 M Tris-HCl, pH 8.5. The column fractions were dialyzed and tested in the RFC cell

proliferation assay, and the biologically active fractions pooled for further purification. The G-100 pool was then applied to a Hydroxylapatite chromatography column (Bio-Gel HTP) equilibrated in 0.01 M sodium phosphate, pH 7.2. Following stepwise elution of the adsorbed material with 0.1 M sodium phosphate, pH 7.2 and 1.0 M NaCl in 0.01 M sodium phosphate, pH 7.2, the fractions were dialyzed against double distilled water and bioassayed using chondrocytes. The HTP fractions exhibiting mitogenic activity were pooled and applied to a DEAE Affi-gel Blue chromatography column equilibrated with 0.02 M Tris-HCl, pH 7.2. The adsorbed material was eluted by the stepwise addition of 0.15 M NaCl in equilibration buffer, 1.0 M NaCl in equilibration buffer and finally 0.5 M sodium thiocyanate (NaCNS). The column fractions were dialyzed against double distilled water and their mitogenic activity tested in the RFC assay. Active DEAE-Affigel Blue fractions were pooled and applied to a PBE chromatofocusing column. The chromatofocusing gel, the samples and all buffers were degassed before use. The column was equilibrated in 0.025 M ethanolamine, pH 9.4. Before applying the sample, 2 ml of Sephadex G-75 was layered on top of the gel to slow sample entry into the gel. Following sample application and adsorption to the column, an additional 5 ml of ethanolamine was added. The sample was eluted with

Polybuffer 96-HCL, pH 6.5. This buffer creates a pH gradient of 9.4 to 6.5 as it passes through the chromatofocusing gel, allowing the material to elute at its isoelectric point. Residual protein was removed from the gel with a 1 M NaCl solution. Fractions were tested for activity in the chondrocyte cell proliferation assay. For future analysis, samples were aliquoted and stored at -20°C.

3.1.2 Heparin-Sepharose Chromatography of hCGF hS-hCGF

To further purify hCGF, chromatofocused hCGF (2 mg) was applied to a heparin-Sepharose chromatography column (1 ml bed volume) equilibrated in 20 mM phosphate buffer, pH 7.4 (Too et al., 1987). A gradient of 0.5-3.0 M NaCl was used to elute proteins from the column. The fractions were dialyzed against 0.02 M phosphate buffer, pH 7.4 containing 20% (v/v) ethylene glycol and tested for mitogenic activity by [3H]-thymidine incorporation and cell proliferation assays. All samples were stored at -20°C.

3.1.3 Human Pituitary Growth Factor (hPGF)

A human pituitary growth factor, hPGF, was purified to homogeneity by Dr. J.M. Rowe in this laboratory (Rowe et al., 1986b). Briefly, 1200 human pituitaries were homogenized in 0.15 M NH_4SO_4, pH 5.5, stirred for 2 h and centrifuged for 40 min at 30,000 x g. The supernatant was added to heparin-Sepharose and stirred 1 h, after which the resin was allowed to settle and the supernatant was decanted. The resin was poured into a column and equilibrated with 10 mM phosphate buffer, pH 7.4 containing 0.5 M NaCl and 20% (v/v) ethylene glycol. The column was developed with a gradient of 0.5-3.0 M NaCl in 10 mM phosphate buffer containing 20% ethylene glycol. Biologically active fractions were pooled and dialyzed twice against phosphate buffer containing 20% ethylene glycol. This dialyzed material was then reapplied to the heparin-Sepharose column. The column was washed with equilibration buffer and the activity eluted with a 0.5-3.0 M NaCl gradient. Fractions with biological activity were pooled for further purification.

A Copper-Sepharose affinity column was equilibrated in 10mM phosphate buffer, pH 7.4 containing 0.5 M NaCl and 20% ethylene glycol before application of the heparin-Sepharose pool. The column was washed with

equilibration buffer containing 1 mM imidazole and developed with a gradient of 1-10 M imidazole. The mitogenic fractions were pooled, diluted 1:10 in ammonium formate (0.05M, pH 6.5) and applied to a column of CM-52. The column was sequentially eluted with 0.1-0.7 M ammonium formate. Biologically active fractions of CM-52 purified hPGF were stored at -20°C diluted in DMEM/10% FBS.

3.2 Cell Culture

3.2.1 Rabbit Fetal Chondrocytes (RFC)

3.2.1.1 Preparation of Chondrocytes

Chondrocytes were obtained from the knee cartilage of rabbit fetuses (day 21 gestation) as previously described (Kellett et al., 1981). Briefly, the cartilage was minced and digested with 0.25% collagenase in Ham's F-10 medium supplemented with 10% FBS, for 45 min at 37°C. Following centrifugation at 500 x g, the chondrocytes were cultured in Ham's F-10 growth medium (Ham's F-10 supplemented with 10% (v/v) fetal bovine serum, 100 i.u./ml penicillin, 100 ug/ml streptomycin, 4.5 g/L D-glucose and 4mM L-glutamine) in a humidified atmosphere of 95% air, 5% CO_2. Once

confluent, the primary cultures of RFC's were detached by trypsinization and kept frozen in liquid nitrogen in Ham's F-10 medium containing 20% FBS and 10% dimethyl sulfoxide (DMSO).

3.2.1.2 RFC Proliferation

Secondary cultures of RFC's were routinely grown in Ham's F-10 growth medium which was replenished twice weekly. Confluent cultures were trypsinized, centrifuged at 1000 x g for 3 min and the cell pellet resuspended in media. RFC's were plated (day 0) in 35 mm culture dishes (1-2 x 10^4 cells/dish) in 2 ml of F-10 growth medium and allowed to attach overnight. Sterile samples (100 ul volume) were added on day 1 and 3. The cells were trypsinized on day 5 and the cell number determined using a Coulter Counter.

3.2.1.3 RFC Population Doubling Time

The effect of mitogenic stimulation on the RFC population doubling time was determined using the following equations (Gertler et al., 1985):

i) Number of Doublings =

$$\frac{\log \frac{\text{final cell number}}{\text{initial cell number}}}{\log 2}$$

ii) Number of Doublings/Day =

$$\frac{\text{Number of Doublings}}{\text{Number of Days}}$$

iii) Mean Doubling Time (hours) =

$$\frac{24 \text{ hours}}{\text{Number of Doublings/Day}}$$

3.2.1.4 [3H]-Thymidine Incorporation

Confluent RFC secondary cultures were trypsinized, centrifuged, and resuspended in growth media as described for the proliferation assay. The cells were plated in 96-well microtiter plates (1 x 10^4 cells/well) in 100 ul of F-10 growth medium and incubated for 18-24 h. The media was then aspirated and the cells were washed 4 times with serum-free Ham's F-10 containing 0.1% BSA, antibiotics, glucose and glutamine. Mitogen (100 ul) and an equal volume of serum-free media were added and the cells were incubated a further 16-18 h followed by a 4 h pulse with 1 uCi/ml [3H]-thymidine. The media was aspirated and the cell layer washed 4 times with ice cold phosphate-buffered saline (PBS) containing 0.1% BSA and 50mg/L $CaCl_2$. The monolayers were extracted with 100 ul of ice cold 10% TCA and left at 4°C for 15-30 mins. The TCA was aspirated completely and the cell monolayer dissolved in 100 ul of 0.1 N KOH for a minimum of 30 min. The dissolved samples were transferred to scintillation vials and the wells washed twice with 100 ul of double distilled water. Following addition of scintillation fluid, the amount of incorporated radioactivity was determined by liquid scintillation counting in a B-scintillation counter.

3.2.1.5 Cell Morphology

To observe any possible morphological changes in the chondrocytes as a result of addition of mitogens, control and treated cells were photographed. Pictures were taken through a Nikon Inverted Microscope using a Nikon camera and black and white 35 mm Plus-x-Pan film (Kodak). Cells given maximal doses of hPGF, hS-hCGF, or bFGF (Collaborative Research) as well as control cells were photographed.

3.2.2 Bovine Corneal Endothelial Cells (BCE)

3.2.2.1 Preparation of Endothelial Cells

Bovine corneal endothelial cells, established in culture according to the method of Gospodarowicz et al. (1977), were generously provided by Dr. R.P.C. Shiu or Dr. Y. Sato. Following primary endothelial cell culture in Medium 199 supplemented with 20% FBS, antibiotics, FGF (Collaborative Research, 100 ng/ml) and 5% (w/v) T-40 Dextran, confluent flasks were trypsinized and the cells stored in liquid nitrogen.

3.2.2.2 BCE Proliferation Assay

BCE cell cultures were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% FBS, 100 i.u./ml penicillin and 100ug/ml streptomycin, containing 500 ng/ml EGF and 100 ng/ml FGF (Collaborative Research), in a humidified atmosphere of 95% air, 5% CO_2. The media was changed twice weekly and the cells were passaged at confluency. Following trypsinization, the cells were transferred to 35 mm dishes (1×10^4 cells/dish) in 2 ml of DMEM/15% FBS growth medium and allowed to attach overnight. To remove the serum, the media was aspirated and the cell monolayers washed once with DMEM containing only antibiotics. Two ml of fresh DMEM containing 1% FBS and antibiotics plus the samples (100 ul) was then added to each dish. A second sample addition was given on day 3. On day 5, triplicate cultures were trypsinized and the cells counted with a Coulter Counter.

3.2.2.3 BCE Population Doubling Time

The time required for the BCE cells to undergo cell division in the presence or absence of mitogen was

calculated as described for chondrocyte cell growth (Section 3.2.1.3).

3.2.2.4 [3H]-Thymidine Incorporation

The BCE monolayers were trypsinized and plated in 24-multiwell plates (3 x 10^5 cells/well) in 1 ml of DMEM growth medium. Twenty-four hours later, 1 ml of fresh media was added to the cultures. When the cells reached a subconfluent state, the wells were washed once with DMEM, the media changed to 2 ml of DMEM containing 0.1% BSA and antibiotics, and incubated a further 40-48 h. The media was aspirated and replaced with 1 ml of fresh serum-free media and samples (100 ul) were added. Following a 12 h incubation with [3H]-thymidine, the media was aspirated and the monolayer washed twice with ice cold PBS. The cells were extracted with 1 ml of ice cold 10% TCA and incubated on ice for 30 min. The TCA was removed and the monolayers briefly exposed to a second TCA addition. The TCA was aspirated thoroughly and the wells washed twice with an ethanol/ether (3:1) mixture. The cell monolayer was dissolved in 1 M NaOH (0.5 ml/well) overnight. Following neutralization with 0.5 ml/well of 1 M HCl, 0.5 ml of sample was transferred to scintillation vials and the amount of incorporated radioactivity determined in a B-scintillation counter.

3.2.2.5 Cell Morphology

Photographs of BCE cells were taken to observe any morphological changes resulting from treatment of the cells with hCGF. Procedures used were as described for rabbit fetal chondrocytes (Section 3.2.1.5).

3.2.3 Human Breast Cancer (T-47D) Cell Proliferation Assay

T-47D cells were maintained in DMEM containing 10% FBS, 100 i.u./ml penicillin, 100 ug/ml streptomycin, 10 ug/ml porcine insulin, 4.5 g/L D-glucose and 4 mM L-glutamine in a humidified atmosphere of 95% air, 5% CO_2. The medium was changed twice weekly and the cells passaged at confluency. For bioassay, confluent cells were trypsinized, centrifuged at 1000 x g for 3 min and the cell pellet resuspended in fresh growth media (without insulin). Cells were plated in 35 mm dishes (1 x 10^4 cells/dish) in DMEM/10% FBS growth medium. At 24 h intervals, 3 dishes were trypsinized and counted. Once the cells had undergone one cell division (usually within 72 h), the cell monolayers were washed once with DMEM supplemented with antibiotics, and 2 ml of fresh DMEM/1% FBS media (containing glucose, glutamine and

antibiotics) was added to the cultures. Samples (100 ul) were added at this time (day 1) and again on day 3. On day 7, the cells were trypsinized, syringed through an 18 gauge needle (to prevent cell clumping), and the cell number determined.

3.2.4 Human Foreskin Fibroblast (MCH6) Proliferation Assay

The MCH6 fibroblasts (MCH6; Montreal Children's Hospital, tissue sample 6), were surgically removed from an eighteen month old patient and have subsequently been maintained in culture or in liquid nitrogen. These cells have been maintained (in Dr. K. Wrogemann's Laboratory) in human McCoy's medium supplemented with 10% FBS and antibiotics. For experimental use, the cells were cultured in DMEM supplemented with 10% FBS, 4mM glutamine, 100 i.u./ml penicillin and 100 ug/ml streptomycin in a humidified atmosphere of 95% air, 5% CO_2, with media changes every 2-3 days. Confluent monolayers were trypsinized and plated in 35 mm culture dishes (1 x 10^4 cells/dish) in 2 ml of DMEM growth medium. Following overnight attachment, the cells were washed once with DMEM containing only antibiotics, then incubated with DMEM supplemented with 2.5% FBS, glutamine and antibiotics. Samples (100 ul) were added on day 1

and 3. On day 6 the cultures were trypsinized and the cell number counted.

3.2.5 Mouse Embryonic Fibroblast (Balb/c3T3, clone A31) Proliferation Assay

The Balb/c3T3 fibroblasts were maintained in culture in a similar manner to the MCH6 fibroblasts, with certain differences. The cells were only allowed to achieve 60-70% confluency prior to passage. For bioassay, a plating density of 2 x 10^4 cells/35 mm dish and DMEM supplemented with 1% FBS were used. Samples (100 ul) were added day 1 and day 3 and the assay was counted on day 5.

3.2.6 Rat Lymphoma (Nb_2) Cell Proliferation Assay

The growth of the Nb_2 cells was analyzed according to the method of Tanaka et al. (1980). Suspension cultures of Nb_2 lymphoma cells were routinely grown in Fischer's media supplemented with horse serum (HS; 10%), fetal bovine serum (FBS; 10%), 2-mercaptoethanol (2-ME; 10^{-4} M), sodium bicarbonate ($NaHCO_3$; 0.86%), penicillin (50 U/ml) and streptomycin (50 ug/ml) in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. Approximately 18-24 h prior to use in a bioassay, Nb_2 cell growth was arrested by incubation of

the cells in Fischer's/10% horse serum media (FH). On day 1, the cells were plated (1 x 10^5 cells/35 mm culture dish in 2 ml of FH media and the samples (50 ul) added. The cell number was determined on day 3 using a Coulter Counter.

3.3 Immunological Analysis of hCGF

3.3.1 Immunodot Blot Analysis

Antigen samples were diluted in Tween-20 Tris-buffered saline (TTBS; 20 mM Tris, 500 mM NaCl, 0.05% Tween-20, pH 7.5) containing 0.03% (w/v) rabbit serum. Two techniques were used to apply samples to nitrocellulose filters:

i) manual application -- a grid pattern (0.9 cm x 0.9 cm) was drawn on the filters with a soft pencil (Jahn et al., 1984). Sheets with 6 squares (2 x 3) were used. The filters were rinsed in TTBS for 10 min and air-dried for 5 min before use. Samples (2 ul) were spotted on the centre of each square;

ii) Biorad Dot-Blot Apparatus -- a nitrocellulose filter cut to the size of the apparatus was rinsed in TTBS and air-dried before use. The filter was securely clamped between the two plexiglass plates of the dot-blot apparatus. Samples (25 ul) were applied to the wells,

followed by 2-3 mins of suction to maximize protein adsorption to the filter. The wells were then washed with sample buffer, the suction was reapplied, and the membrane was removed from the blotting apparatus.

For both methods of sample application, all subsequent steps were carried out at room temperature (RT) with gentle shaking. Filters were incubated for 30 min in 3% rabbit albumin-TTBS to block any non-specific binding sites (i.e. quench). The blots were then incubated overnight with antisera to FGF or normal rabbit serum (1:1000 in 1% rabbit albumin-TTBS). The filters were rinsed in double distilled water and washed twice for 10 min each in TTBS. After a further 2 h incubation with horse radish peroxidase-conjugated goat anti-rabbit second antibody (GAR-HRP), at 1:1500 in 1% BSA-Tris buffered saline (TBS; 20 mM Tris, 500 mM NaCl, pH 7.5), the blots were developed. The filters were washed as described above and placed in HRP colour development solution (HRP reagent/0.15% H_2O_2 in TBS) until spots were visualized on the nitrocellulose. To stop further colour development, the filters were rinsed several times in double distilled water.

3.3.2 Western Blot Analysis

3.3.2.1 SDS-Polyacrylamide Gel Electrophoresis

The SDS-polyacrylamide gel electrophoresis was performed in 6-20% linear gradient acrylamide slab gels (Laemmli, 1970). Samples were adjusted to a final concentration of 2% SDS, 10% (v/v) glycerol, 5% (v/v) B-mercaptoethanol, and bromophenol blue (tracking dye); then boiled for 5 mins. Electrophoresis was carried out, with cooling, at 25 mA/slab gel for 4 h.

3.3.2.2 Electrophoretic Transfer of Proteins to Nitrocellulose

Transfer of the proteins to nitrocellulose was conducted according to the method of Towbin et al. (1979), with modifications. The electrophoretic transfer and the immuno-detection solutions used were from the Bio-Rad Immuno-blot (GAR-HRP) assay kit, with minor modifications. Following electrophoresis, the gels were washed in transfer buffer containing 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3 for 20 min. The nitrocellulose was carefully wetted by floating it in transfer buffer for 5 min.

A "sandwich" was then formed, consisting of the

following layers: i) a porous pad soaked in cold (4°C) transfer buffer; ii) one layer of filter paper; iii) the SDS-polyacrylamide gel; iv) the nitrocellulose sheet, cut to the size of the gel; v) a second layer of filter paper; and finally vi) another porous pad (Burnette, 1981). The sandwich was assembled in a Hoeffer transfer grid which was clamped tightly closed and immediately inserted (with the nitrocellulose towards the anode) into the Hoeffer transfer cell containing 4 L of cold transfer buffer. Electrophoretic transfers were performed at 800 mA/100 V (0-4°C, 1 h) using the Hoeffer Transfer Apparatus.

3.3.2.3 Immunological Detection of Proteins on Nitrocellulose

To prevent non-specific binding, the filters were soaked in 5% BSA (fatty acid and globulin-free) in Tris-buffered saline (TBS; 20 mM Tris, 500 mM NaCl, pH 7.5), for 30 min. The blots were then incubated overnight in first antibody (1:200) in 1% BSA-TBS. Following a rinse in double distilled water and two - 10 min washes in TBS, the filters were incubated for 2 h with HRP-conjugated goat anti-rabbit second antibody (1:1500) in 1% BSA-TBS. The filters were washed as described above and placed in HRP colour development

solution (0.05% HRP colour development reagent, 16.7% ice cold methanol, 0.015% H_2O_2 in TBS), and covered in dark plastic until bands were visualized. All steps in the above procedure were conducted at room temperature with gentle shaking.

3.4 Rat Pituitary Organ Culture

3.4.1 Preparation of Pituitary Tissue

Both male and female Sprague Dawley rats were used in the pituitary organ culture experiments. The animals were killed by decapitation; the pituitary glands were removed; washed 4 times with sterile serum-free Ham's F-10 medium; and minced into 1 mm^3 fragments. The pituitary pieces were placed on sterile siliconized lens paper rafts. The rafts were then floated in culture dishes containing 2 mls of serum-free Ham's F-10 supplemented with antibiotics. Following a 2-3 h pre-incubation period to allow for removal of residual blood, the pituitary rafts were ready for use in various experiments.

3.4.2 Rat Pituitary/RFC Co-Culture

Rabbit fetal chondrocytes were plated as previously described (Section 3.2.1.2). Following overnight cell attachment, the pre-incubated rat pituitary rafts were floated in the chondrocyte cultures establishing a co-culture system. On day 5, the pituitary rafts were removed, the RFC's were trypsinized, and the cell number was determined.

3.4.3 Collection and Assay of Conditioned Media

Conditioned media from two sources was collected: i) Rat Pituitary Organ Culture conditioned media; and ii) Rat Pituitary/Rabbit Fetal Chondrocyte Co-Culture conditioned media. To collect the rat pituitary organ culture conditioned media, one, two, or three pituitaries per culture dish were incubated in serum-free Ham's F-10 containing glucose, glutamine and antibiotics. Media was collected after 48, 72 and 144 h of incubation. The media was immediately filtered through a 0.45 u millipore filter and kept at -20^{o}C.

The co-culture conditioned media was collected on day 5 of the RFC proliferation assay. Following collection of this conditioned media, the chondrocytes were washed twice with serum-free Ham's F-10 ,

trypsinized and counted. In both media collections, control conditioned media was produced by incubating lens paper rafts in the appropriate media, and in the latter case, also in the presence of chondrocytes. Aliquots of both conditioned media preparations were tested in the RFC proliferation and [3H]-thymidine uptake assays.

3.4.4 Pituitary Cell Attachment

Duplicate culture dishes, each containing one rat pituitary raft and 2 mls of Ham's F-10 growth media were incubated until day 5 of a corresponding RFC assay. The media was collected, the cells were trypsinized and the cell number was counted.

3.5 Human Pituitary Tumor Serum Analysis

3.5.1 Patient Serum Samples

The serum samples utilized in these studies were collected over a period from 1983-1984. These samples had been stored at -20^{o}C since their collection. Two serum samples from each patient were analyzed: i) blood

which leaked into the surgical site during removal of the tumour; and ii) peripheral serum. Prior to application to individual heparin-Sepharose columns, each sample was centrifuged at 1500 rpm for 10 min; filtered through a 0.8 u filter; and the protein concentration was recorded.

3.5.2 Heparin-Sepharose Partial Purification of Human Pituitary Tumor and Serum Samples

The heparin-Sepharose columns consisted of a siliconized glass Pasteur pipet with a small wad of glass wool inserted at the base. The gel was equilibrated in 0.02 M sodium phosphate buffer, pH 7.2, containing 0.5 M NaCl, then poured into the pipets (300 ul bed volume/column). The human pituitary tumor conditioned medium as well as the serum samples (diluted 1:3 with column buffer), were applied to individual columns. The conditioned medium was passed through the column only once. In the case of the serum samples, the unadsorbed fraction was collected and this material was reapplied to the column. Two - 1 ml washes with 3 M NaCl in 0.02 M sodium phosphate buffer, pH 7.2, were then collected. The concentration of all fractions was recorded. The samples were then dialyzed against 0.01 M NH_4HCO_3 containing 0.1% BSA for 48 h, followed by a further 24 h of dialysis against DMEM containing 0.1% BSA. The mitogenic activity of the column fractions and the

untreated serum samples were tested in the RFC proliferation assay.

3.6 Swarm Rat Chondrosarcoma *In Vivo* Study

3.6.1 Tumor Maintenance and Transplantation

The transplantable rat chondrosarcoma used in the following study was originally maintained in 150 to 200 g male Buffalo rats. They were excised 8 weeks later after the animals were overdosed with ether. Tumor tissue exhibiting no signs of necrosis was washed three times in serum-free Ham's F-10 medium supplemented with antibiotics; thinly sliced; and then forced through a sterile stainless steel fine mesh wire screen into a beaker of medium. The resultant slurry was then rinsed three times (centrifuged 1000 x g for 5 mins). One ml aliquots of this preparation (100 mg tumor) were injected subcutaneously (sc) into both flanks of either the experimental animals or into animals used for tumor maintenance (McCumbee and Lebovitz, 1980b).

3.6.2 Experimental Animals

Normal and hypophysectomized (HPX) male Sprague

Dawley rats weighing approximately 150 g were housed in wire-bottomed cages in a room having a 12-h photoperiod and a temperature range of 21-23°C. They were allowed free access to food and water. Under these conditions, only those HPX rats that showed no weight gain during an initial equilibration period were used. At the end of the experiment, each animal was examined for remnants of pituitary tissue, to verify complete hypophysectomy.

3.6.3 Injection Procedure

All animals were injected sc twice daily (9:00 and 21:00) for a total of 4 weeks. The control and experimental treatments given are listed in Table 1. The hCGF preparation used was a crude HTP pool. Injection volume for all treatments was 0.5 mls. Once a week, the rats were weighed and their nose-to-tail lengths measured. At the end of the study, final body weights and nose-to-tail lengths were recorded. The animals were then decapitated and the wet weights of the chondrosarcoma tissue as well as several organ wet weights (liver, lung, kidney, spleen and testes) were measured.

3.7 Statistical Analysis

Statistical analyses were completed using a one way analysis of variance (Hewlett-Packard, 1967).

TABLE 5: Treatment Protocol for Swarm Rat Chondrosarcoma Study*

Normal Rats (n)	Treatment
2	Injectable Water **
3	100 ug hGH
3	250 ug hCGF

Hypopophysectomized Rats (n)	Treatment
4	Injectable Water
4	10 ng hGH
4	100 ug hGH
6	250 ug hCGF

* The animals were injected twice daily for four weeks.

** The Injectable Water consisted of sterile water containing 0.02 M Ammonium Bicarbonate.

4. RESULTS

The hCGF used throughout the following studies consisted of three preparations of increasing purity: chromatofocused (CF-hCGF); heparin-Sepharose purified chromatofocused hCGF (hS-hCGF); and finally, homogeneous human pituitary growth factor (hPGF). The chromatofocused material was isolated according to the procedures of Kasper (Ph.D. Thesis, 1984). The hS-hCGF eluted from heparin-Sepharose with 1.5 M NaCl and has a MW of 18-20 kD (Too et al., 1987). Pure hPGF with MW of 18 kD and an isoelectric point of 7.5, is homologous to basic FGF at the amino-terminus (Rowe et al., 1986b).

4.1 The Effect of hCGF on the Growth of Mesodermal Cells in vitro

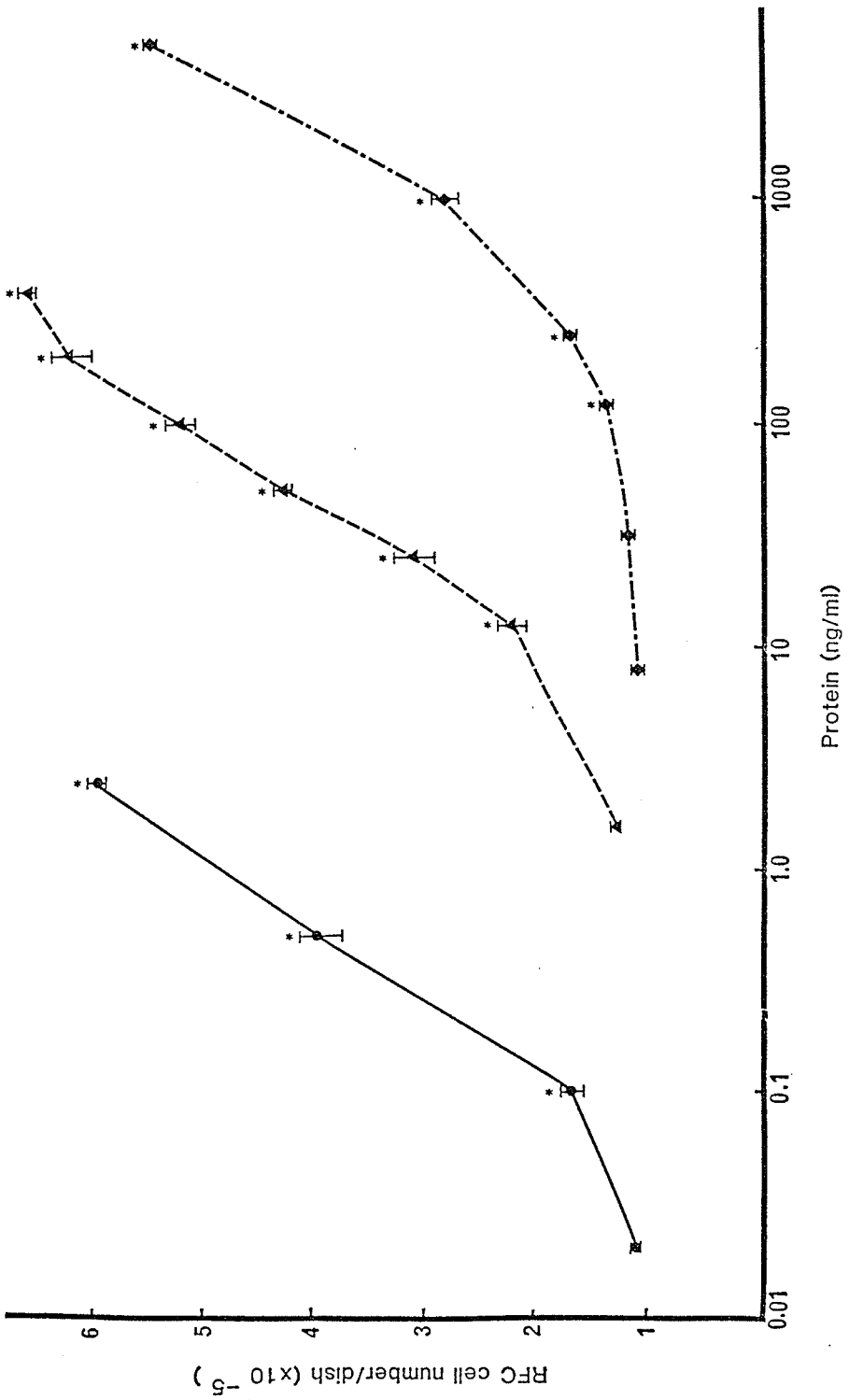
4.1.1 Rabbit Fetal Chondrocytes

Dose-response curves for the three hCGF preparations are shown in Figure 2. As reported previously, application of CF-hCGF to heparin-Sepharose resulted in an approximately 100-fold purification of the mitogenic activity (Too et. al., 1987). The level of stimulation achieved with hPGF is consistent with that observed by Rowe et al. (1986), with a significant increase in cell number (1.6-fold) occurring in the presence of only 0.1 ng/ml of mitogen (Figure 2). The human PGF is ~100-fold more potent than hS-hCGF in stimulating RFC prolifera-

FIGURE 2. The effect of several preparations of hCGF at various stages of purification on the proliferation of RFCs.

CF-hCGF (◆—·—·—◆); hS-hCGF (▲— — —▲); and hPGF (●————●).

The data represent the mean values of triplicate or duplicate (CF-hCGF) dishes (SD < 4% of the mean). *P<0.01 for the difference in cell number from control (100 ul of Ham's F10/0.1% BSA).


RFC cell number/dish (x10 $^{-5}$)
1
2
3
4
5
6
0.01
0.1
1.0
10
100
1000
Protein (ng/ml)

tion. Addition of 0.5 ng/ml of hPGF caused a significant effect on thymidine uptake by the chondrocytes (Figure 3). Compared to the CF-hCGF material, hS-hCGF and hPGF were 25- and 500-fold more potent stimulators of DNA synthesis.

The effect of pituitary FGF, EGF, TGF-alpha and IGF-II on the growth rate and the initiation of DNA synthesis in RFCs was compared to that of hS-hCGF (Figure 4 A,B). Potent mitogenic activity was demonstrated upon the addition of either human pituitary-derived hS-hCGF or bovine pituitary-derived FGF to the bioassays, with significant ($P<0.01$) stimulation occurring at low concentrations (0.5-5 ng/ml) of either mitogen. Although EGF and TGF-alpha had no effect of chondrocyte proliferation, stimulation of DNA synthesis was observed in the presence of these growth factors. A similar stimulation of [3H]-thymidine incorporation (~600%) was observed with EGF and TGF-alpha at all doses tested. Addition of IGF-II also significantly ($P<0.01$) stimulated DNA synthesis at concentrations above 100 ng/ml (the effect of IGF-II on RFC cell number was not examined due to insufficient material).

Addition of hPGF to RFC cultures caused dramatic morphological changes (Figure 5 A,B). In the absence of mitogen, the cells were large and spread apart in the dish. The hPGF treated cells were smaller with a rounded or fusiform shape. The round cells were still undergoing

FIGURE 3. The effect of several preparations of hCGF at various stages of purification on the incorporation of [3H]-thymidine into RFCs. CF-hCGF (●—·—·—●); hS-hCGF (▲— — —▲); and hPGF (◆————◆). The data represent the mean of triplicate determinations (SD < 7% of the mean). *P<0.01 for the difference from control (100 ul of Ham's F10/0.1% BSA).

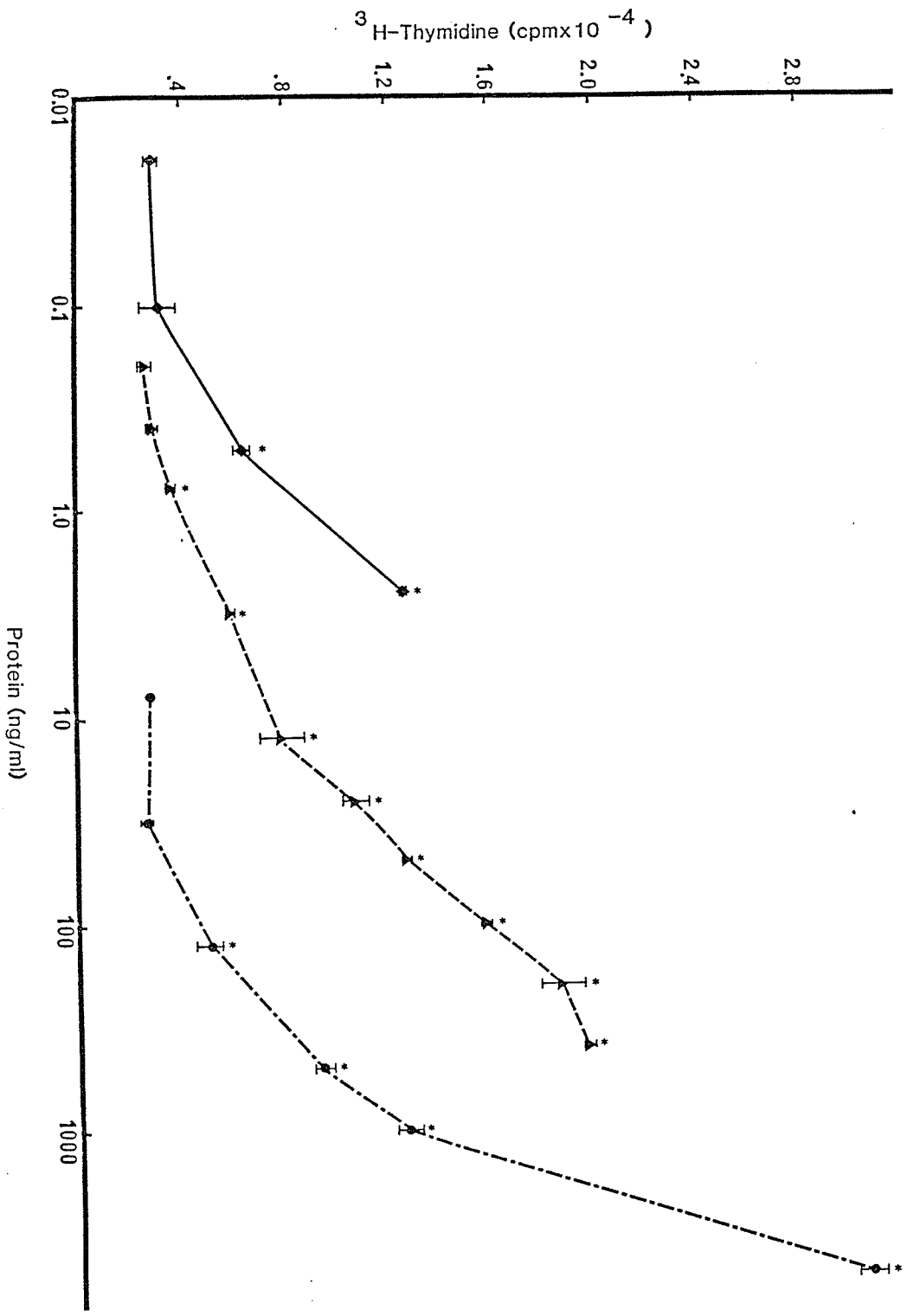
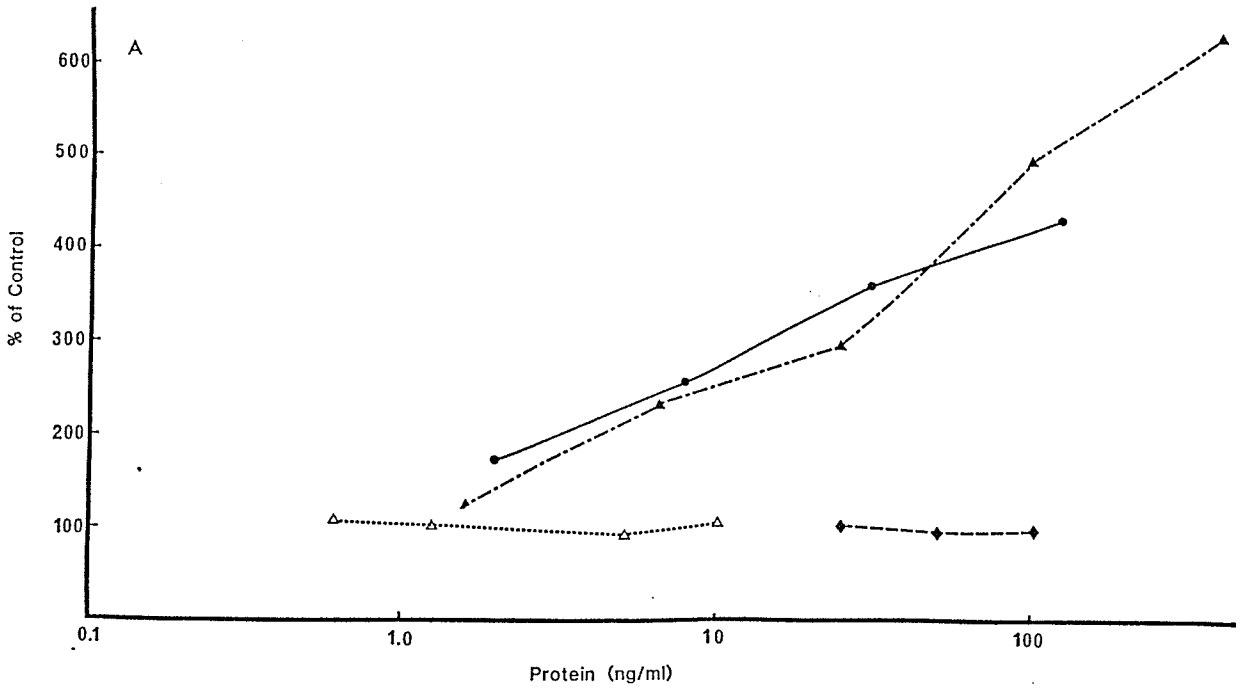

^{3}H-Thymidine (cpmx10^{-4})
.4
.8
1.2
1.6
2.0
2.4
2.8
0.01
0.1
1.0
10
100
1000
Protein (ng/ml)

FIGURE 4. The effect of growth factor addition on the growth of RFCs.

A. Cell Proliferation.

B. [3H]-Thymidine Incorporation.

hS-hCGF (▲—·—·—▲); FGF (●————●); EGF (△·····△); TGF-alpha (◆– – – –◆) and IGF-II (◇————◇). Points are the means of triplicate determinations (SD < 10% of the mean). *P<0.01 for the difference from control (A. RFCs maintained in Ham's F-10/10% FBS; B. RFCs maintained in serum-free Ham's F-10/0.1% BSA).


A
600
500
400
300
200
100
% of Control
0.1
1.0
10
100
Protein (ng/ml)

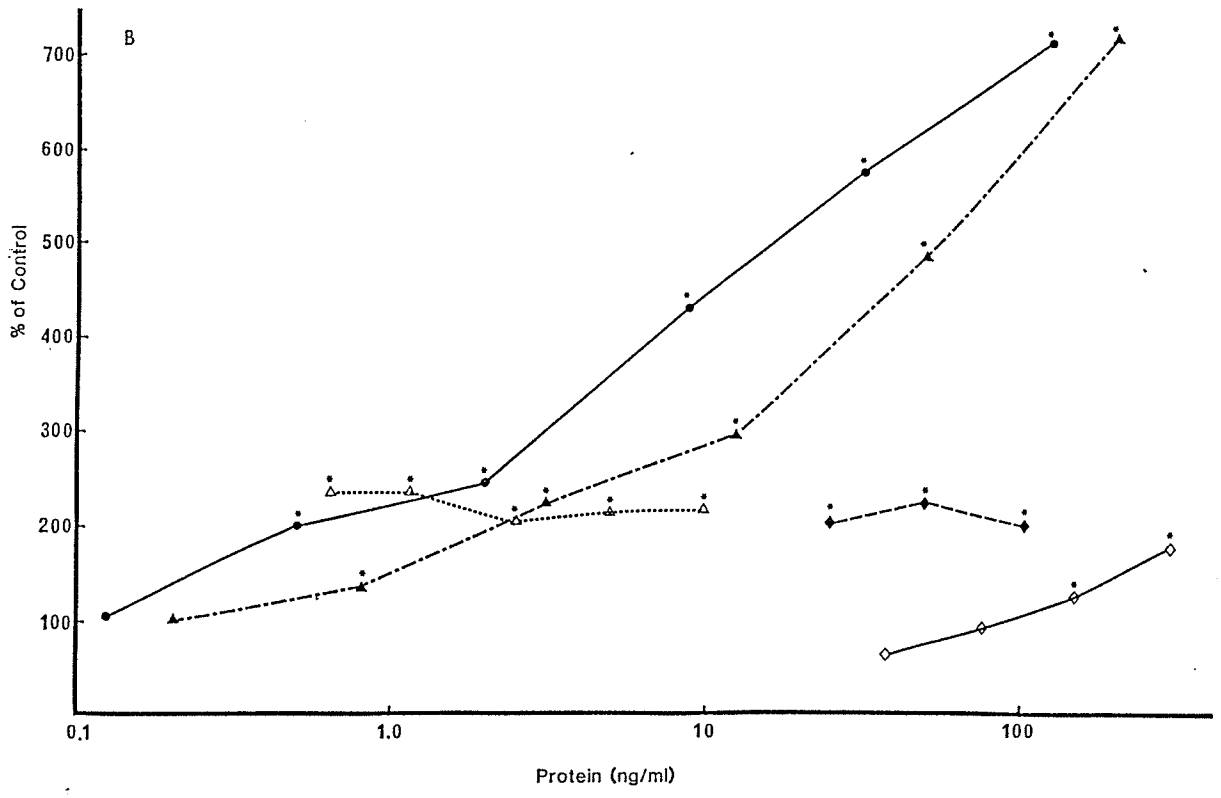
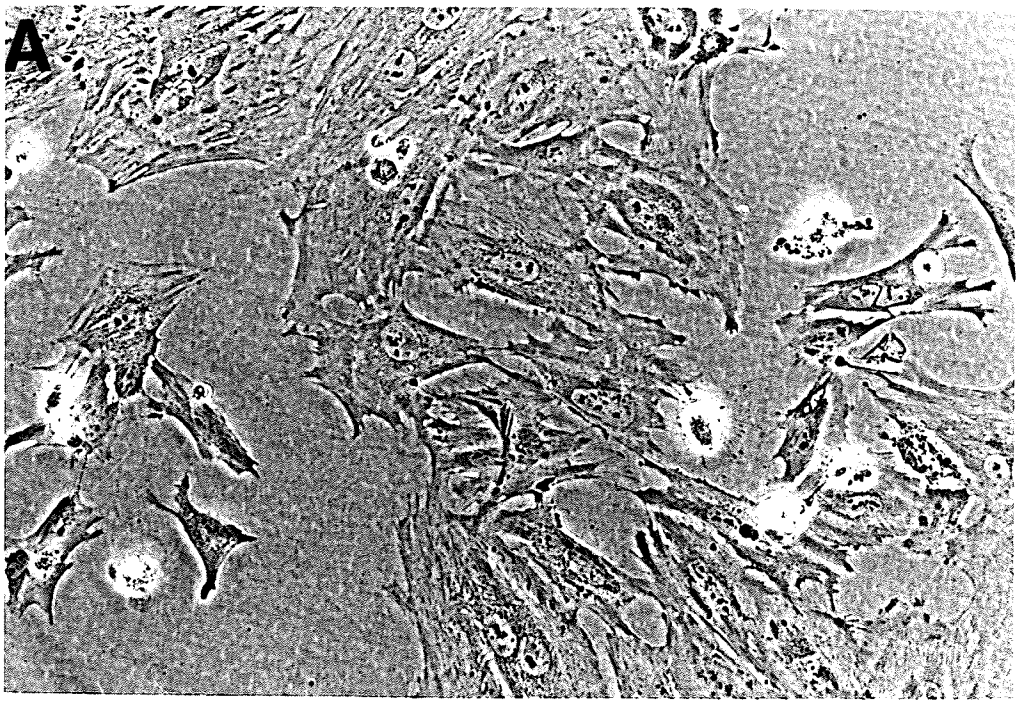

B
700
600
500
400
300
200
100
% of Control
0.1
1.0
10
100
Protein (ng/ml)

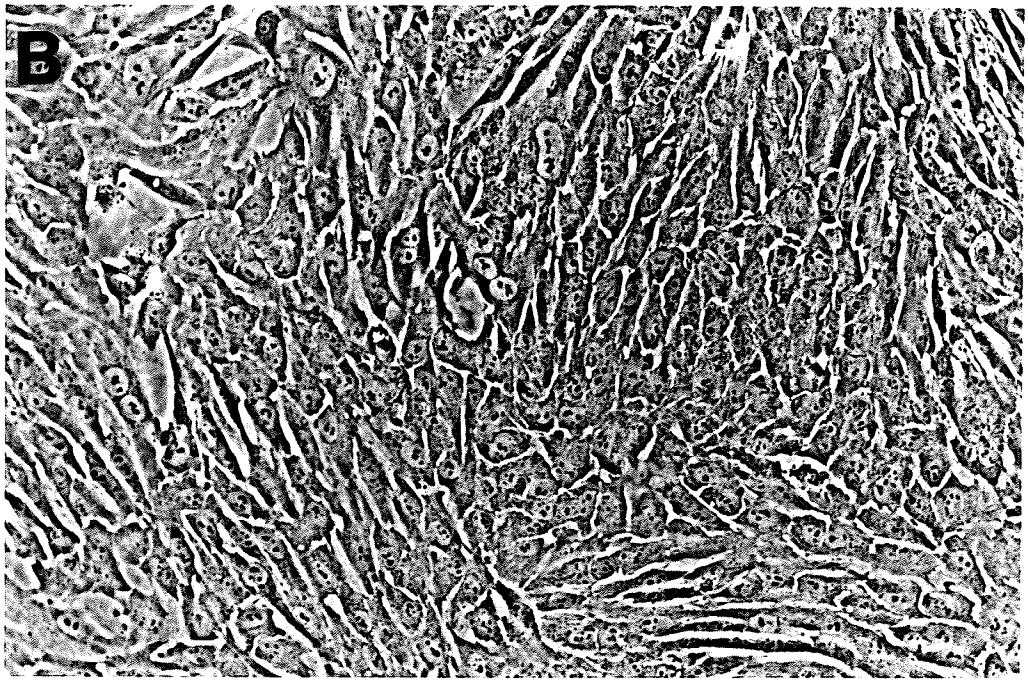
FIGURE 5. The morphology of RFCs in the presence and absence of hPGF.

A. Control RFC cultures.

B. RFCs treated with 2.5 ng/ml hPGF.

Pictures were taken on day 5 of the assay. (Magnification x 20).


A


B

cell division and only loosely attached to the flask whereas the cells with a fusiform shape had attached to the surface of the dish. Binucleate nuclei were present in both groups. Similar morphological differences were observed in the presence of hS-hCGF, CF-hCGF and bovine pituitary FGF.

4.1.2 Bovine Corneal Endothelial Cells (BCE)

To study the mitogenic activity of hCGF on the rate of BCE cell proliferation, it was necessary to reduce the serum level since the cells grew very rapidly in the presence of 15% or 10% FBS (Figure 6). Therefore, to observe mitogenic activity, the cells were grown in DMEM containing 1% FBS. The cells were plated at 1×10^4 cells/dish. The final control cell number was 2.9×10^4 cells/dish, indicating that the cells were able to grow at this serum concentration and were consequently responding to the added mitogen, not simply to added nutrients.

The three preparations of hCGF were tested for their mitogenic effects on the BCE cells (Figures 7 and 8). Maximal stimulation of cell proliferation was obtained with 1 ug/ml of CF-hCGF, 100 ng/ml of hS-hCGF and ~1 ng/ml of hPGF (Figure 7). As little as 0.1 ng/ml of hPGF caused significant (1.3-fold) stimulation of cell growth; half-maximal stimulation was elicited with ~0.15 ng/ml. Dose-response analysis of thymidine uptake (Fig-

FIGURE 6. The effect of serum concentration on the growth rate of BCE cells. Cells were initially plated in DMEM supplemented with 15% FBS. Following overnight cell attachment, the cultures were washed with serum-free medium and incubated in DMEM containing 0.1% (◈——◈); 1% (▲·····▲); 10% (△-—-—△); or 15% (●———●) FBS. Cell number was determined daily for triplicate cultures (SD < 5% of the mean).

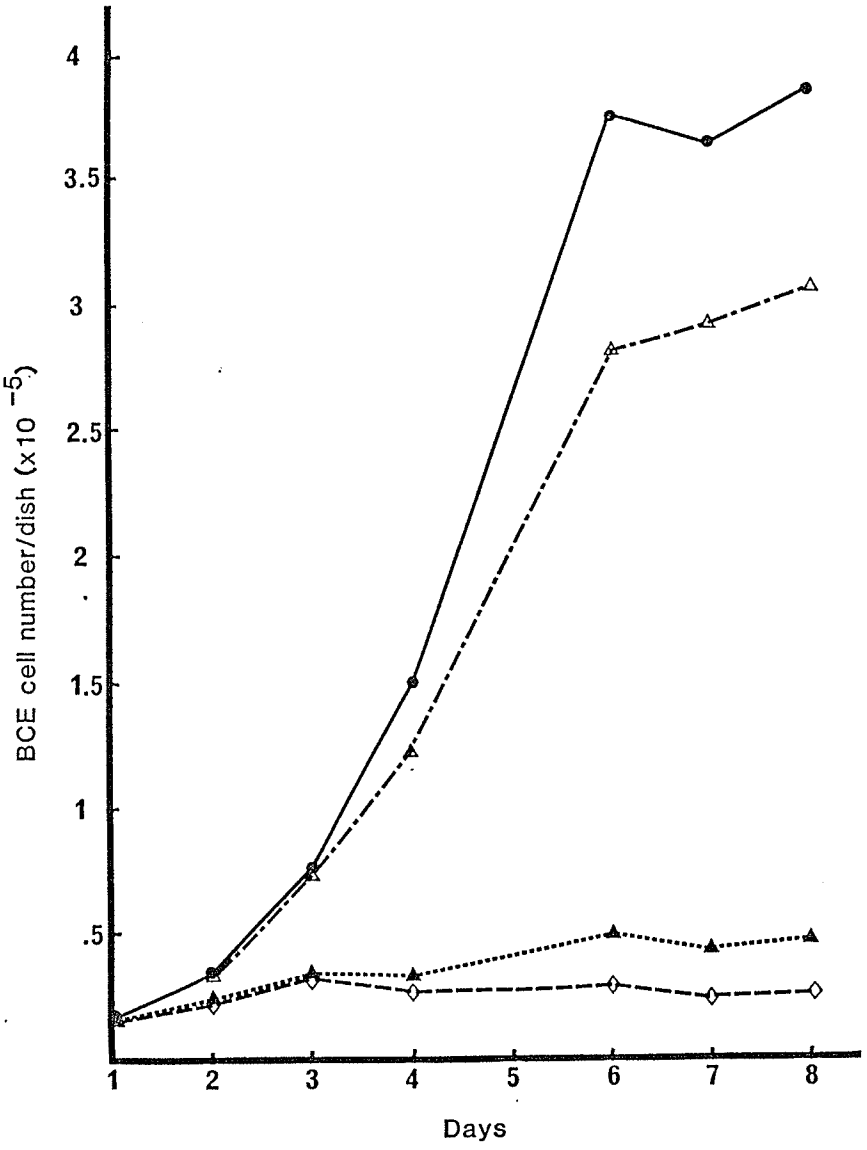

4
3.5
3
2.5
2
1.5
1
.5
BCE cell number/dish (x10 $^{-5}$)
1
2
3
4
5
6
7
8
Days

FIGURE 7. The effect of several preparations of hCGF at various stages of purification of hCGF on the proliferation of BCE cells. CF-hCGF (▲——▲); hS-hCGF (△——△); and hPGF (●—·—·—●). The assay was completed in DMEM/1% FBS. The data represent the mean values of triplicate cultures (SD < 4% of the mean). **P<0.05, *P<0.01 for the difference in cell number from control (100 ul of DMEM/0.1% BSA).

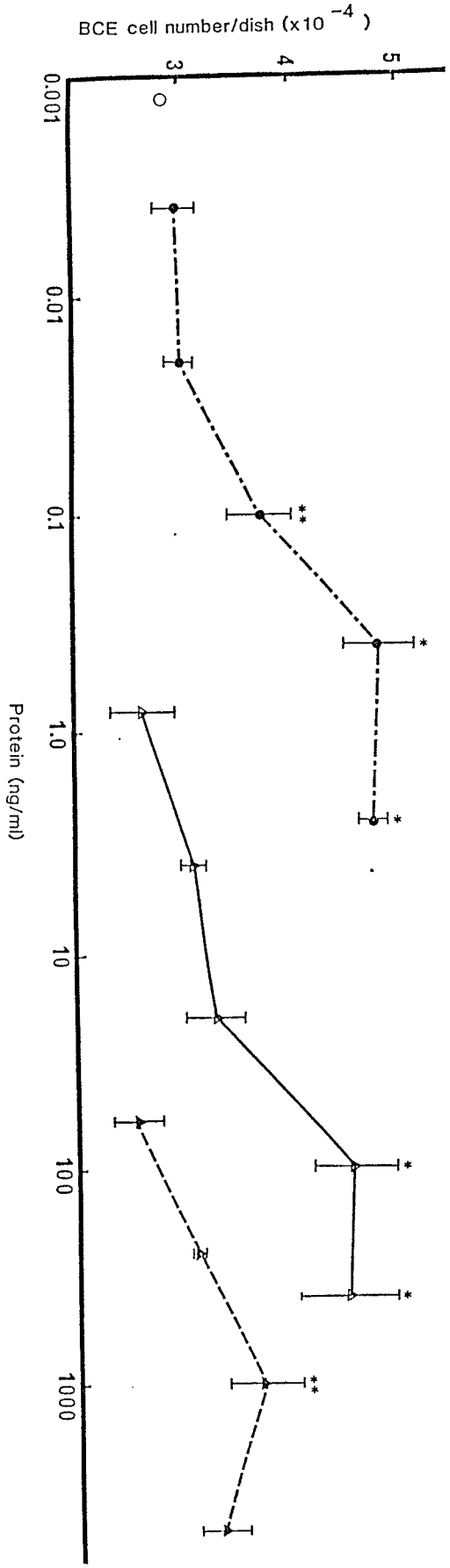
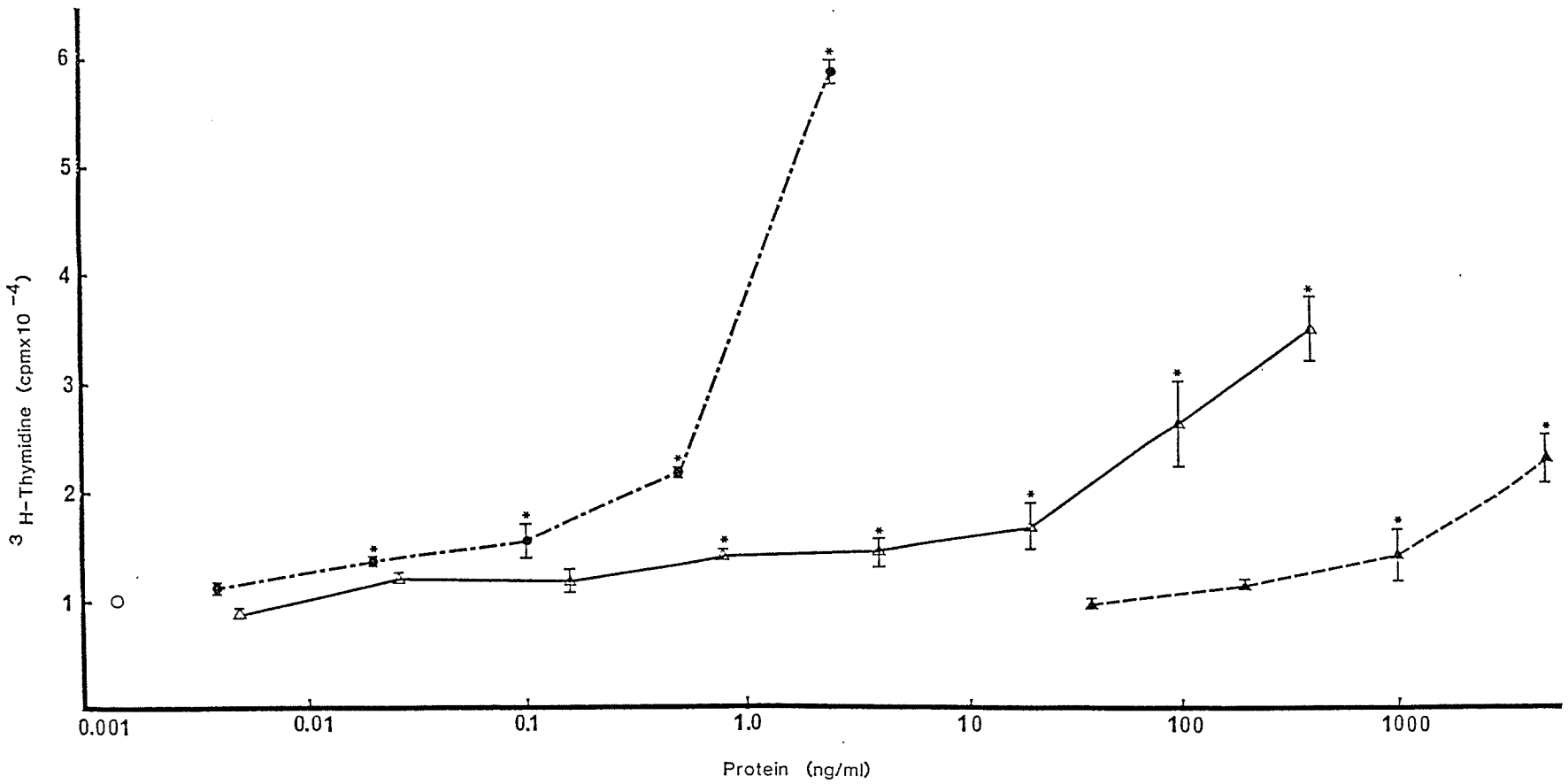

BCE cell number/dish (x10 $^{-4}$)
3
4
5
0.001
0.01
0.1
1.0
10
100
1000
Protein (ng/ml)
**
*
*
*
*
**

FIGURE 8. The effect of several preparations of hCGF at various stages of purification on the incorporation of [3H]-thymidine into BCE cells. CF-hCGF (▲– – – –▲); hS-hCGF (△————△); and hPGF (●–·–·–●). The data represent the mean of triplicate determinations (SD < 9% of the mean). *P<0.01 for the difference from control (100 ul of DMEM/0.1% BSA).


6
5
4
3
2
1
0
^{3}H-Thymidine (cpmx10^{-4})
0.001
0.01
0.1
1.0
10
100
1000
Protein (ng/ml)

ure 8) demonstrated an ~100-fold increase in mitogenic activity for the hS-hCGF preparation as compared to CF-hCGF; hPGF was similarly 100-fold more active than hS-hCGF.

The relative mitogenicity of hS-hCGF, FGF and EGF were also compared (Figure 9 A,B). EGF was a potent mitogen for the corneal endothelial cells, eliciting a significant response at concentrations as low as 0.01 and 1 ng/ml (thymidine uptake and cell growth assays, respectively). Although FGF was slightly more mitogenic than hS-hCGF, both preparations were less mitogenic than EGF in both bioassays.

The effect of hCGF on the doubling time of chondrocytes and endothelial cells was determined (Table 6). The addition of partially purified hS-hCGF or pure hPGF significantly reduced the doubling time of both cell types. In the case of hS-hCGF, two protein concentrations have been given since the BCE cells were maximally stimulated with 100 ng/ml of growth factor, whereas this concentration was submaximal for the chondrocytes: 400 ng/ml, the highest dose of hS-hCGF tested, achieved slightly less than maximal RFC growth (Figure 2). The slight increase in the doubling time of the RFCs observed in the presence of hPGF compared to hS-hCGF (400 ng/ml), although not significant, may reflect the greater fold-stimulation achieved with this concentration of hS-hCGF.

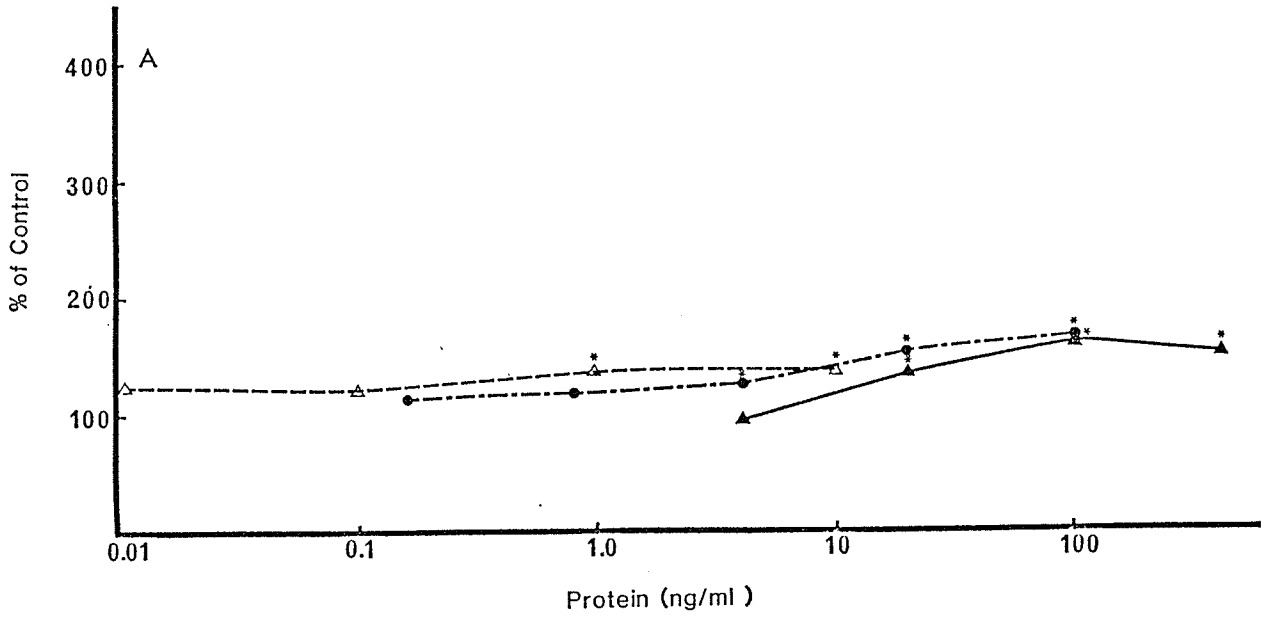
The pure hPGF caused a more marked decrease in

FIGURE 9. The effect of growth factor addition on BCE cell growth.

A. Cell proliferation.

B. [3H]-Thymidine Incorporation.

hS-hCGF (▲——▲); FGF (●-—-—●); and EGF (△— — —△). Points are the means of triplicate determinations (SD < 8% of the mean). *P<0.01 for the difference from control.


A
400
300
200
100
% of Control
0.01
0.1
1.0
10
100
Protein (ng/ml)

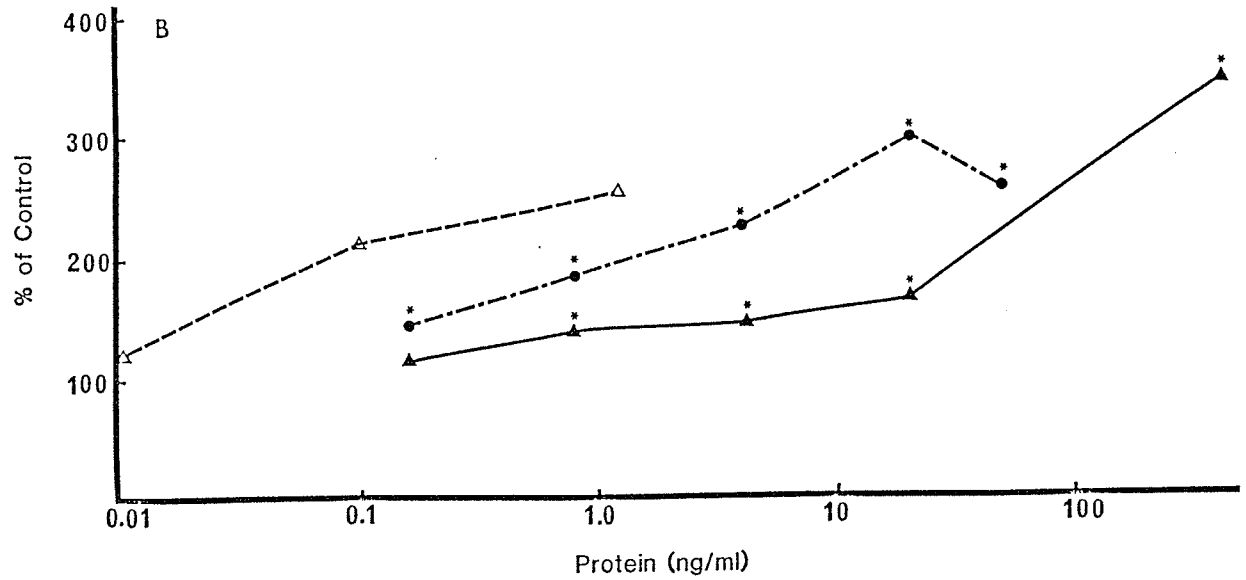

B
400
300
200
100
% of Control
0.01
0.1
1.0
10
100
Protein (ng/ml)

TABLE 6: Comparison of the effect of hCGF on chondrocyte and endothelial cell proliferation and doubling time.

TREATMENT	FOLD STIMULATION		DOUBLING TIME (h)	
	RFC	BCE	RFC	BCE
CONTROL**	0	0	46.2	139.2
SERUM	0	2.8	47.3	57.8*
hS-hCGF				
100 ng/ml	4.9	1.5	26.1*	90.6*
400 ng/ml	6.2	-	24.6*	-
hPGF				
2.5 ng/ml	5.7	1.7	25.2*	82.9*

the doubling time of BCE cells than the partially purified hS-hCGF and produced a somewhat greater fold stimulation of these cells (Table 6). It is interesting to note that although the endothelial cells are more sensitive to hCGF than the chondrocytes, hCGF addition causes a greater fold stimulation of the RFCs. The effects of serum at the concentrations normally used for culturing these cells (i.e. 10% FBS for the RFCs; 15% FBS for the BCE cells), demonstrates the need to reduce the serum level to study the response of the BCE cells to mitogenic stimulation. Since the BCE cells grew more rapidly in the presence of 15% FBS than upon addition of hCGF, this mitogenic activity would have been masked at high serum levels. In Table 6, the control and serum values given for the RFC cells were both determined using Ham's F10/ 10% FBS since the RFC proliferation assay is routinely conducted in the presence of 10% serum. The 1 h difference in the doubling time is not significant and probably reflects intra-assay variation in cell counts.

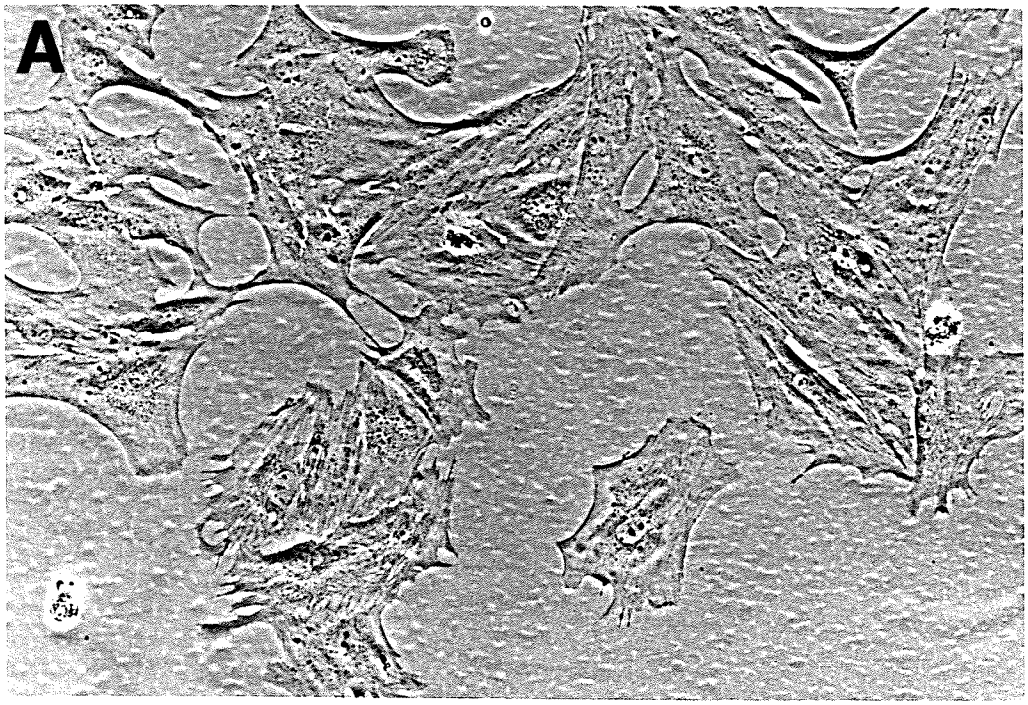
Addition of hPGF to BCE cells also changed their morphology (Figure 10 A,B). BCE cells maintained in the absence of hPGF appeared larger and spread further apart than those maintained in the presence of hPGF. At subconfluency, hPGF treated cultures grew in monolayers. Close examination of the hPGF stimulated cells suggested the appearance of some undergrowth, a characteristic of FGF addition that has

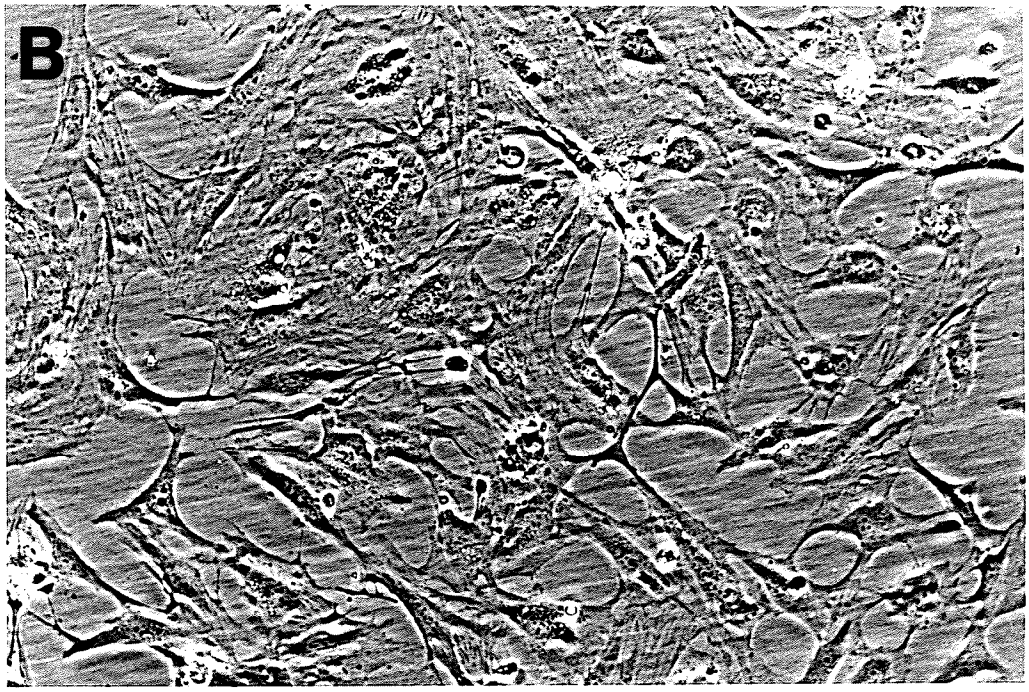
FIGURE 10. Morphology of BCE cells in the presence and absence of hCGF.

A. Control BCE cultures.

B. BCEs treated with 2.5 ng/ml hPGF.

Pictures were taken on day 5. (Magnification x 20).


A


B

been reported for aortic endothelial cells (Gospodarowicz et al., 1978).

4.1.3 Human Foreskin Fibroblasts (MCH6)

The MCH6 fibroblasts used in these studies are routinely cultured in either Human McCoy's medium or DMEM (both supplemented with 10% FBS and antibiotics) (personal communication, F.Pereira, D. Wrogeman's laboratory). Since DMEM was being used to culture the Balb/c3T3 cells and the T-47D cells, the MCH6 cells were cultured in this medium as well. To optimize assay conditions, the growth rates of the MCH6 cells at different plating densities (Figure 11) and in media supplemented with 10, 1, or 0.1% FBS and in serum-free medium (Figure 12) were measured. A plat-ing density of 1×10^4 cells/dish was chosen since at this density the cells grew well but did not achieve confluency over 7 days of culture. When grown in 10% FBS, the cells proliferated quite rapidly, whereas in 0.1% FBS and 1% FBS and in serum-free media the cells grew very little. In a subsequent assay, addition of hS-hCGF (100ng/ml) to cultures supplemented with 2.5% FBS resulted in the greatest fold stimulation of cell growth (data not shown) and this serum concentration was used for the dose-response curves for the hCGF preparations.

Addition of CF-hCGF, hS-hCGF and hPGF to the MCH6 fibroblasts caused a small (1.3-, 1.4- and 1.4-

FIGURE 11. Growth rate of MCH6 fibroblasts plated at different cell densities. $1x10^4$ (▲·····▲); $2x10^4$ (▲—·—·—▲); and $4x10^4$ (●————●) cells/dish. Cells were grown in DMEM/10% FBS and the cell number of triplicate dishes was determined daily (SD < 10% of the mean).

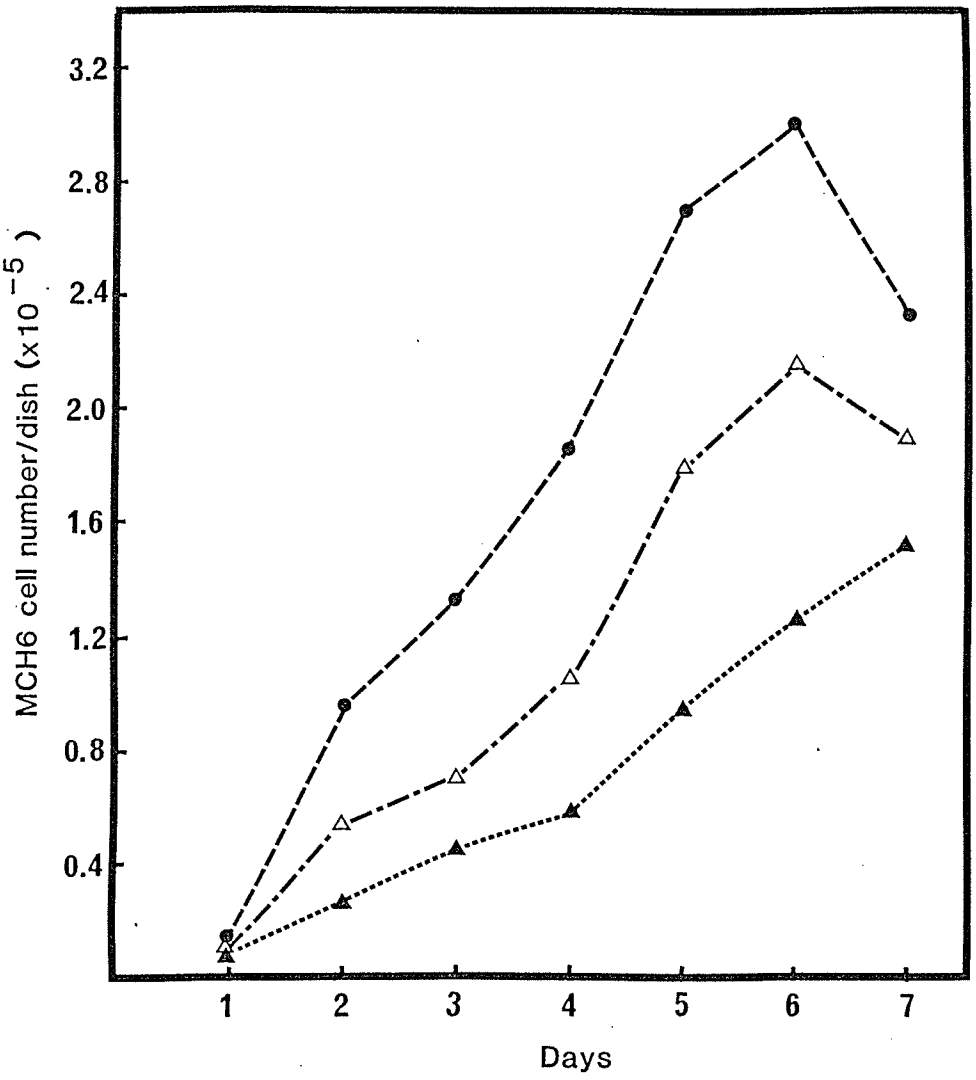
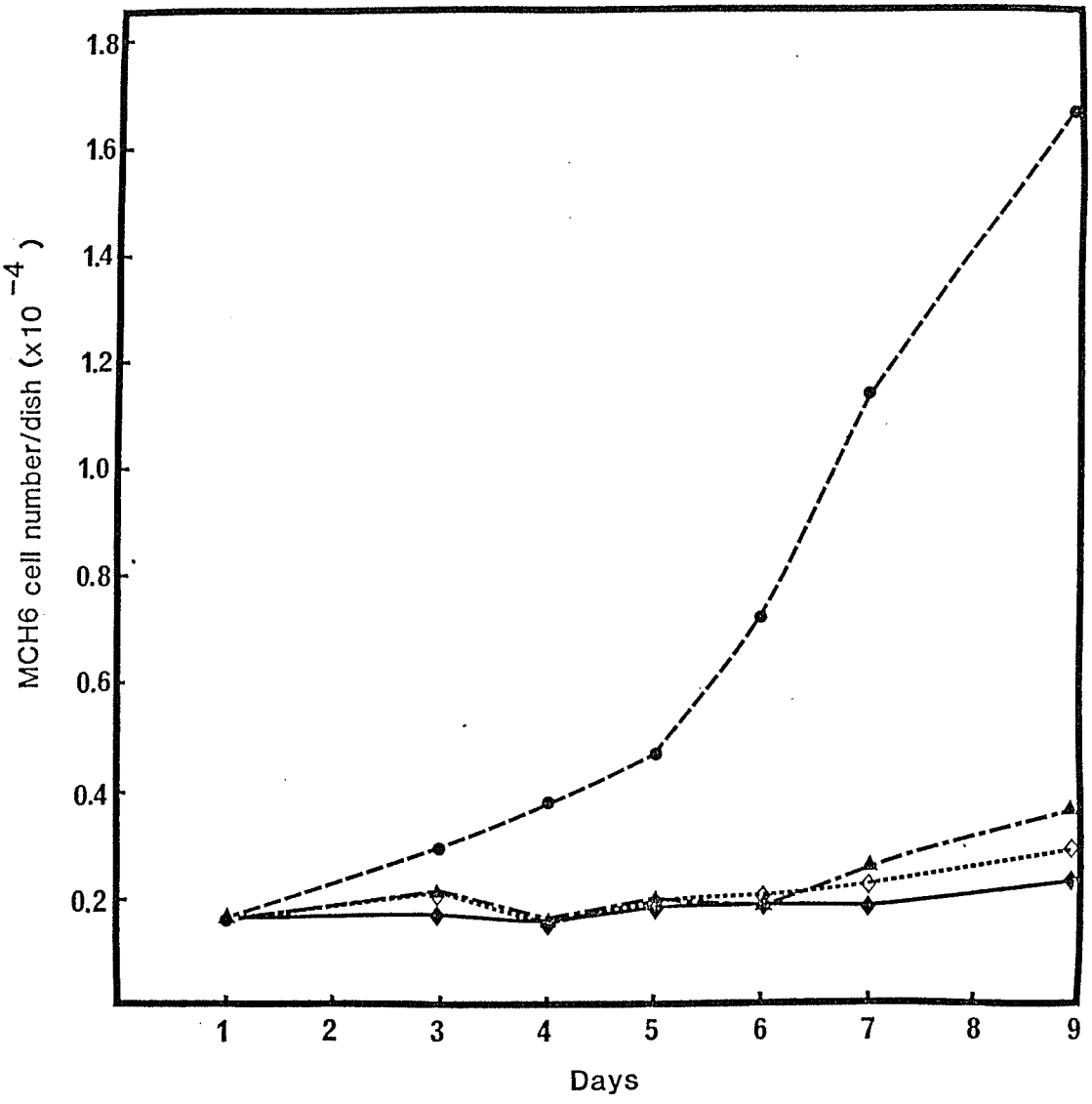

MCH6 cell number/dish (x10 $^{-5}$)
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
1
2
3
4
5
6
7
Days

FIGURE 12. Growth rate of MCH6 cells in DMEM supplemented with various serum concentrations. Cells were plated at 1 x 10^4 cells/dish in DMEM/10% FBS. Following overnight attachment, the cultures were rinsed with serum-free medium and incubated in medium containing 10% FBS (●———●); 1% FBS (▲-·-·-▲); 0.1% FBS (◇·····◇); or 0.1% BSA (◆——◆). The cell number of triplicate cultures was determined daily (SD < 10% of the mean).


MCH6 cell number/dish (x10 $^{-4}$)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
1
2
3
4
5
6
7
8
9
Days

fold, respectively) increase in cell number (Figure 13). hCGF was effective at low doses, with 0.1 ng/ml of hPGF, 10 ng/ml of hS-hCGF, and 60 ng/ml of CF-hCGF capable of stimulating fibroblast growth. The addition of 10 ng/ml of bovine FGF to this assay also produced a 1.3-fold stimulation (data not shown).

4.1.4 Balb/c3T3 Cells

The ability of several preparations of hCGF to stimulate Balb/c3T3 fibroblast proliferation was examined. Preliminary growth curves to establish efficient plating densities (Figure 14) and serum concentrations (Figure 15) were completed. A plating density of 2×10^4 cells/35 mm dish was utilized for subsequent assays, since cells plated at higher densities grew very rapidly while the growth rate of lower density cultures was not consistent (data not shown). Maintenance of the cells in media supplemented with 5% or 10% FBS resulted in rapid cell proliferation whereas cells maintained in 0.1% FBS or in serum-free medium did not divide. Cells grown in 1% FBS or 2.5% FBS underwent one or several cycles of cell division, respectively. To assay hCGF mitogenic activity in Balb/c3T3 cells, medium supplemented with 1% FBS was used.

In the first assay, all growth factor preparations (i.e. hPGF, hS-hCGF, and CF-hCGF) caused inhibition of cell growth at all the concentrations tested

FIGURE 13. The effect of several preparations of hCGF at various stages of purification on the proliferation of MCH6 fibroblasts. CF-hCGF (▲———▲); hS-hCGF (△—·—△); and hPGF (●·····●). The assay was completed in DMEM/2.5% FBS. The data represent the mean values of triplicate cultures (SD < 6% of the mean). **P<0.05, *P<0.01 for the difference from control (100 ul DMEM/0.1% BSA).

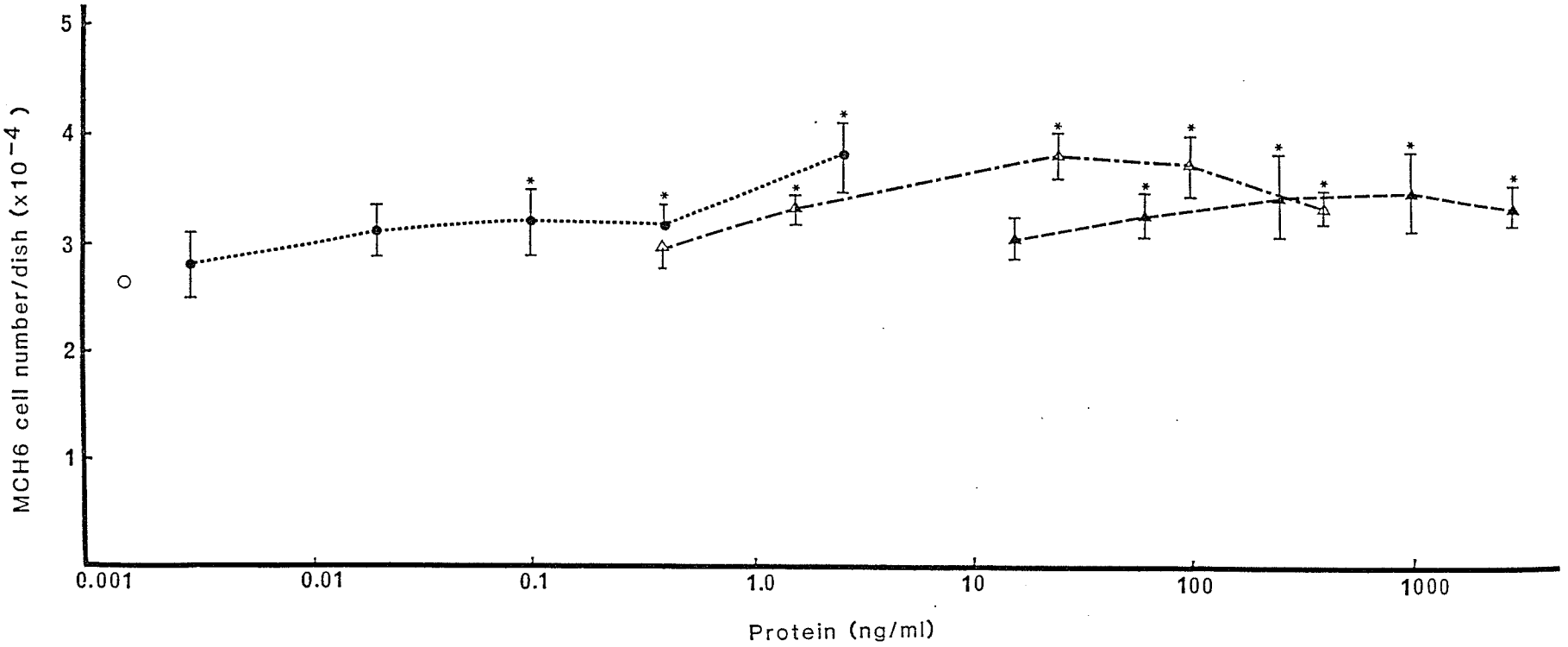

MCH6 cell number/dish (x10⁻⁴)
5
4
3
2
1
0.001
0.01
0.1
1.0
10
100
1000
Protein (ng/ml)

FIGURE 14. Growth rate of Balb/c3T3 cells plated at different initial cell densities. $1x10^4$ (▲.....▲); $2x10^4$ (▲—·—·—▲); and $4x10^4$ (●————●) cells/dish. Cells were grown in DMEM/10% FBS and the cell number of triplicate dishes was determined daily (SD < 10% of the mean).

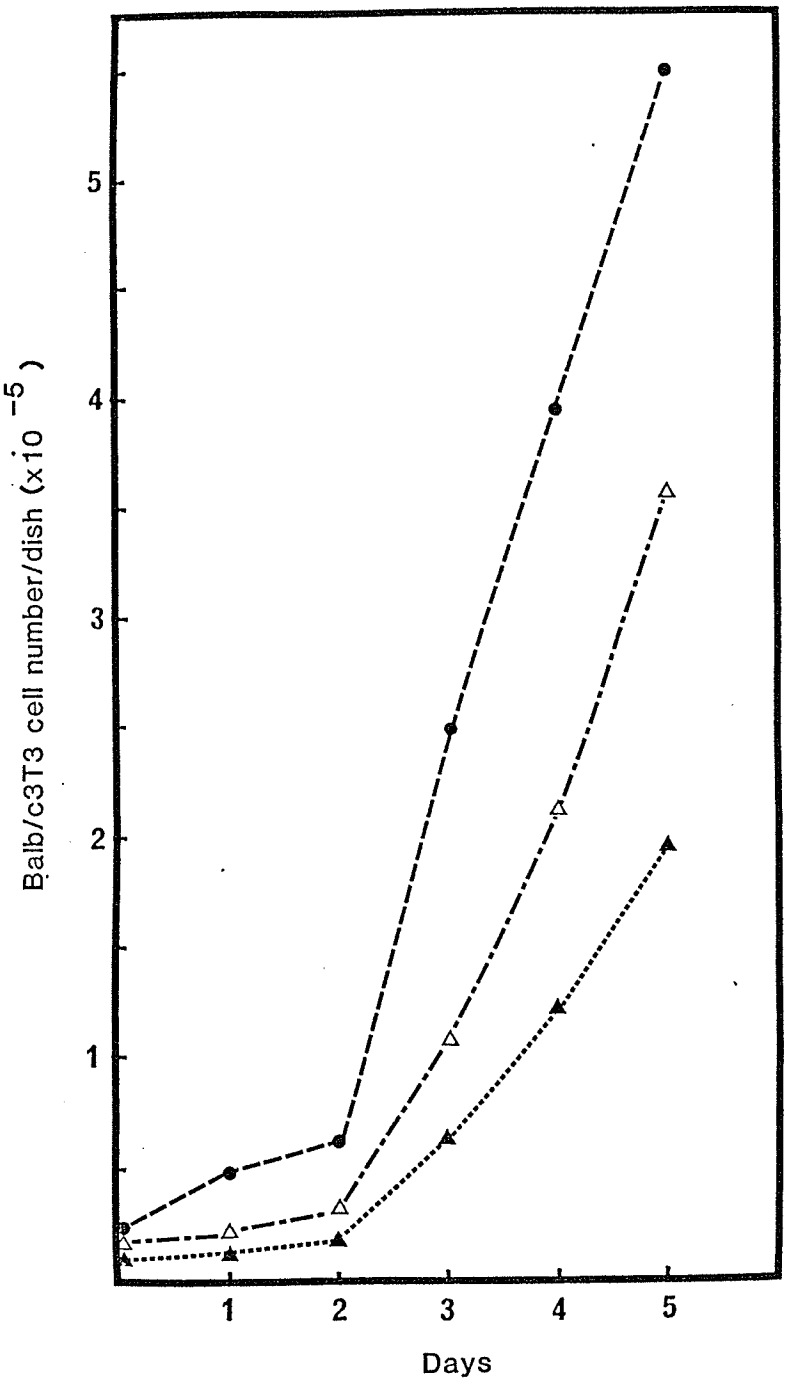
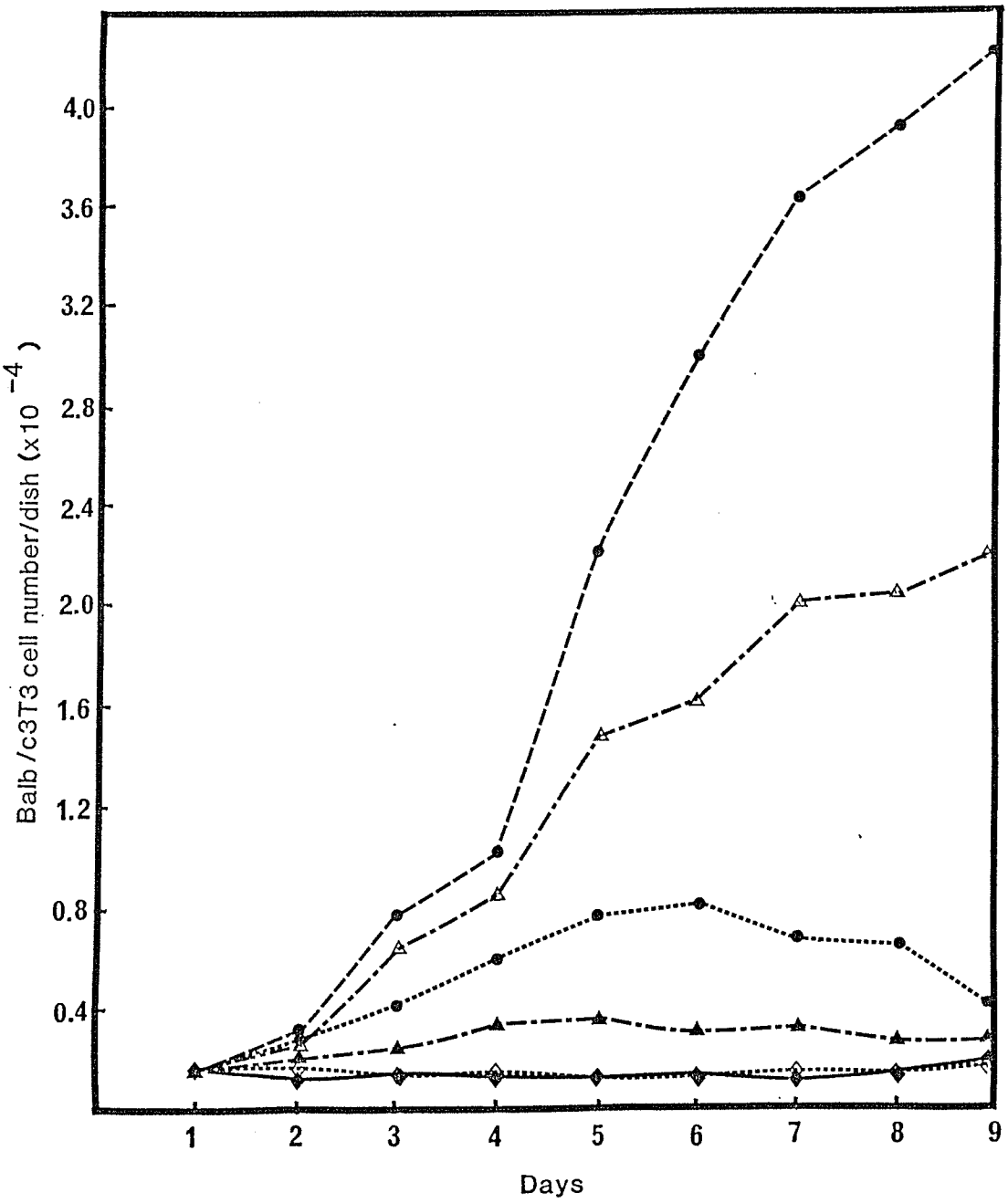

Balb/c3T3 cell number/dish (x10 $^{-5}$)
5
4
3
2
1
1
2
3
4
5
Days

FIGURE 15. Growth rate of Balb/c3T3 cells in DMEM supplemented with various serum concentrations. Cells were plated at 2 x 10^4 cells/dish in DMEM/10% FBS. Following overnight attachment, the cultures were rinsed with serum-free medium and incubated in medium containing 10% FBS (●——●); 5% FBS (△-·-△); 2.5% FBS (●·····●); 1% FBS (▲-·-▲); 0.1% FBS (◇·····◇); or 0.1% BSA (◆——◆). The cell number of triplicate dishes was determined daily (SD < 10% of the mean).


4.0
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
Balb/c3T3 cell number/dish (x10 $^{-4}$)
1
2
3
4
5
6
7
8
9
Days

(Figure 16 A). Microscopic examination suggested a toxic effect of higher protein concentrations; many cells were floating or rounded up and only loosely attached to the culture dish. The concentration range initially tested in the Balb/c3T3 was the same as that tested and shown to be mitogenic in the other cell types tested. Since protein factors are sometimes toxic at higher concentrations, a second series of hCGF dose-response curves with a concentration range of 10 ng/ml to 0.1 fg/ml were completed.

At very low concentrations, (1 fg/ml to 1 pg/ml) a small (1.2) but significant stimulation of Balb/c3T3 growth was observed (Figure 16 B). Above 1 pg/ml, hPGF again inhibited cell growth. However, even the second, lower doses of hS-hCGF and CF-hCGF did not stimulate cell growth.

4.1.5 T-47D Human Breast Cancer Cells

The effects of hS-hCGF and hPGF on the proliferation of T-47D human breast cancer cells is shown in Figure 17. Preliminary results showed the greatest stimulation of T-47D growth with CF-hCGF (1 ug/ml) in the presence of 1% FBS (data not shown), therefore this serum concentration was utilized for the hCGF dose-response assay.

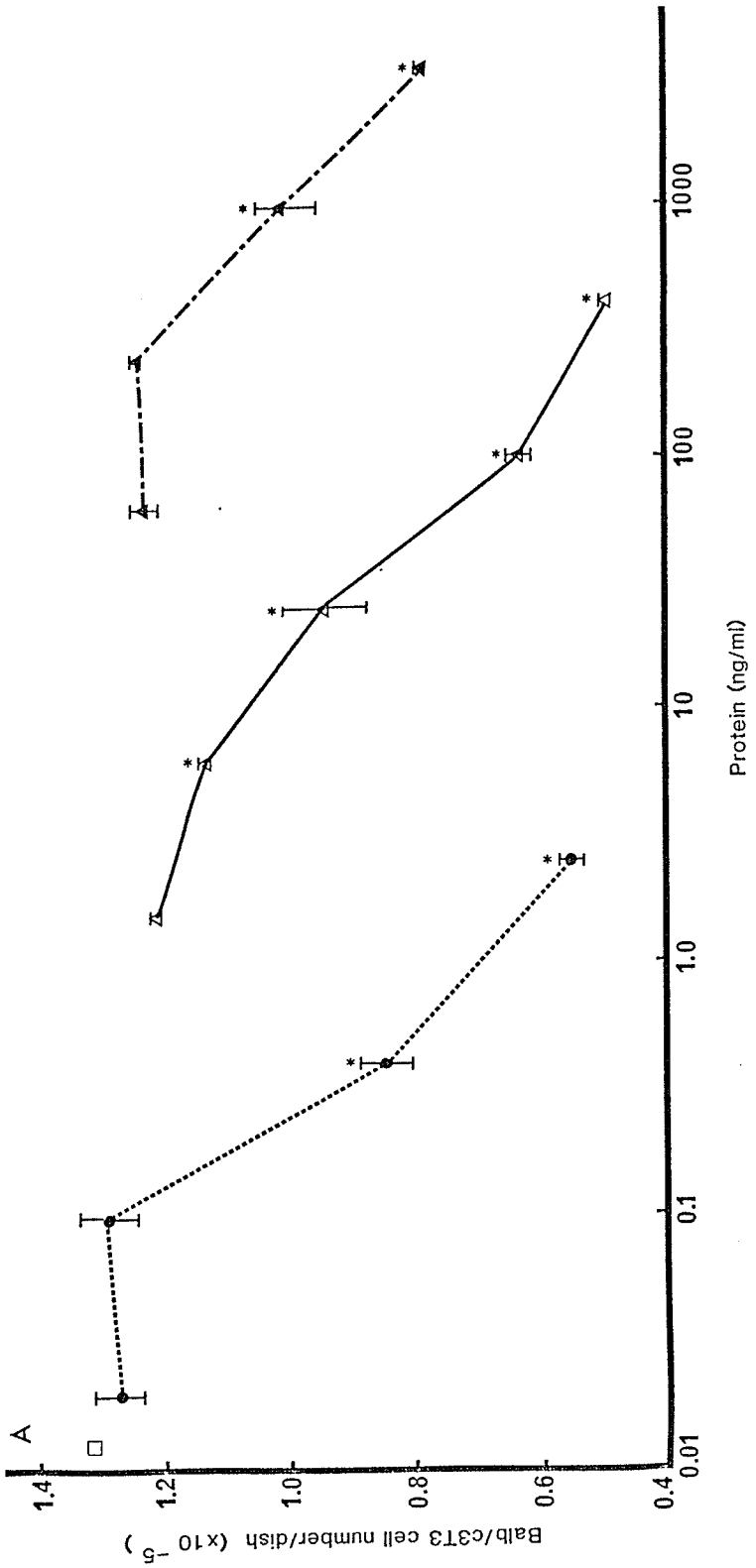
The mitogenicity of hPGF was ~200-fold greater than that observed for hS-hCGF. A significant increase

FIGURE 16. The effect of several preparations of hCGF at various stages of purification on the proliferation of Balb/c3T3 cells.

A. Concentration Range of ng/ml.

B. Concentration Range of pg/ml.

CF-hCGF (▲-·-·-▲); hS-hCGF (△———△); and hPGF (●·····●). Cells were plated in DMEM/10% FBS and incubated overnight. For assay, the media was changed to DMEM/1% FBS. The mean values for triplicate dishes are plotted (SD < 10% of the mean). **P<0.05, *P<0.01 for the difference from control (100 ul of DMEM/0.1% BSA). Cells maintained in DMEM/10% FBS reached a density of (A) 2.2 x 10^5 cells/dish and (B) 3.5 x 10^5 cells/dish.


A
Balb/c3T3 cell number/dish (x10 $^{-5}$)
1.4
1.2
1.0
0.8
0.6
0.4
0.01
0.1
1.0
10
100
1000
Protein (ng/ml)

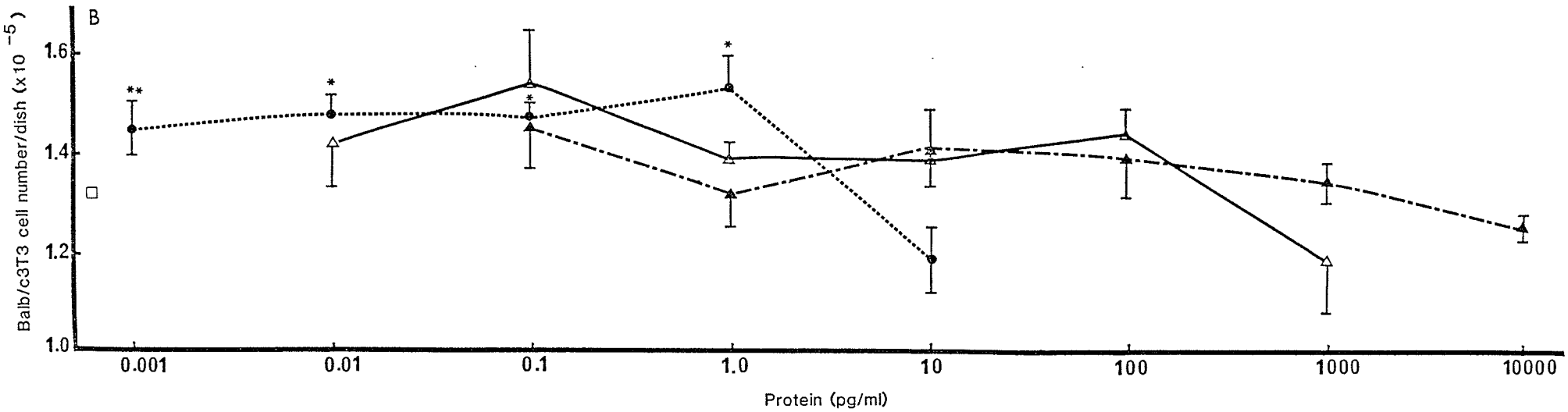
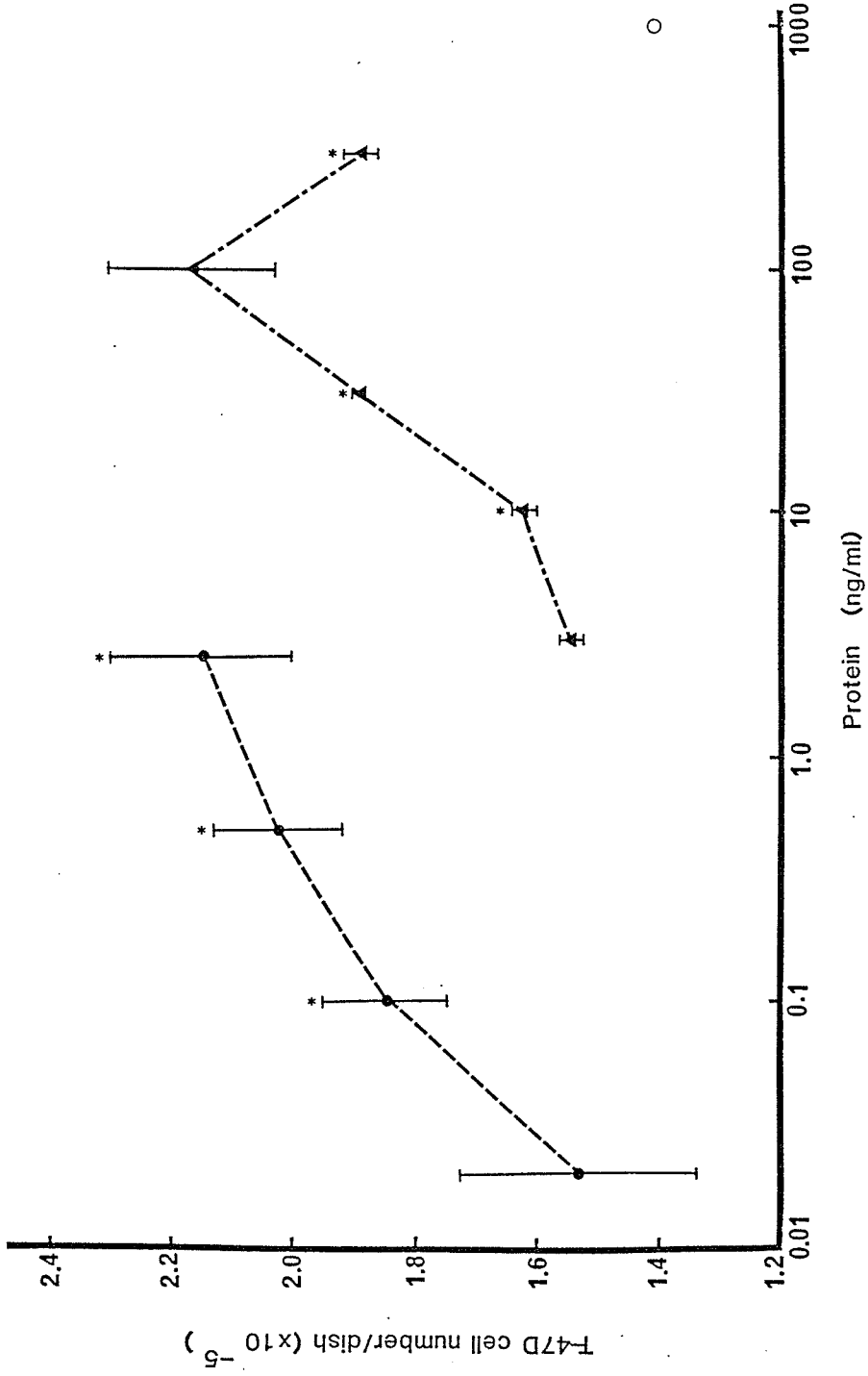

B
Balb/c3T3 cell number/dish (x10 $^{-5}$)
1.6
1.4
1.2
1.0
**
*
*
*
0.001
0.01
0.1
1.0
10
100
1000
10000
Protein (pg/ml)

FIGURE 17. The effect of hS-hCGF and hPGF on the proliferation of T-47D human breast cancer cells. hS-hCGF (▲-·—·—▲); and hPGF (●———●). The assay was completed in DMEM/1% FBS. The data represent the mean values of triplicate (CF-hCGF) or duplicate (hS-hCGF) (SD < 9% of the mean). *P<0.01 for the difference from control (100 ul of DMEM/0.01% BSA added to cells cultured in DMEM/1% FBS).


T-47D cell number/dish (x10 $^{-5}$)
2.4
2.2
2.0
1.8
1.6
1.4
1.2
0.01
0.1
1.0
10
100
1000
Protein (ng/ml)
*
○

in cell growth was observed with 0.1 ng/ml of hPGF. Addition of 2.5 ng/ml of hPGF or 100 ng/ml of hS-hCGF produced a 1.5-fold increase in cell number. In the same assay, addition of 1 ug/ml of CF-hCGF caused a 1.3-fold increase in cell number (data not shown).

As indicated in the BCE results, the increase in T-47D growth in response to hCGF addition was not simply due to a lack of nutrients. Plated at an initial cell density of 1.2×10^4 cells/dish, control dishes obtained a final cell density of 1.4×10^5 cells/dish, demonstrating the presence of sufficient nutrients in the medium.

4.1.6 Rat Lymphoma (Nb_2) Cells

Screening of several relatively impure preparations of hCGF revealed its potent mitogenic effects in the Nb_2 cell assay (Figure 18). As little as 1 ng/ml of CF-hCGF caused significant ($P<0.05$) stimulation of Nb_2 cell growth.

Stimulation of Nb_2 cell proliferation by lactogenic hormones (GH, PRL) has been well documented (Gout et al., 1980; Tanaka et al., 1980). For this reason, the effects of two steps in hCGF purification, a crude G-100 preparation and CF-hCGF were tested in the Nb_2 assay in the presence and absence of monoclonal antibodies to hGH and hPRL (Figure 19 A,B). Antiserum to hGH significantly prevented activity of low

FIGURE 18. Dose-response curves of Nb_2 cells cultured in the presence of several preparations of hCGF at various stages of purification. Samples (50 ul in FH medium) were added 18-24 h following arrest of cell growth and cell number was determined on day 3. G-100 (●——●); HTP (●-·-·-●); DEAE Affigel Blue (●·····●); CF-hCGF (●——●); and hGH (◇——◇). The data represent the mean values of duplicate dishes (SD < 4% of the mean). **P<0.05, *P<0.01 for the difference in cell number from control (50 ul FH medium only).

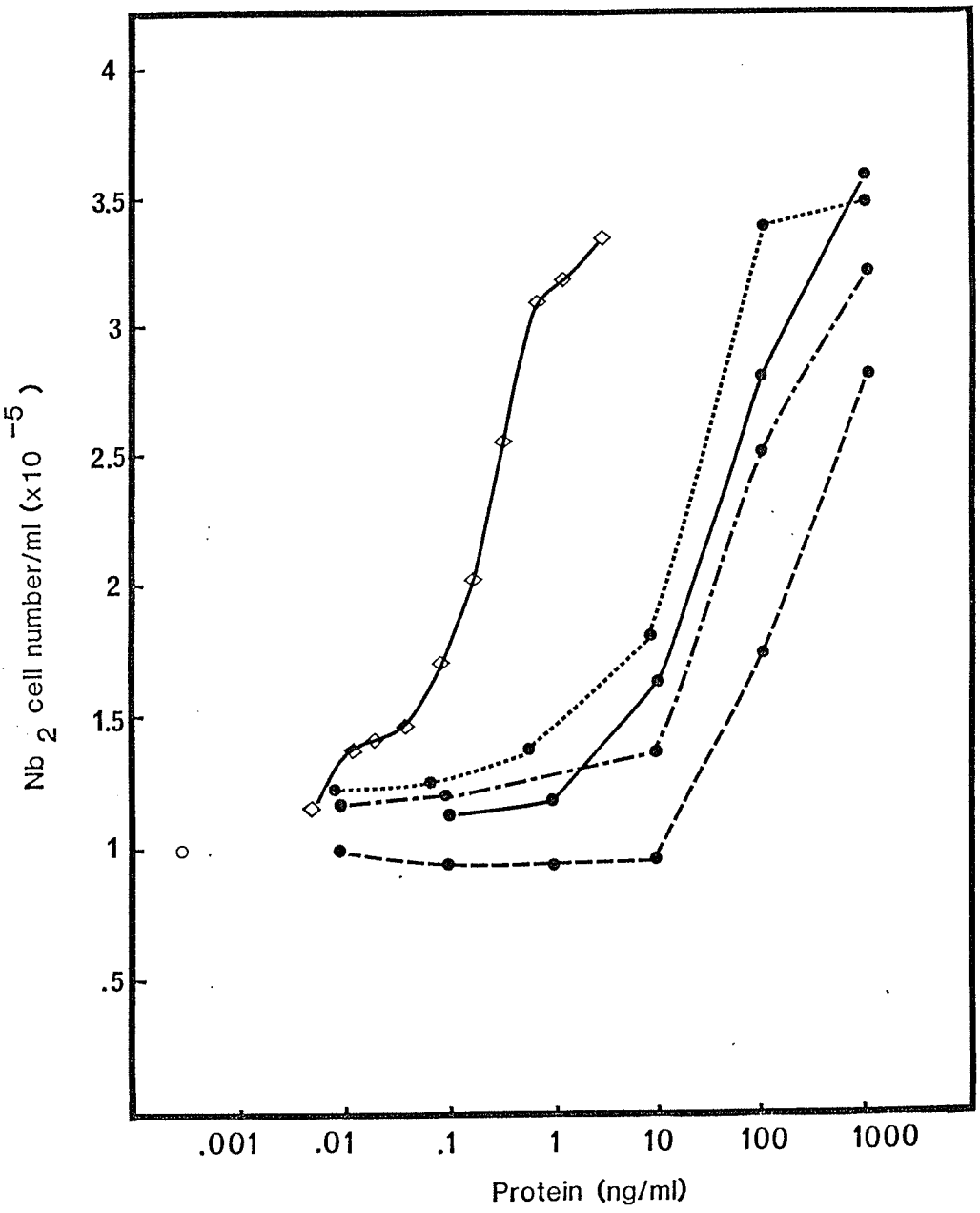
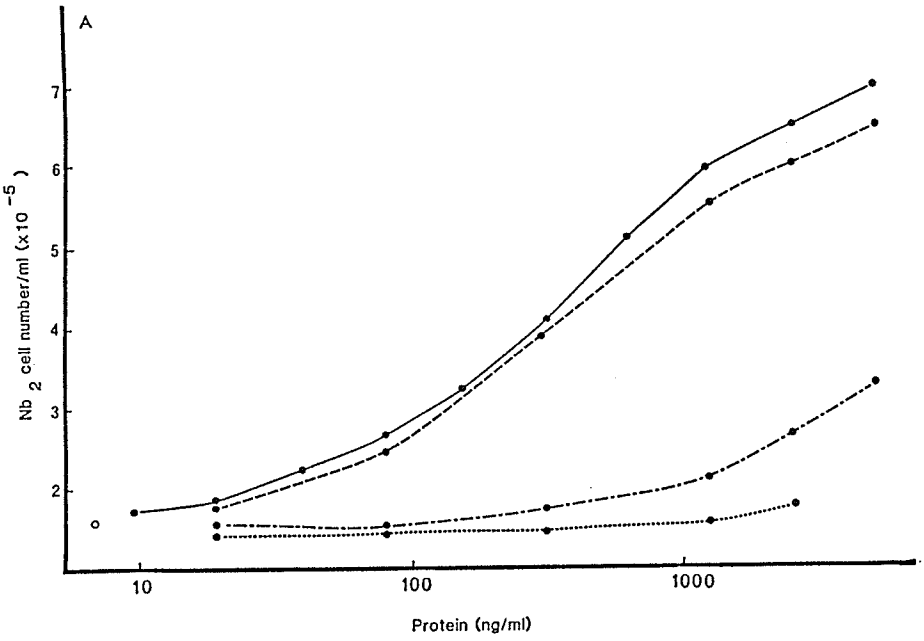

4
3.5
3
2.5
2
1.5
1
.5
Nb 2 cell number/ml (x10 -5)
.001
.01
.1
1
10
100
1000
Protein (ng/ml)

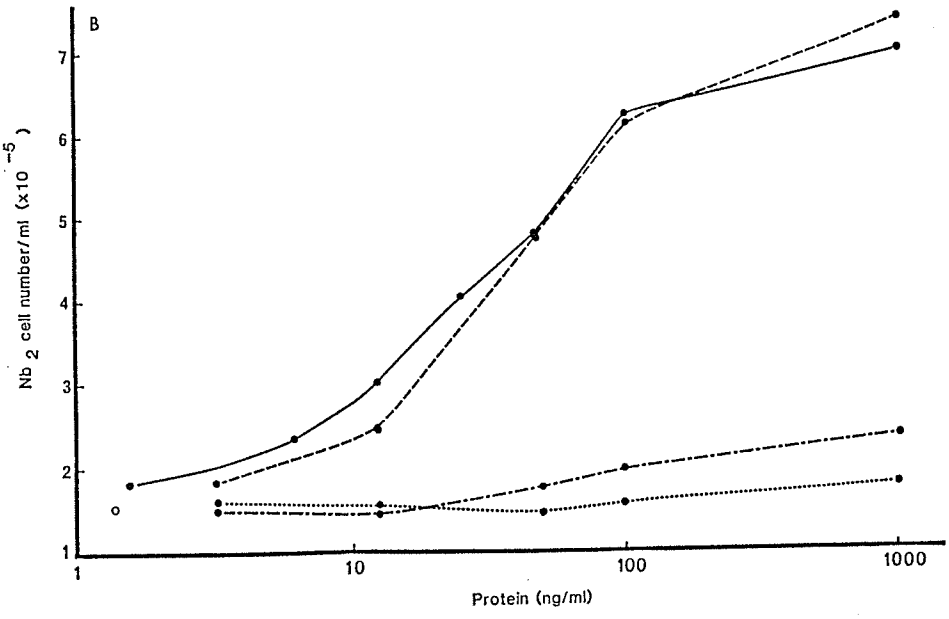
FIGURE 19. The effect of antiserum to hGH and/or antiserum to hPRL on the stimulation of NB_2 cell growth by (A) G-100 and (B) CF-hCGF preparations of hCGF. Cultures were incubated for 72 h with hCGF in the presence (added before growth factor) or absence of the antisera. The final concentration of antisera was 1:4000.

A. G-100 (●——●); G-100 and anti-hGH (●-·-·-·-●); G-100 and Anti-hPRL (●— — —●); G-100 and anti-hGH/Anti-hPRL (●·····●).

B. CF-hCGF (●——●); CF-hCGF and anti-hGH (●-·-·-·-●); CF-hCGF and anti-hPRL (●— — —●); CF-hCGF and anti-hGH/Anti-hGH (●·····●).

The data represent the mean values of duplicate cultures (SD < 10% of the mean). **P<0.05, *P<0.01 for the difference in cell number from control.


A
Nb 2 cell number/ml (x10 -5)
7
6
5
4
3
2
10
100
1000
Protein (ng/ml)


B
Nb 2 cell number/ml (x10 -5)
7
6
5
4
3
2
1
1
10
100
1000
Protein (ng/ml)

concentrations (2.5 ug/ml) of the G-100 pool, but only partially blocked activity above this protein concentration. This antisera significantly ($P<0.01$) inhibited CF-hCGF stimulation of Nb_2 cell proliferation at all growth factor concentrations tested; although higher amounts (100-1000 ng/ml) of CF-hCGF caused slight stimulation of the cells. In contrast, anti-hPRL antibodies were unsuccessful in blocking the activity of either preparation. The combination of the two antisera completely abolished the effects of both hCGF pools. Dose-response curves of hGH and hPRL in the presence and absence of antiserum to hGH and/or hPRL were completed in the same assay (data not shown) for comparison. Addition of either antiserum separately or in combination had no effect on Nb_2 cell growth (data not shown).

Radioimmunoassay of the G-100 and CF-hCGF pools confirmed the presence of hGH in these preparations (~5 ng/ml and <1 ng/ml, respectively).

Thus, the proliferative effects of hCGF on Nb_2 cells appears to be the result of hGH contamination in these preparations rather than stimulation of the cells by the pituitary mitogen. The hS-hCGF had no effect in the NB_2 assay (C. Too, personal communication).

Table 7 summarizes the effects of the hS-hCGF preparation on the proliferative response of several mesodermal cell types. The influence of this FGF-like mitogen on a variety of cells is considered an important

TABLE 7: Comparison of the response of several cell types to hS-hCGF.

CELL TYPE	ORIGIN	RESPONSE	EC_{50}*
CHONDROCYTE**	Fetal Rabbit	+++ (6x)***	35 ng/ml
ENDOTHELIAL	Bovine Cornea	++ (1.6x)	40 ng/ml
T-47D	Human Breast Cancer	++ (1.6x)	30 ng/ml
MCH6	Human Foreskin Fibroblasts	+ (1.4x)	1 ng/ml
Balb/c3T3	Mouse Embryonic Fibroblasts	+ (1.2x)	<1 pg/ml
Nb_2	Rat Lymphoma	-	-

* Values are estimated

** The proliferative response of the RFCs was considerably greater than that of the other cell types tested

*** Maximal fold stimulation

factor in the regulation of normal and neoplastic growth of tissues (which are composed of several mitogen-responsive cell types).

4.2 Immunological Analysis of hCGF

Based on amino-terminal sequence analysis, the first eleven residues of hPGF are identical (Rowe et al., 1986b) to that reported for bFGF (Esch et al., 1985a). Since human pituitary hS-hCGF has a similar MW (18-20kD) and elutes from heparin-Sepharose with a similar (1.5 M) NaCl concentration, to that reported for bovine pituitary bFGF, the relationship of this mitogen to bFGF was also examined using immunological techniques.

Polyclonal antibodies to synthetic bovine pituitary bFGF peptides conjugated to BSA (the gift of Dr. Andrew Baird, The Salk Institute, San Diego, CA) were screened for cross-reactivity to hS-hCGF. The bovine bFGF antisera tested by immuno-dot blot were:

anti bFGF (1-10)
anti bFGF (1-24)
anti bFGF (16-24)
anti bFGF (30-50)
anti bFGF (72-87)

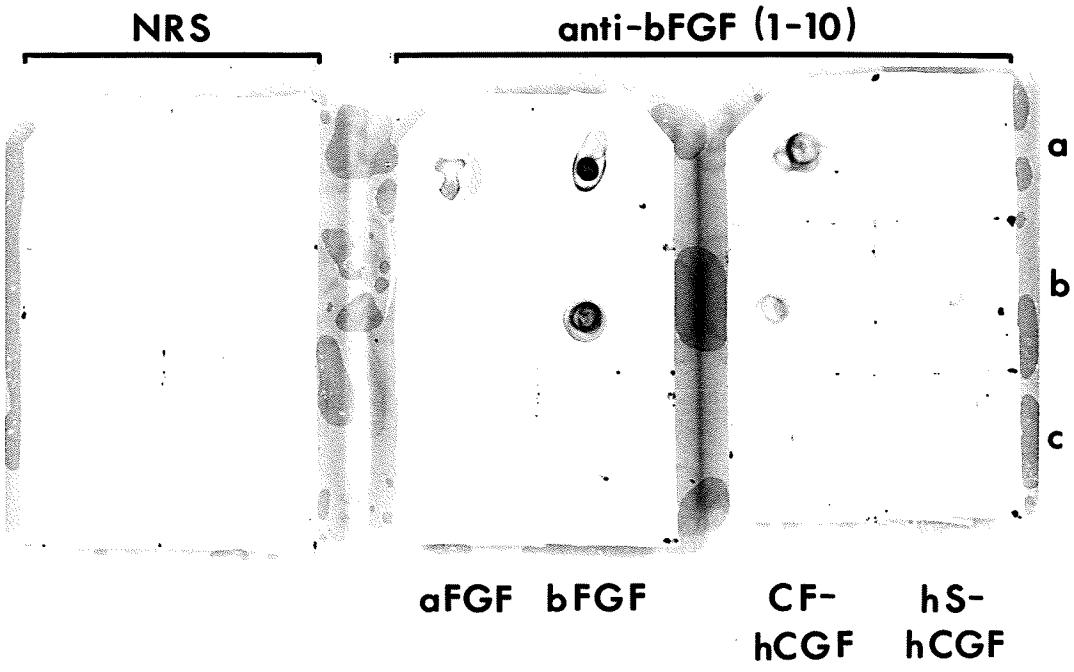
Antisera to bovine brain anti aFGF (1-15) and aliquots of aFGF and bFGF were also provided.

4.2.1 Immunodot Blot Analysis

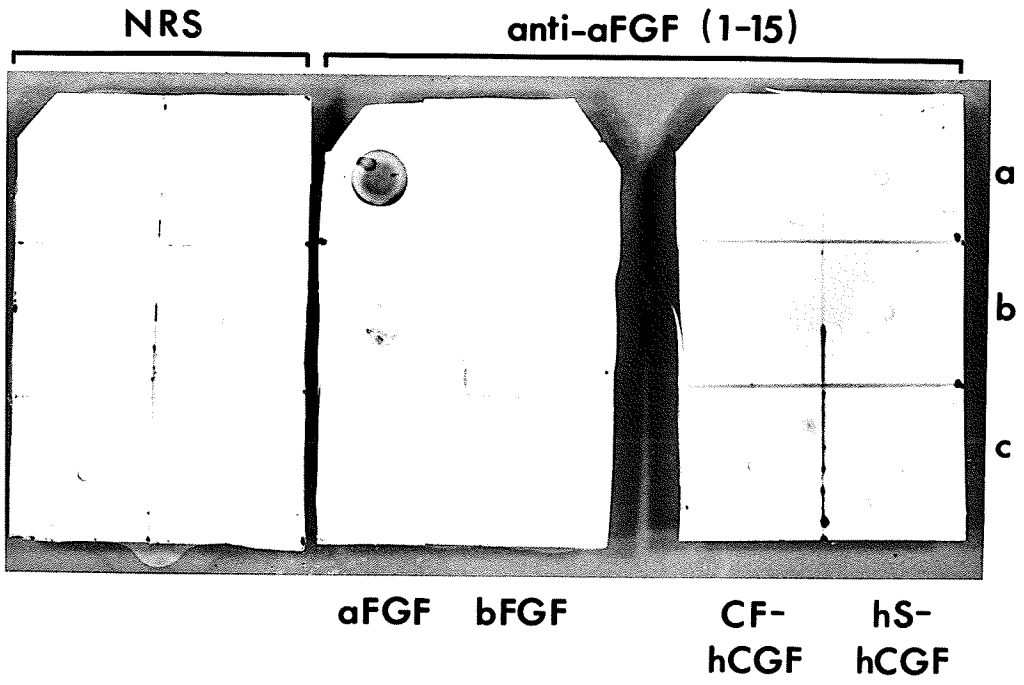
Figure 20 (A,B) shows the cross-reactivity of

FIGURE 20. Immuno-dot Blot analysis: cross-reactivity of human CF-hCGF and hS-hCGF, and bovine aFGF and bFGF (Dr. Baird) to (A) anti bFGF (1-10) and (B) anti aFGF (1-15). The antigen concentrations were a) 30 ng, b) 3 ng and c) 0.3 ng with the exception of CF-hCGF (a, 100, b, 10 and c, 1 ng). The antibodies and NRS were at 1:1000 dilutions.

A


NRS
anti-bFGF (1-10)
a
b
c
aFGF
bFGF
CF-hCGF
hS-hCGF

B


NRS
anti-aFGF (1-15)
a
b
c
aFGF
bFGF
CF-hCGF
hS-hCGF

hS-hCGF as well as CF-hCGF to anti bFGF (1-10) and anti aFGF (1-15). The bFGF and aFGF samples provided by Dr. Baird were used for comparison. Both CF-hCGF and hS-hCGF cross-reacted to bFGF antibodies but did not bind to aFGF antibodies. The samples in this assay had been applied to the nitrocellulose manually, causing the somewhat diffuse appearance of the spots.

In the second analysis, the Biorad dot-blot apparatus was used to apply the antigens to the nitrocellulose. Commercial bovine pituitary FGF (Collaborative Research) was used for comparison to hS-hCGF since the biological activity of this preparation had been examined in the RFC and BCE cells. At antisera dilutions of 1:1000, FGF cross-reacted to all of the bFGF antisera preparations (Figure 21). In contrast, hS-hCGF did not cross-react with anti bFGF (30-50), but did react to the other bFGF antisera tested.

4.2.2 Western Blot Analysis

Samples of hPGF, hS-hCGF, bovine bFGF (Collaborative Research), bovine bFGF (Dr. Baird), and BSA were applied to a 6-20% linear gradient gel. Following transfer to nitrocellulose, the membranes were incubated with anti bFGF (1-24), anti bFGF (69-87) or normal rabbit serum (NRS). The results are shown in Figure 22. Although all the mitogens cross-reacted with both bFGF antisera, certain differences between human and bovine

FIGURE 21. Immuno-dot Blot analysis: cross-reactivity of hS-hCGF and bovine bFGF (Collaborative Research) to bovine bFGF antisera. Lane a) hS-hCGF, 60 ng; b) bovine bFGF, 30 ng; c) bovine bFGF, 100 ng. Each nitrocellulose blot was incubated with a different bovine bFGF antisera or NRS (1;1000 dilution).

Blot A, NRS; Blot B, anti bFGF (1-24);

Blot C, anti bFGF (16-24); Blot D, antibFGF (30-50);

Blot E, anti bFGF (69-87); Blot F, anti bFGF (72-87)

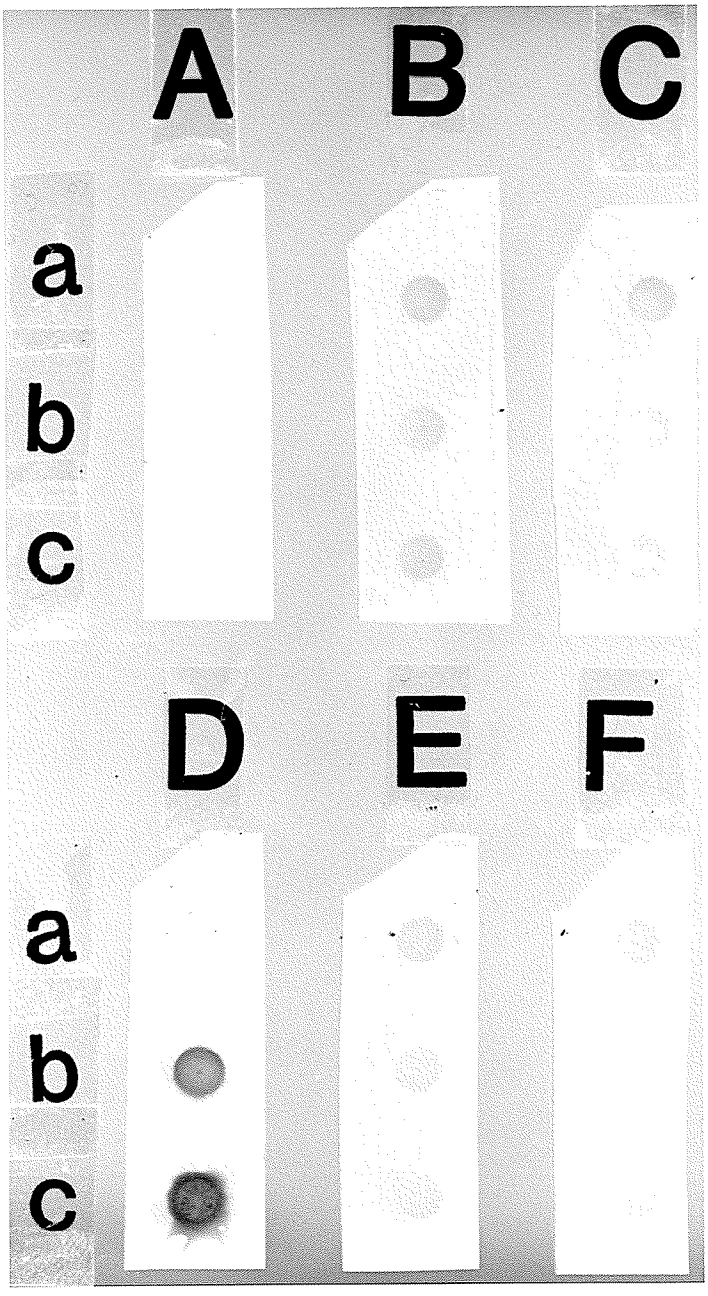
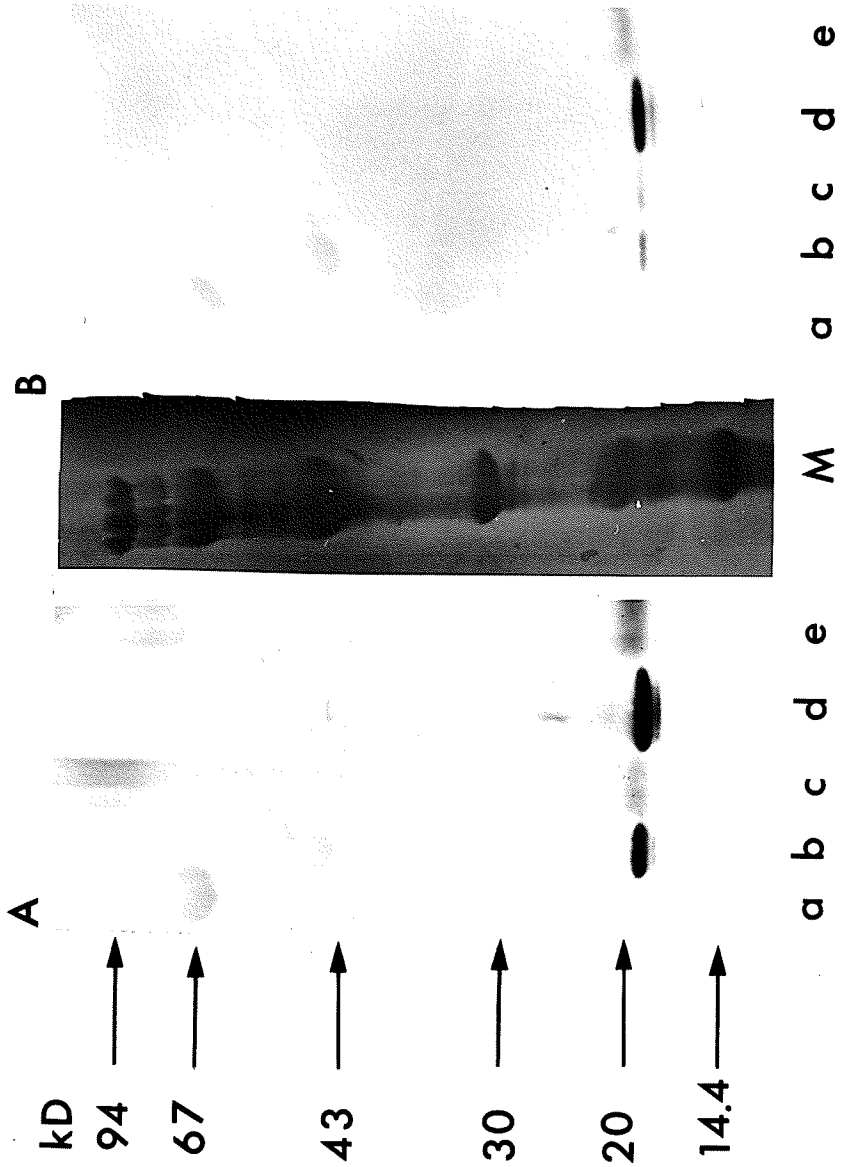

A
B
C
a
b
c
D
E
F
a
b
c

FIGURE 22. Western Blot: cross-reactivity with bovine bFGF antisera. A) anti bFGF (1-24) and B) anti bFGF (69-87). Lane a) BSA, 15 ug; b) bovine bFGF (Collaborative Research), 15 ug; c) hS-hCGF, 15 ug; d) bovine bFGF (Dr. Baird), 100 ng; e) hPGF, 25 ng. Antisera were diluted 1:200.


A
B
kD
94
67
43
30
20
14.4
a b c d e
M
a b c d e

preparations were observed. Cross-reaction of the two bovine FGF samples with either antibody produced a doublet of MW 18-20 kD, with the larger band exhibiting the stronger affinity for the antisera. The single band produced by hS-hCGF and hPGF incubation with the antisera was more diffuse and showed less affinity for these antisera than the bovine FGFs. Comparison of the hS-hCGF band visualized on the Western blot with amido black stained MW markers (M) simultaneously transferred to nitrocellulose showed that, similar to bovine bFGF, hS-hCGF (and hPGF) had a MW of 18-20 kD.

4.3 Rat Pituitary Organ Culture Studies.

Fibroblast Growth Factor has been purified from bovine and human pituitary glands and from rat chondrosarcoma. Immunoreactive FGF has been detected in several rat tissues including brain, pituitary, liver, kidney, spleen and thymus and in rat serum where hypophysectomy decreases the circulating levels of the mitogen (Mormede et al., 1985). Cultured bovine anterior pituitary cells release immunoreactive FGF into the culture medium (Baird et al., 1985). However, whether FGF is actually released from pituitary cells into the circulation in vivo and into conditioned medium in vitro (or if its presence in these places is due to cell lysis or leakage) is a subject of much debate. To study this

question, the human pituitary/RFC co-culture system previously described by S. Kasper in our laboratory, was modified and elaborated upon for use with a much more accessible tissue, the rat pituitary.

Figure 23 shows the effect of one, two, or three rat pituitaries on the proliferation of RFC cells. Since maximal (3.3-fold) stimulation was observed in the presence of only one pituitary, this was the top dose utilized in following experiments. The increase in cell number could only be attributed to RFC growth, rather than to attachment and growth of pituitary cells, since the cell counts determined when the human pituitary rafts were incubated alone were negligible (0.5×10^4 cells/dish).

The conditioned medium collected from this co-culture experiment was mitogenic in a second (consecutive) RFC bioassay as well (Figure 24), reflecting release of the mitogen from the pituitary tissue. The slight increase in stimulation observed with the 3 pituitary conditioned medium was not significantly different from the results obtained with 1 or 2 pituitaries.

Since maximal stimulation of RFC proliferation was achieved in the presence of one rat pituitary, dose-response curves of pituitary tissue from individual animals were completed. The pituitaries were minced and tissue corresponding to approximately 1/2, 1/4 and 1/8 of the gland was incubated with the RFCs. To detect any sex

FIGURE 23. Mitogenic activity present in one, two or three rat pititaries co-cultured with RFCs. Pituitaries were added on day 1 of a RFC proliferation assay. Cell number was determined on day 5.

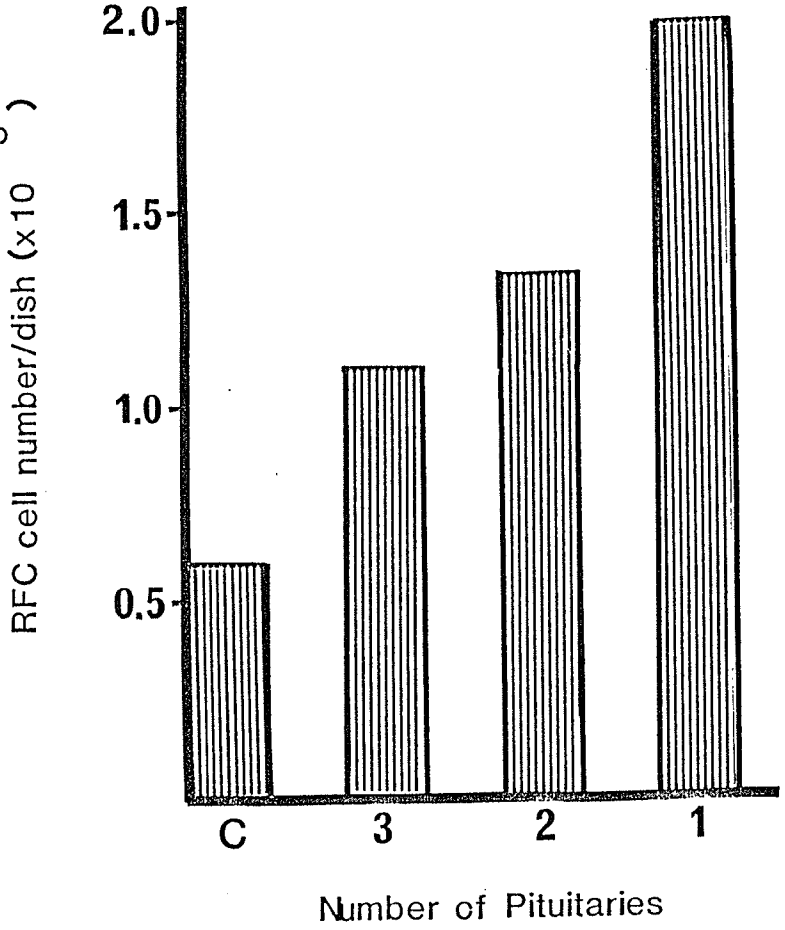
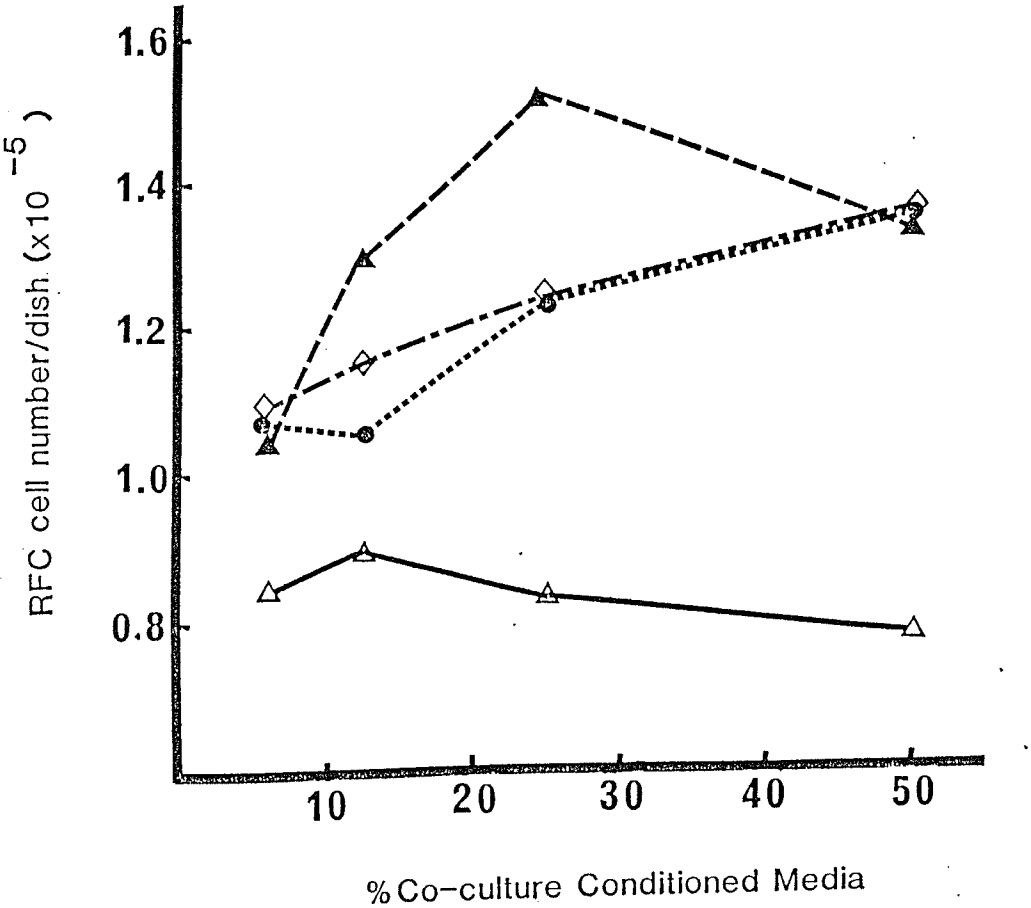

2.0
1.5
1.0
0.5
RFC cell number/dish (x10 -5)
C
3
2
1
Number of Pituitaries

FIGURE 24. Mitogenic activity of rat pituitary/RFC co-culture conditioned media. Conditioned medium was collected from RFCs cultured in the presence of 3 (▲——▲); 2 (◇—·—·—◇); or 1 (●·····●) rat pituitary gland. Control (△————△) conditioned medium was obtained by incubating a lens paper raft with RFCs.


RFC cell number/dish (x10 -5)
1.6
1.4
1.2
1.0
0.8
10
20
30
40
50
% Co-culture Conditioned Media

differences, pituitaries from female and male rats were tested individually. Figure 25 represents the combined data of several animals for each sex. Significant stimulation ($P<0.01$) over control cells was observed in all cultures; the degree of stimulation was similar for male versus female rats. The stimulatory effect of the rat pituitary/RFC co-culture conditioned medium suggested that mitogenic activity was being released from the rat pituitaries into the culture medium. Two approaches were taken to test this hypothesis.

The first procedure consisted of collecting media conditioned by rat pituitary tissue and testing its mitogenic potential. Rat pituitary conditioned medium was collected at 48 h intervals for a period of six days. The effect of conditioned medium from male and female pituitaries on thymidine uptake in RFCs was measured (Figure 26 A, B). The majority of the mitogenic activity was found in the first (i.e. 48 h) collection, with little or no activity remaining by day 6. In this experiment, a significant difference in RFC stimulation was observed in the male versus female 48 h conditioned medium, but was not apparent in later collections. These results confirmed the presence of mitogenic activity in pituitary conditioned medium but did not provide information as to the mechanism of release of the mitogen (i.e. due to cell lysis or secretion).

A second series of experiments were undertaken

FIGURE 25. The effect of rat pituitary fragments on the proliferation of RFCs. A) Male; B) Female; C) Control The number of animals per dose is indicated within each bar. *P<0.01 for the difference from control RFCs.

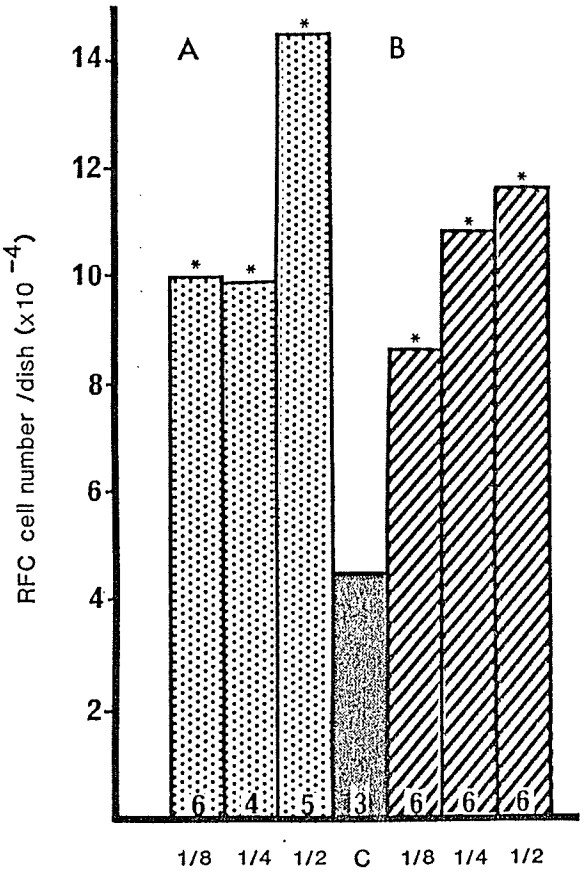
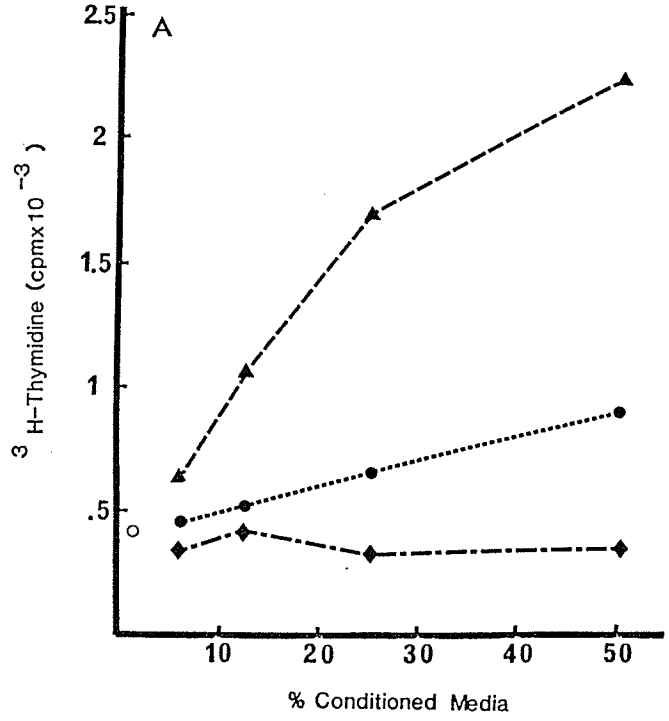

A
B
14
12
10
8
6
4
2
RFC cell number /dish (x10 $^{-4}$)
6
4
5
3
6
6
6
1/8
1/4
1/2
C
1/8
1/4
1/2

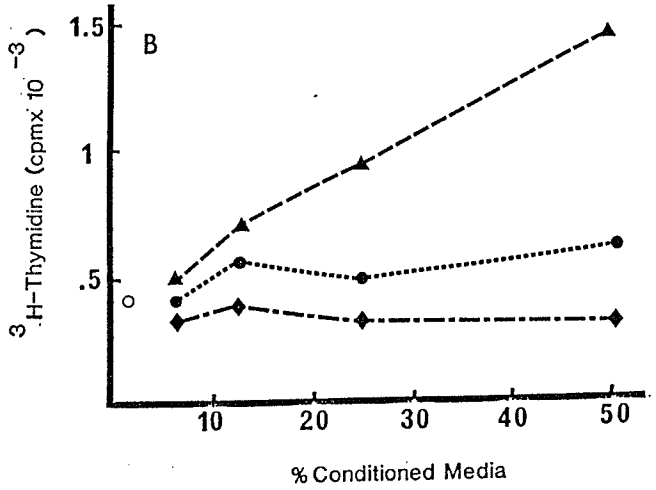
FIGURE 26. Dose-response curves of rat pituitary conditioned medium for each day of collection. Pituitaries from male and female rats were minced, placed on sterile lens paper rafts and floated in Ham's F10/0.1% BSA. Media was collected at 48 h intervals. For assay, samples were diluted in F10/0.1% BSA (200 ul final volume/well).

A. Male: day 2 (▲———▲); day 4 (●·····●); and day 6 (◆—·—·—◆).

B. Female: day 2 (▲———▲); day 4 (●·····●); and day 6 (◆—·—◆).

Values are the mean of triplicate counts (SD < 10% of the mean).


A
2.5
2
1.5
1
.5
0
^{3}H-Thymidine (cpmx10^{-3})
10
20
30
40
50
% Conditioned Media


B
1.5
1
.5
0
^{3}H-Thymidine (cpmx 10^{-3})
10
20
30
40
50
% Conditioned Media

to examine the actual mechanism of release of mitogenic activity. For these studies, the rat pituitary rafts were incubated with RFCs for six days; removed and placed in culture medium overnight (to remove residual conditioned medium from the previous assay); and finally placed on freshly plated RFCs on day 1 of a second RFC proliferation assay. In one experiment, this process was repeated with a third consecutive assay.

The conditioned medium collected from the first co-culture experiment, when added to a new chondrocyte assay, still contained potent mitogenic activity (Figure 27). Since no difference was observed in the male versus female pituitary tissue responses, the data has been combined. In the presence of 1/2 pituitary tissue, the RFC cell number increased 2.9-fold in the first incubation and 3.9-fold following a second incubation period. The pituitary tissue rafts (1 pituitary/dish) used to assay pituitary cell attachment during the first co-culture experiment, also exhibited potent mitogenic activity in this second assay.

To ascertain the general area within the pituitary which is responsible for the mitogenic activity, the glands were separated into anterior and posterior regions and dose-response curves of the relative activities completed. As indicated in Figure 28, the anterior pituitary contains the majority of the mitogenic activity present in this tissue. When these

FIGURE 27. The effect of rat pituitary/RFC co-culture added to a second RFC proliferation assay. The value indicated within the bar is the number of animals/group. *P<0.01 for the difference from control (C; one lens paper raft floating in RFC culture dishes).

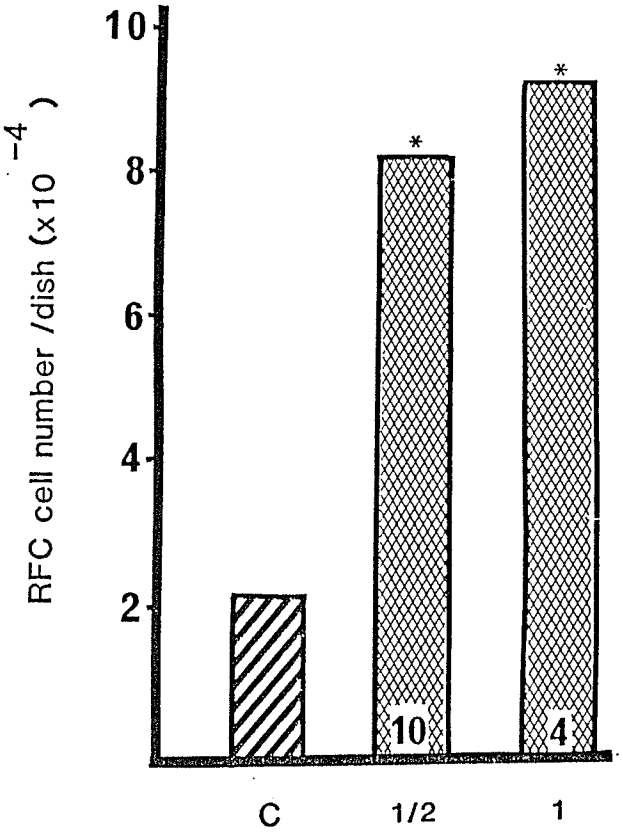
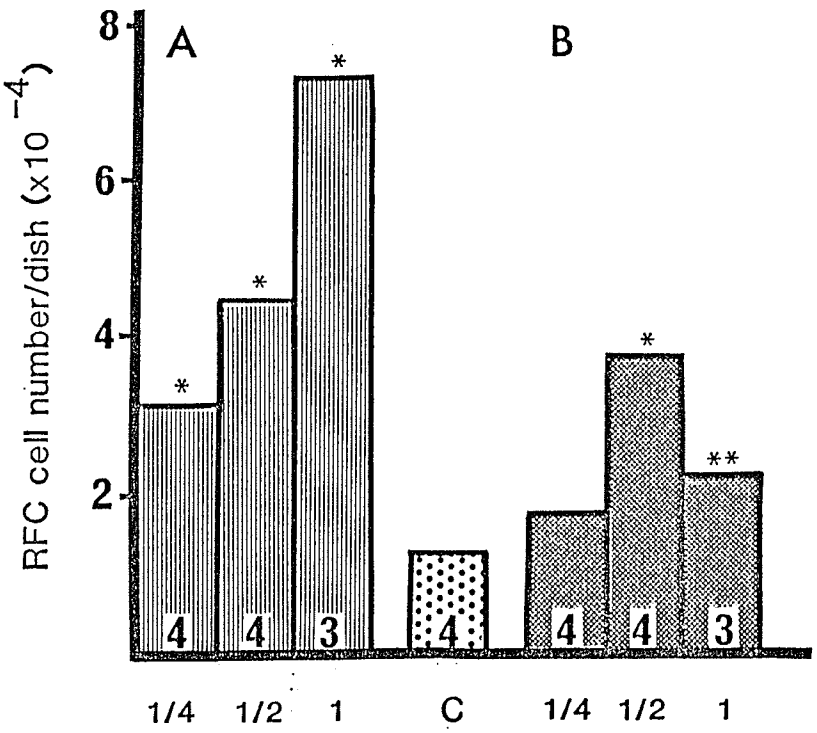

RFC cell number /dish (x10 -4)
10
8
6
4
2
*
*
10
4
C
1/2
1

FIGURE 28. Comparison of the mitogenic activity in rat anterior pituitary and posterior pituitary co-cultured with RFCs. (A) Anterior Pituitary; (B) Posterior Pituitary. The value given within each bar is the number of animals/group. **P<0.05, *P<0.01 for the difference from control (C).


RFC cell number/dish (x10 $^{-4}$)
8
6
4
2
A
B
*
*
*
*
**
4
4
3
4
4
4
3
1/4
1/2
1
C
1/4
1/2
1

tissue fragments were co-cultured with three consecutive sets of RFC cultures (Figure 29), the anterior pituitary tissue still exhibited significant stimulation of cell growth. In contrast, the slight stimulation observed with one posterior pituitary in the first co-culture, was no longer visible in the latter two assays.

The ability of rat pituitary tissue maintained in organ culture for over 3 weeks to stimulate cell proliferation further suggested that not only was the pituitary mitogen being released into the medium, but that this prolonged activity was probably the result of de novo synthesis within the pituitary tissue.

4.4 Human Pituitary Tumor Studies

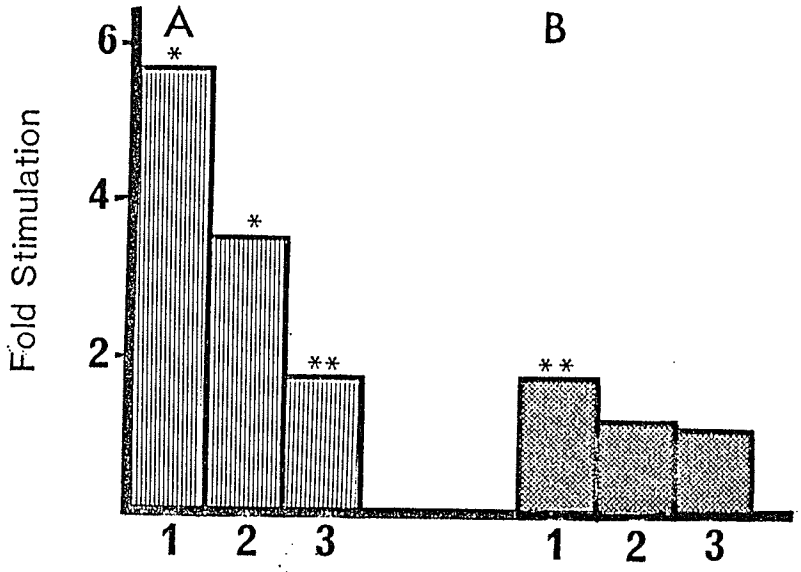
Immunoreactive bFGF has been detected in the serum of normal women and in normal male rats (Baird et al., 1986). Very high levels of several hormones (i.e. GH, PRL, TSH, LH, and FSH) have been reported in the pituitary vascular bed (Samaan and Leavens, 1981). Our laboratory has previously reported that conditioned medium of human pituitary tumor fragments as well as serum from the pituitary vascular bed stimulated RFC proliferation in vitro (Kasper, 1984; Kasper and Friesen, 1986). Preliminary characterization suggested that this mitogenic activity was similar to hCGF. We have recently shown that hCGF is a heparin-binding mitogen and that

FIGURE 29. Mitogenic activity in rat anterior and posterior pituitaries following consecutive co-cultures with RFCs. Following each RFC assay, the pituitary fragments were incubated overnight in culture medium before addition to a fresh RFC assay.

A. Anterior Pituitary (one/dish).

B. Posterior Pituitary (one/dish).

The numbers under the bars correspond to the number of the consecutive assay.


A
B
6
4
2
Fold Stimulation
*
*
**
**
1
2
3
1
2
3

this property can be used for its successful purification (Too et al., 1987). The present investigation was undertaken to determine if the mitogenic activity present in human pituitary tumors and in pituitary-associated serum (and possibly as yet undetected activity in peripheral serum) samples from these patients could be partially purified using heparin-Sepharose chromatography.

Figure 30 illustrates the presence of mitogenic activity in human pituitary tumor conditioned medium fractionated over a heparin-Sepharose column. An increase in thymidine uptake by RFCs was produced by both the unadsorbed as well as the 3M NaCl fractions, suggesting incomplete adsorption of growth factor to the heparin-Sepharose column. In contrast, addition of up to 130 ul (~100 ug/ml) of unprocessed conditioned media and up to 250 ul (~25 ug/ml) of the 3M NaCl column fraction had no effect in the RFC proliferation assay (data not shown). This study was completed prior to the observation that ~98% of the mitogenic activity present in human pituitary tissue will bind to heparin-Sepharose when passed over the column several times (Too et al., 1987). The pituitary tumor conditioned medium used in this study was only run over the heparin-Sepharose once. Unfortunately, the remainder of this material was insufficient to re-purify (with several passages over heparin-Sepharose) and re-test this response.

Serum from four patients with pituitary tumors

FIGURE 30. Mitogenic activity of human pituitary conditioned medium before and after heparin-Sepharose purification.

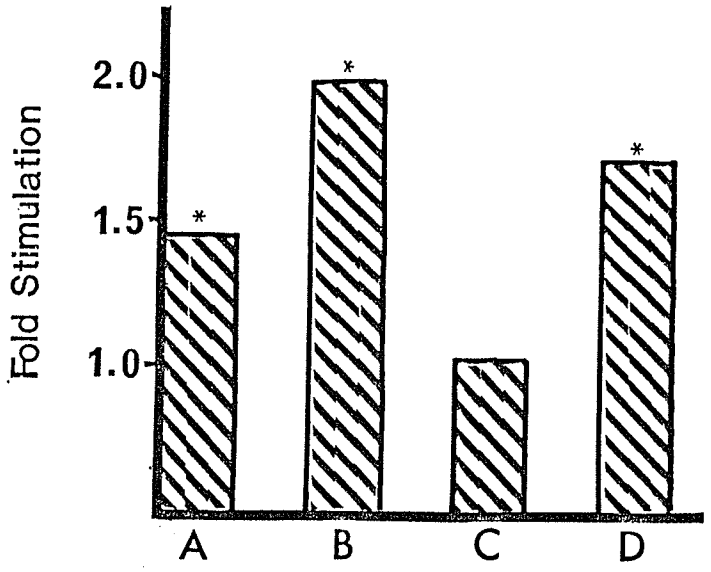
A. Precolumn, unfractionated sample;

B. Unadsorbed fraction;

C. Material eluted with 0.5 M NaCl;

D. Material eluted with 3 M NaCl.

Samples were assayed at 50% (A,B) or 100% (C,D) (v/v) conditioned medium. Control conditioned medium was produced by floating a lens paper raft in culture medium for an equal length of time and applying this material to heparin-Sepharose. Values are the mean of triplicate determinations (SD < 4% of the mean). *$P<0.01$ for the difference from control (50% or 100% control conditioned medium).


Fold Stimulation
2.0
1.5
1.0
*
*
*
A
B
C
D

were chromatographed over heparin-Sepharose and eluted with 3 M NaCl. Both serum from the site of removal of the tumor and peripheral serum samples were analysed for each patient (Figure 31 A-D). In the case of one patient, cystic fluid from the tumor was also tested. The serum controls for the assay were normal male and female peripheral serum chromatographed over heparin-Sepharose. Each of the samples was passaged over heparin-Sepharose twice. There was no difference in effect observed from control cells (i.e. RFCs cultured in Ham's F10/10% FBS) with either of the normal samples (data not shown).

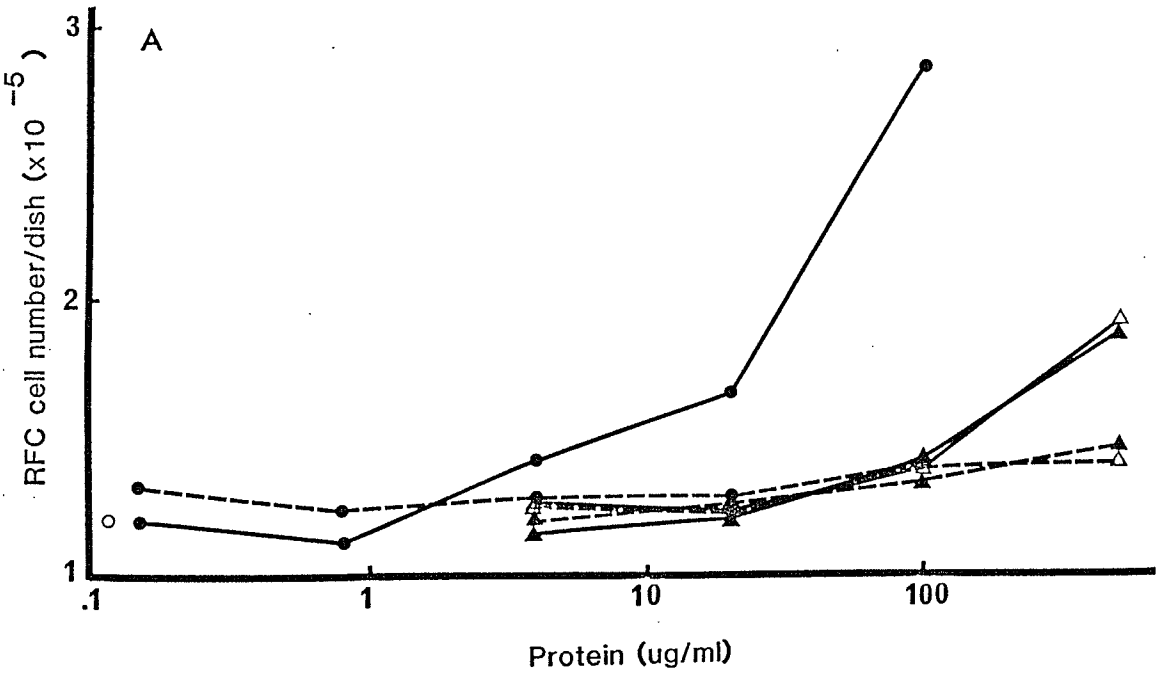
Mitogenic activity was observed in the pituitary serum of 3 of the 4 patients (A,B,C). Heparin-Sepharose partial purification of two samples (A and B) resulted in 20- and 5-fold stimulation of cell growth over that observed for unfractionated pituitary serum from the same patient. The majority of the mitogenic activity was heparin-binding, although some activity was observed in the unadsorbed fraction from patient A.

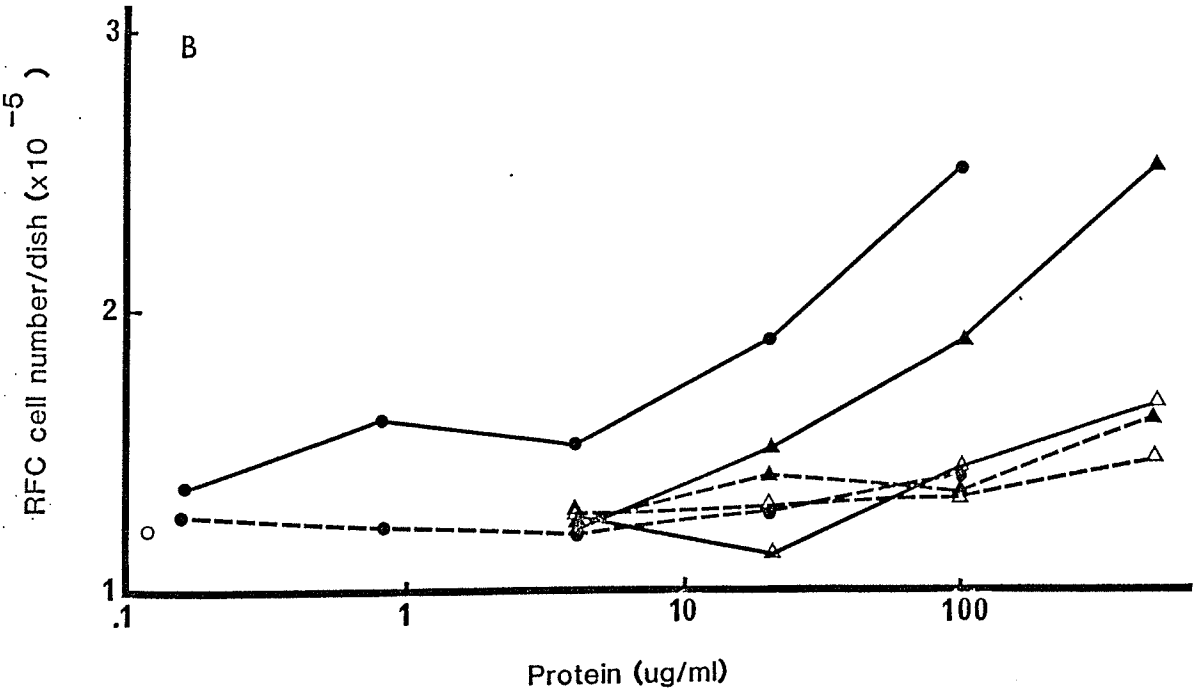
Significant stimulation of cell growth was also observed in the unfractionated peripheral serum of all patients (top dose) over control normal peripheral serum. The 3 M NaCl fraction (100 ug/ml) of these samples also contained significant mitogenic activity when compared to control serum. The level of stimulation observed with peripheral serum was much less than that observed with

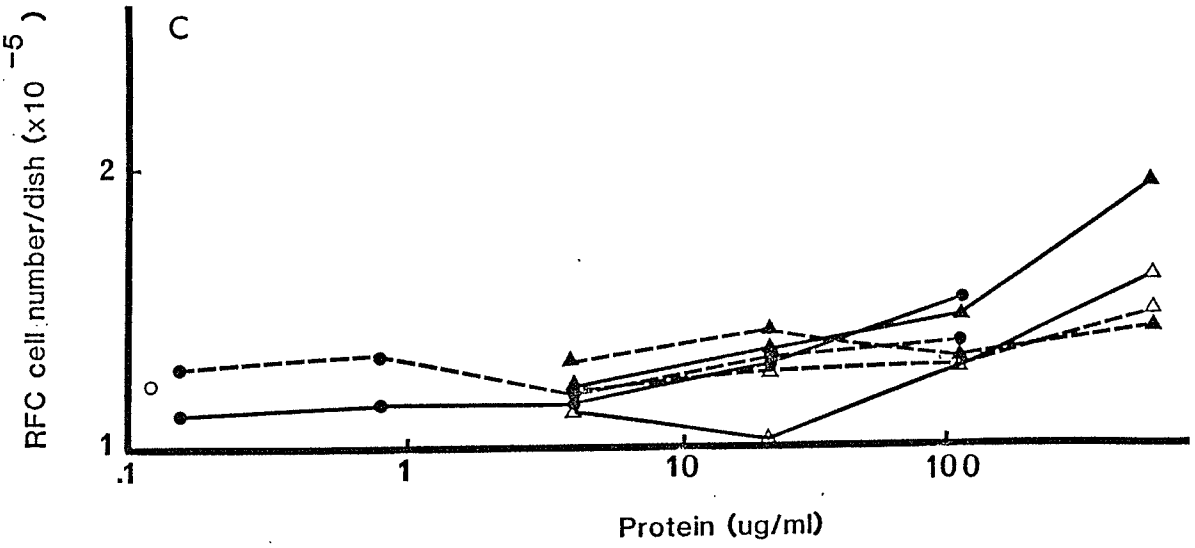
FIGURE 31. Mitogenic activity of the pituitary and peripheral serum samples obtained from patients with pituitary tumors before and after heparin-Sepharose purification. A. female, non-functional adenoma; B. female, prolactinoma; C. female, GH-secreting; D. male, prolactinoma.

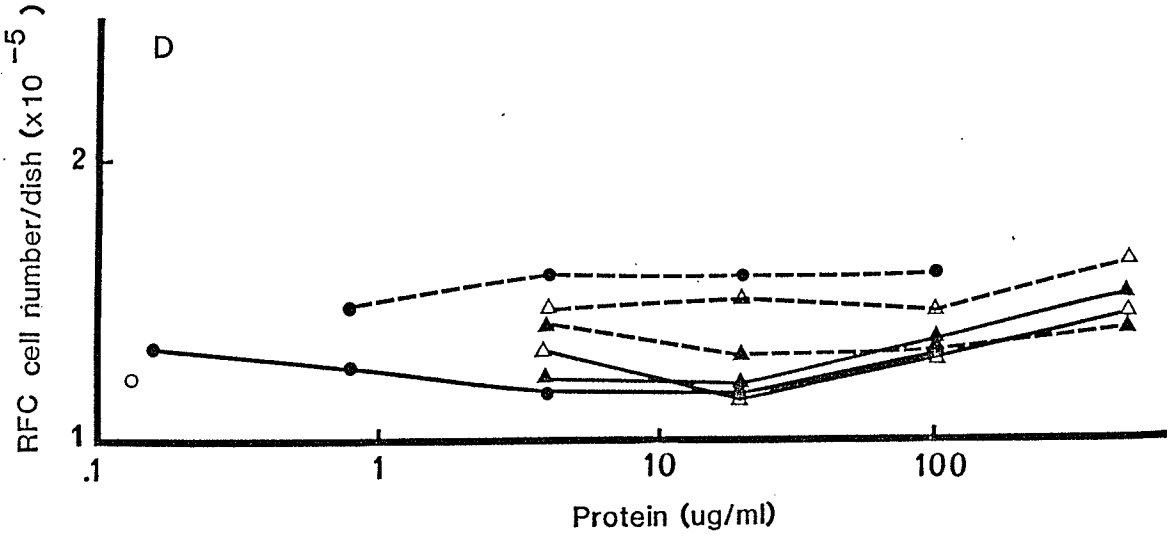
I. Pituitary Serum Sample: Precolumn (▲——▲); Unadsorbed (△——△); and 3 M Wash (●——●).

II. Peripheral Serum Sample: Precolumn (▲– – –▲); Unadsorbed (△– – –△); and 3 M Wash (●– – –●).


A
RFC cell number/dish (x10 -5)
3
2
1
o
.1
1
10
100
Protein (ug/ml)


B
RFC cell number/dish (x10 -5)
3
2
1
o
.1
1
10
100
Protein (ug/ml)


C
RFC cell number/dish (x10 -5)
2
1
.1
1
10
100
Protein (ug/ml)


D
RFC cell number/dish (x10 -5)
2
1
.1
1
10
100
Protein (ug/ml)

equal concentrations of pituitary serum in all but one patient. Both the unfractionated pituitary and peripheral serum of patient D exhibited little biological activity. However, the peripheral serum 3 M NaCl fraction caused a 1.3-fold increase in RFC cell number and was significantly ($P<0.01$) more potent than the 3 M fraction of the pituitary serum at all concentrations tested (Figure 31, D). A sample of pituitary tumor cystic fluid also taken from patient D did not stimulate RFC growth (data not shown). Likewise, no effect was observed with a sample of pituitary tumor conditioned medium from patient C (data not shown).

These results show that the mitogenic activity present in pituitary and peripheral serum of patients suffering from various types of pituitary tumors is heparin-binding, and as such, is FGF-like.

4.5 Swarm Rat Chondrosarcoma *In Vivo* Study

The Swarm rat chondrosarcoma was used as an experimental model to study the effects of hCGF *in vivo* on cartilage tumor growth. Normal and hypophysectomized (HPX) Sprague Dawley rats were inoculated (sc) with 100 mg of tumor slurry/flank and injected twice daily with hCGF, hGH or injectable water. The hCGF used for injection was partially purified using hydroxylapatite chromatography. The most active HTP fractions were

pooled, dialysed against double distilled water and concentrated to a final protein concentration of 500 ug/ml. Since this tumor was known to be GH-responsive, 200 ug/day of hGH was injected into normal and HPX rats for comparison to hCGF activity. Several HPX rats received 20 ng/day of hGH since this amount of hGH contaminant (as measured by RIA) was detected in the HTP pool. The hGH used in this experiment was purified in our laboratory (98% pure compared to WHO standards). Control animals received injectable water, the vehicle used to solubilize hGH. All animals received injections twice daily (9:00 and 21:00) for a total of 4 weeks (see Table 5).

The effects of hCGF and hGH on two parameters of growth, i.e. body weight gain and increased nose-to-tail length, are shown in Figures 32 and 33. No significant difference in these measurements were observed in normal animals injected with either hGH or hCGF. Daily injection of 200 ug of hGH caused significant increase in nose-to-tail length and weight gain in the HPX rats. The hCGF injection also caused an increase in body weight gain but, due to the large variation observed in the hGH (a, 20 ng/day) group this effect was not significant. The increase in nose-to-tail length measured in the hCGF-treated HPX rats, although significantly different ($P<0.05$) from control animals, was not different from those injected with hGH.

FIGURE 32. Comparison of the effects of hGH and hCGF on Body Weight Gain in male Sprague Dawley rats.

A. Normal Rats

B. HPX Rats

C. Injectable water

(a) 20 ng hGH/day; (b) 200 ug hGH/day, hCGF, 500 ug HTP pool/day. The number of animals/treatment is indicated within the bars. The data represent the mean ± SD of the measurements. **P<0.05.

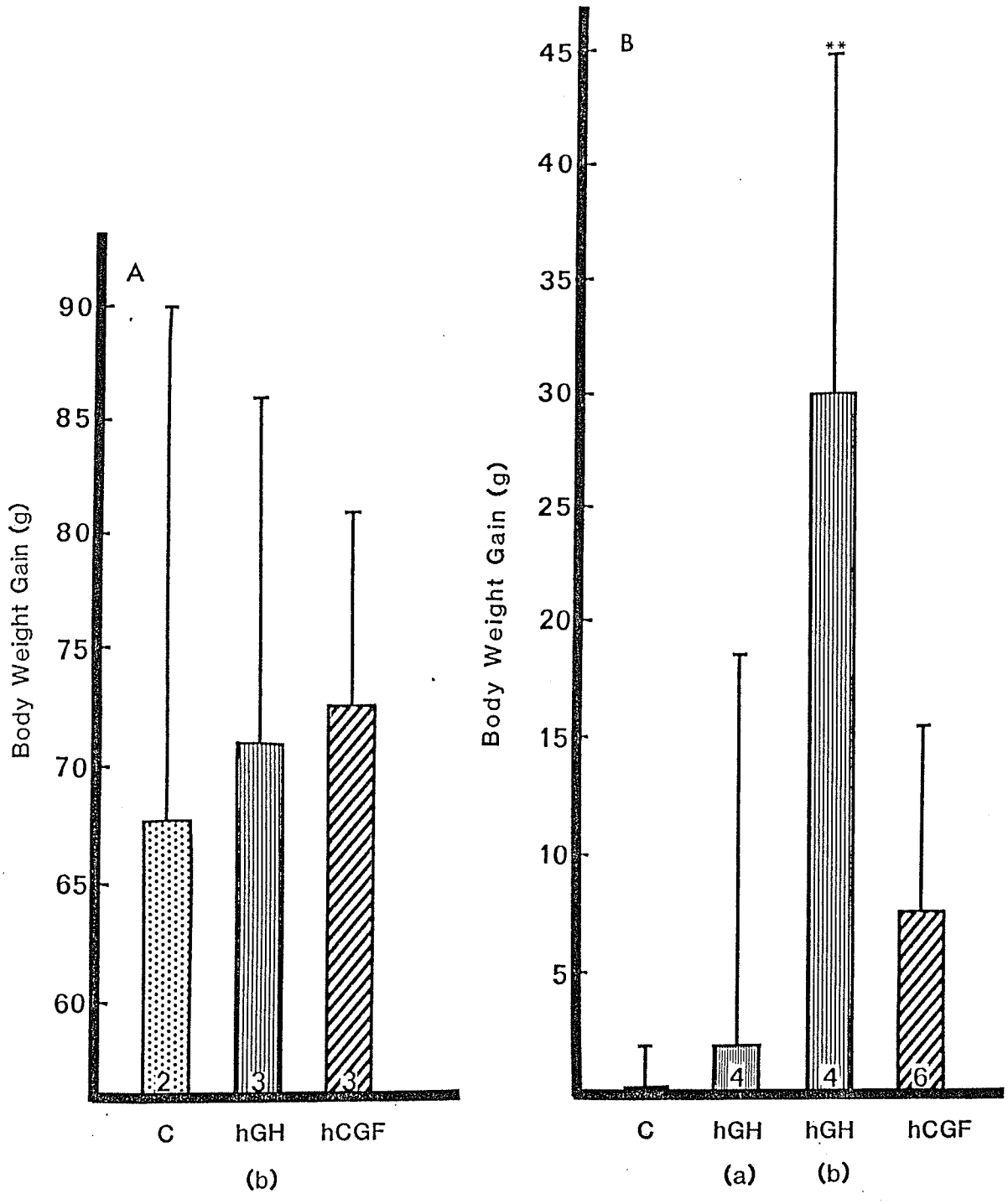

A
Body Weight Gain (g)
90
85
80
75
70
65
60
2
3
3
C
hGH
hCGF
(b)
B
Body Weight Gain (g)
45
40
35
30
25
20
15
10
5
**
4
4
6
C
hGH
hGH
hCGF
(a)
(b)

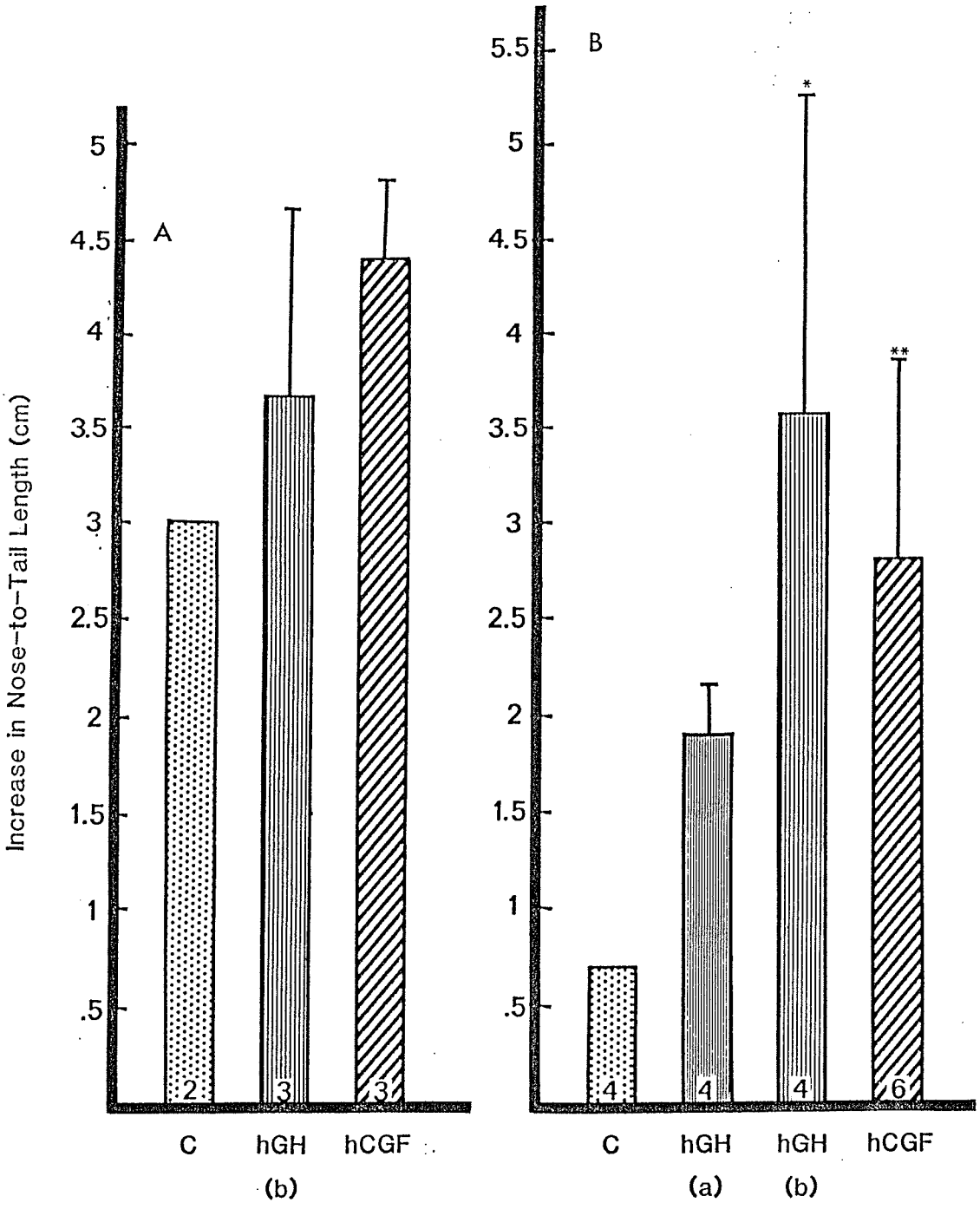
FIGURE 33. Comparison of the effects of hGH and hCGF on Increased Nose-to-Tail Length.

A. Normal Rats

B. HPX Rats

C. Injectable water

(a) 20 ng hGH/day; (b) 200 ug hGH/day; hCGF, 500 ug/day. The number of animals/treatment is indicated within the bars. The data represent the mean ± SD of the measurements. *P<0.01, **P<0.05.


Increase in Nose-to-Tail Length (cm)
A
B
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
.5
*
**
2
3
3
4
4
4
6
C
hGH
hCGF
C
hGH
hGH
hCGF
(b)
(a)
(b)

When considering either growth measurement, some stimulation of the HPX rats by hCGF did occur. However, the large standard deviation within each group makes interpretation of these results difficult. Several factors may have contributed to the observed variability. The number of animals in each group was small, causing individual values to be highly significant. A second factor could be the impurity of the hCGF preparation; the growth factor concentration may have been too low to produce substantial stimulation as was seen with the highly purified hGH. Stringent monitoring of temperature variation and feeding habits of the sensitive HPX rats is also required since changes in either factor can causes dramatic weight fluctuations. Larger treatment groups would also help to alleviate the variability.

The wet weights of several body organs were also measured to detect hGH or hCGF effects on the growth of these tissues. There were no significant differences in the weight of liver, kidney, and spleen from control values for the normal or HPX groups (data not shown). The growth of two tissues, the chondrosarcoma and the testes, were stimulated by hCGF.

A comparison of the effects of hCGF and hGH on testicular weight is shown in Figure 34. In the HPX rats treated with hCGF, due to large standard deviations, hCGF did not significantly increase testicular weight although the mean weight was greater than that observed for other

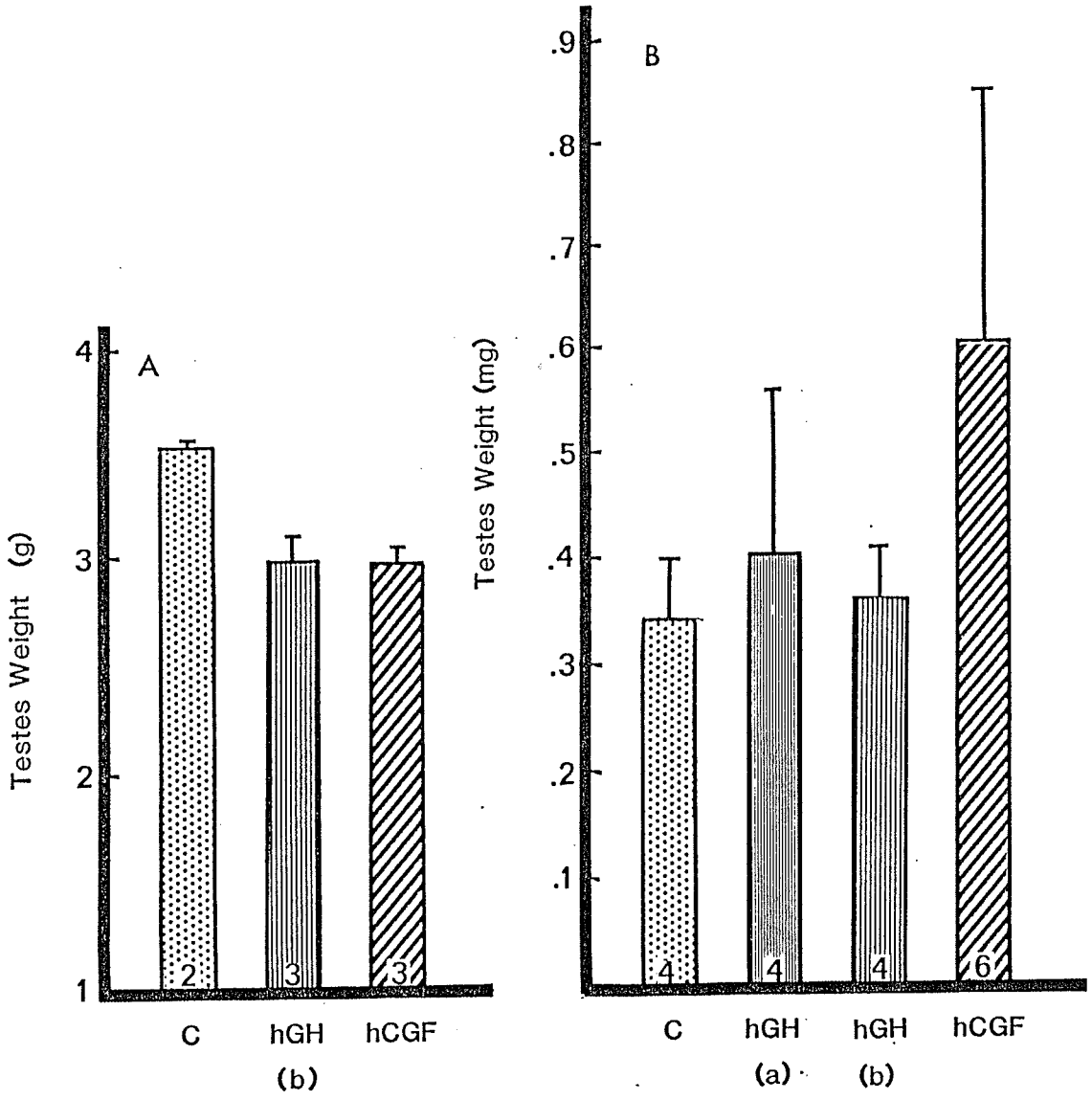
FIGURE 34. Comparison of the effects of hGH and hCGF on Testicular Weight.

A. Normal Rats

B. HPX Rats

C. Injectable water

(a) 20 ng hGH/day; (b) 200 ug hGH/day; hCGF, 500 ug/day. The data represent the mean ± SD of the measurements.


A
Testes Weight (g)
4
3
2
1
2
3
3
C
hGH
hCGF
(b)
B
Testes Weight (mg)
.9
.8
.7
.6
.5
.4
.3
.2
.1
4
4
4
6
C
hGH
hGH
hCGF
(a)
(b)

groups. No difference in testicular weight was observed in the normal groups.

The effects of hCGF and hGH on chondrosarcoma growth are shown in Figure 35. In normal rats, hCGF caused a three-fold increase in tumor weight over hGH treated animals whereas tumor growth was inhibited by hGH. The appearance of the chondrosarcoma tissue was quite different in the hGH versus hCGF treated normal animals. Whereas the tumor was localized in well-defined pink nodules with interseptal vascularization in the hCGF-treated animals, following hGH treatment, the tissue was diffuse and poorly vascularized.

This preliminary study suggests two possible roles for hCGF *in vivo* -- as a mitogenic influence on normal body growth and the etiology of cartilage tumor growth. Ueno *et al*. (1987) have recently found bFGF in testicular tissue. Such a role for FGF has also been suggested by Baird *et al*. (1985) using a mouse chondrosarcoma model. A repeat of this study with purified mitogen and larger treatment groups is required to establish hCGF involvement in normal and neoplastic tissue growth.

FIGURE 35. Comparison of the effects of hGH and hCGF on Chondrosarcoma Weight.

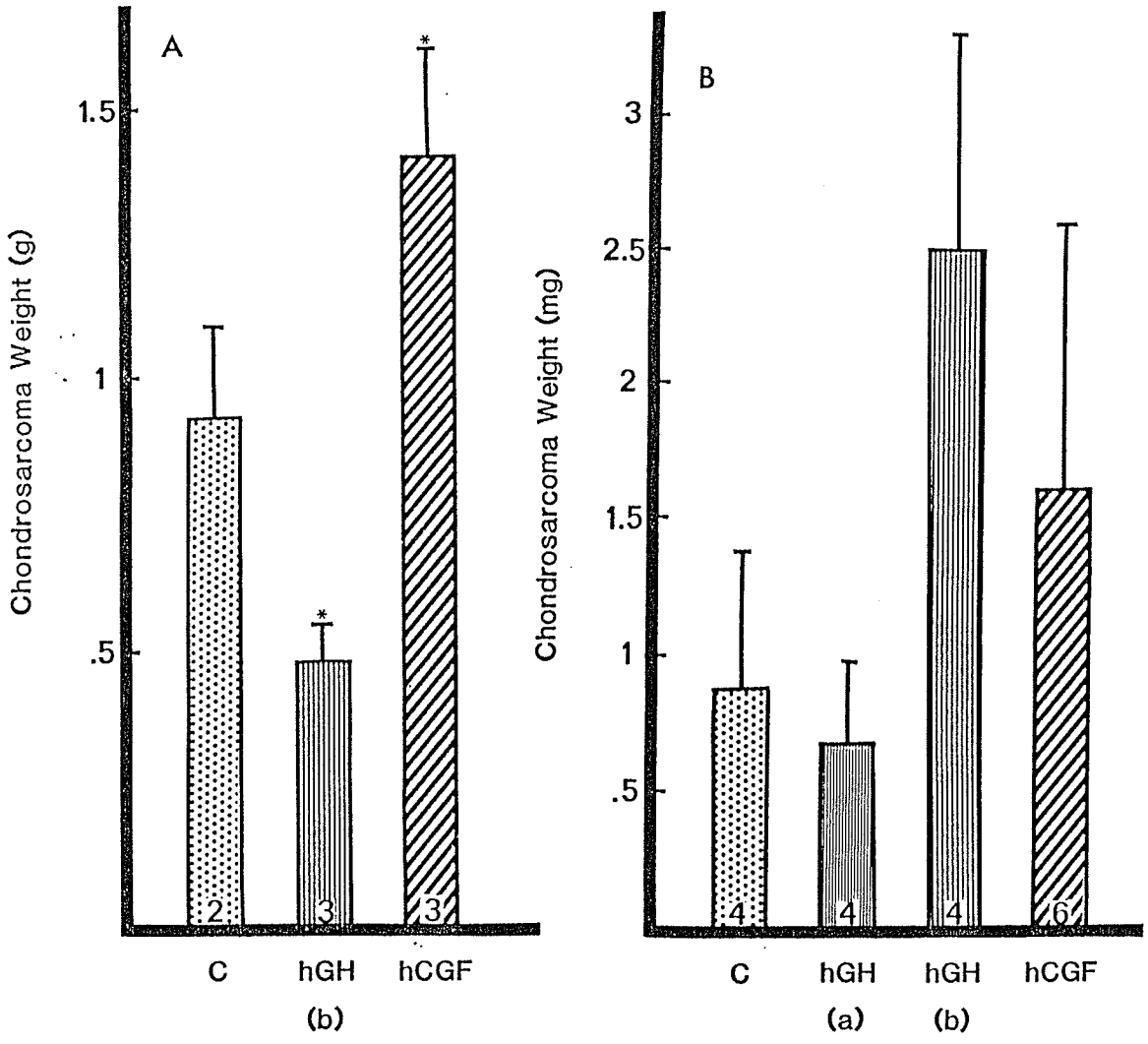
A. Normal Rats

B. HPX Rats

C. Injectable water

(a) 20 ng hGH/day; (b) 200 ug hGH/day; hCGF, 500 ug/day. The data represent the mean ± SD of the measurements.

*P<0.01.


A
Chondrosarcoma Weight (g)
1.5
1
.5
*
*
2
3
3
C
hGH
hCGF
(b)
B
Chondrosarcoma Weight (mg)
3
2.5
2
1.5
1
.5
4
4
4
6
C
hGH
hGH
hCGF
(a)
(b)

5. DISCUSSION

5.1 The Effects of hCGF on the Growth of Mesodermal Cells *In Vitro*

The rabbit fetal chondrocyte (cell growth) bioassay has been previously utilized, in our laboratory, to examine the presence of mitogenic activity in several human tissues including brain (Kellett *et al.*, 1981); pituitary (Kasper *et al.*, 1982, Kasper and Friesen, 1986; Rowe *et al.*, 1986b; Too *et al.*, 1987) and mammary tumor (Rowe *et al.*, 1986a). Since hS-hCGF and hPGF share similar MWs and heparin affinity, and the amino-terminus of hPGF is identical to that reported for bFGF, the *in vitro* actions of the hCGF preparations on the cell types examined in the present report are bFGF-like.

Addition of hCGF to chondrocytes not only stimulated their growth but also altered the appearance of the cells. The fusiform (fibroblastic) configuration of some of the cells that had been treated with hPGF has also been reported for subconfluent cultures of rabbit costal chondrocytes in the presence of FGF (Kato and Gospodarowicz, 1985), whereas a round shape has previously been used to describe rapidly dividing chondrocytes stimulated with a crude pituitary extract *in vitro* (Corval *et al.*, 1972). Thus, the effects of hPGF on chondrocyte morphology were similar to those described

for bovine FGF stimulation.

Gospodarowicz and Mescher (1977) demonstrated the ability of FGF and EGF to stimulate cell proliferation and thymidine uptake in adult rabbit ear chondrocytes. In their studies, EGF stimulation of chondrocyte proliferation was inversely affected by serum concentration. In the presence of 1% serum, EGF caused an 8-fold increase in cell number, whereas, EGF did not stimulate cell growth in 10% serum. The effects of EGF on fetal chondrocyte growth were compared to the published effects of this mitogen on the growth of adult chondrocytes. Since this study was completed in our RFC proliferation assay, 10% FBS was present. Thus, the lack of effect of EGF on RFC proliferation suggests a similarity in EGF action in adult and fetal cartilage <u>in vitro</u>.

Addition of TGF-alpha to RFCs maintained in 10% FBS also had no effect on cell proliferation. In contrast, addition of either EGF (0.6-10 ng/ml) or TGF-alpha (5-20ng/ml) caused a significant ($P<0.01$) but steady (~6-fold) stimulation of DNA synthesis in the RFCs over control values. This suggests that the EGF and TGF-alpha concentrations used were at the maximum range of the assay with lower concentration of these factors, dose-response curves may have been observed.
Gospodarowicz and Mescher (1977) reported maximal

thymidine uptake by adult rabbit ear chondro-cytes with lng/ml of EGF. As was observed in the RFC prolifera-tion assay, EGF and TGF-alpha have similar effects on DNA synthesis in fetal chondrocytes.

The two mitogens, TGF-alpha and EGF are structurally related proteins (~35% homologous) (Marquardt et al., 1984; Derynck et al., 1984) that compete for the same (i.e. EGF) receptor (DeLarco and Todaro, 1980; Massague, 1983). To date, the effects of TGF-alpha both in vitro and in vivo are indistinguishable from EGF. For example, both EGF and TGF-alpha stimulate fibroblasts and endothelial proliferation in vitro and angiogenesis in vivo (Carpenter et al., 1983; Roberts and Sporn, 1985).

The effects of TGF-alpha on chondrocyte growth in vitro has not been reported previously. As expected, TGF-alpha mimicked EGF in its lack of stimulation of RFC proliferation (in 10% FBS) and in its stimulation of thymidine uptake. In the thymidine uptake assay, since dose-response curves were not observed for either mitogen, further examination of their effects could be conducted.

Roles for both FGF and EGF in chondrocyte growth and differentiation in vitro have been described. TGF-B potentiates the actions of these mitogens. TGF-alpha also stimulated thymidine uptake in RFCs in a

manner similar to that observed for the closely related mitogen EGF. This is the first report of chondrocyte stimulation by TGF-alpha. These mitogens may act in concert to regulate normal cartilage and bone formation via autocrine or paracrine secretion.

Addition of bFGF-like hCGF or EGF to cultures of bovine corneal endothelial cells stimulated cell proliferation and thymidine uptake. Although the cells were responsive to lower doses of EGF than hCGF, hCGF had larger stimulatory effects than EGF, findings consistent with the effects of FGF and EGF reported in the literature (Gospodarowicz et al., 1977).

In vivo, corneal endothelial cells consist of a single layer of flattened cells located on the posterior surface of the cornea, separating the corneal stroma from the aqueous humour. This area is quite avascular with endothelial cells receiving nutrients primarily from the aqueous humour, a source of FGF. In vitro, in a manner similar to FGF, addition of hCGF allowed the cells to maintain their in vivo morphology. The subconfluent monolayer cultures contained many polygonal shaped cells; where the cells contacted each other, they presented a configuration similar to that observed for capillary formation in vivo. The mitogen treated cells were smaller than control cultures, a feature consistent with cells undergoing rapid mitotic activity with less energy

spent on production of cytoplasmic components.

The reduced doubling time observed following hCGF addition to either RFC or BCE cells is in agreement with previous reports for FGF stimulation of mesoderm-derived cells (Gospodarowicz et al., 1987) such as luteal cells (Gospodarowicz et al., 1977b), vascular endothelial cells (Gospodarowicz et al., 1980) and chondrocytes (Kato and Gospodarowicz, 1985) and is thought to result primarily from a shortening of the G1 phase of the cell cycle by this competence factor (Gospodarowicz et al., 1981).

Our laboratory has reported the presence of pituitary-derived mitogenic activity capable of stimulating human breast cancer (T-47D) growth in vitro (Shiu, 1981). Shiu and coworkers have further suggested that the stimulatory effects of estrogens on the growth of T-47D human breast cancer cells both in vitro and in vivo required the presence of a pituitary growth factor (Leung and Shiu, 1981; Dembinski et al., 1985). Our laboratory has also reported that purified preparations of two basic heparin-binding growth factors, human mammary tumor-derived growth factor (hMTGF) and human pituitary growth factor (both of which stimulated RFC and BCE cell proliferation) (Rowe et al., 1986a, b) have very different effects on T-47D cells. A two-fold stimulation of T-47D cell number was observed with hMTGF,

whereas hPGF was not mitogenic in this assay.

Since initial screening of CF-hCGF produced a 1.6-fold stimulation of T-47D cell growth, it was decided to test the activity of our hS-hCGF preparation and to re-test the homogeneous hPGF for comparison in this bio-assay. In this study, hPGF culture was (surprisingly) mitogenic, however the culture method differed from that previously described by Rowe et al. (1986b). The cells were allowed to undergo one cell division before growth factor addition for the following reason: following trypsinization the T-47D cells were demonstrating a prolonged lag phase before entering exponential growth (personal communication, Leigh Murphy). During this lengthy lag phase, the cells may not be responsive to exogenous growth factors, the growth factor may simply be degraded and its mitogenic potential would remain unknown. This explanation appears to have been correct since addition of hS-CGF or hPGF following a 74 h pre-incubation in 10% FBS-supplemented media (and at least one cell division) resulted in significant growth-stimulation by both factors.

One can speculate on the possible role(s) of hMTGF and hPGF in the development of solid human mammary tumors. There is substantive evidence to suggest a role of FGFs in the neovascularization of solid tumors. The purification of bFGF-like heparin-binding activity from

mammary tumor tissue and its ability to stimulate human breast cancer cells in vitro suggests an autocrine or paracrine secretory mechanism for hMTGF. However, classic endocrine stimulation of mammary tumor growth by the pituitary-derived mitogens may also occur. The synergism shown between estrogen and FGF may indicate differing mechanisms of stimulation by FGF-like hPGF in estrogen responsive and non-responsive breast tumors.

Human foreskin fibroblasts have previously been shown to proliferate in the presence of human mammary tumor-derived (hMTGF) and human prostate-derived (PrGF) bFGF-like factors (Rowe et al., 1986; Story et al., 1987, respectively) (Table 1). Both research groups have speculated upon the influence of these factors on the growth of solid tumors in two possible ways -- by promoting the proliferation of the fibrostromal as well as the fibrovascular elements of the tumor. In BPH development, the ability of FGF to stimulate chondrocyte and osteoblast growth further suggests a function of this mitogen in promoting the boney metastasis observed in some patients (Story et al., 1987).

Balb/c3T3 cells are an established cell line of uncertain lineage (i.e. fibroblastic or endothelial) for which FGF is a potent mitogen (Gospodarowicz, 1979). Although recent studies have utilized stimulation of [3H]-thymidine incorporation as an index of DNA synthesis and

subsequent cell growth, early investigations demonstrated the ability of FGF to stimulate the proliferation of quiescent Balb/c3T3 cultures (Gospodarowicz and Moran, 1974a,b). Measurement of Balb/c3T3 proliferation was chosen in this study for comparison to other cell types.

Concentrations of hCGF preparation that stimulated the growth of other mesodermal cells types (i.e. chondrocytes, endothelial cells, breast cancer and foreskin fibroblasts) were inhibitory and even toxic to the Balb/c3T3 cells. Stimulation of these cells with extremely low levels of hPGF (~1 fg/ml) demonstrated the extreme sensitivity of these cells to the growth factor.

In their early studies, Gospodarowicz and Moran (1974) suggested that sparse cultures of 3T3 cells must be quiescent in order to respond to FGF. Their results showed the cells undergoing one cell doubling in the presence of 0.6% calf serum. Preliminary growth curves in the present study, produced a similar response in Balb/c3T3 cultures maintained in the presence of 1% FBS and this serum concentration was chosen for the dose response experiments. However, in this assay, the cells grew more rapidly; control cells underwent several cycles of cell division. Perhaps a greater fold stimulation of hPGF activity and stimulation by partially purified hS-hCGF and CF-hCGF would have occurred if the cells had been truly quiescent.

Nb_2 rat lymphoma cells are dependent on lactogenic hormones such as GH and PRL for proliferation (Tanaka et al., 1980). The presence of another Nb_2 cell mitogen, interleukin-2 (IL-2) has recently been established by members of our laboratory (Croze et al., 1988). Tumor promoting phorbol esters such as 12-0-tetradecanoyl-phorbol ester (TPA) enhance the growth stimulating effects of lactogens and to a lesser extent, IL-2 or Nb_2 cells. (Gertler et al., 1985; Croze et al., 1988). In the present study hCGF did not stimulate Nb_2 cell growth; the activity present in the hCGF preparation was apparently due to hGH contamination.

5.2 Immunological Analysis Of hCGF

The immunological analysis of the three hCGF preparations was undertaken to examine their relationship to bovine bFGF. The pituitary-derived mitogens hS-hCGF, hPGF and CF-hCGF show immunological cross-reactivity with polyclonal antibodies raised to synthe-tic bovine bFGF. The protein recovery of hS-hCGF had been too low to visualize on a silver stained SDS-polyacrylamide gel (data not shown). However, using the Western blotting technique, the MW of heparin-sepharose purified mitogen was shown to be 18 - 20 kD. This result confirmed the estimated MW determined with Sephadex G-100

chromatography (Too et al., 1987). The immunoreactive band of MW 18-20 kD visualized with hPGF was also similar to that reported previously (Rowe et al., 1986b). The MW of CF-hCGF was not determined using immunological detection due to an insufficient quantity of this preparation for analysis.

The doublet at MW 18-20 kD observed with bovine bFGF has also been reported for the cross-reaction of human hepatoma-derived bFGF (Klagsbrun et al., 1986). Since the hS-hCGF and hPGF bands were diffuse, the possibility of a microheterogeneous nature for these factors could not be determined.

When hS-hCGF was incubated with anti-bFGF antibodies corresponding to various regions (and functional domains) of the bFGF (1-146) molecule, hS-hCGF did not cross-react to anti-bFGF (30-50) antibodies. A comparison of the functional domains of bFGF, as described by Esch et al. (1985), with the corresponding bFGF antiserum is shown in Table 8.

Thus, hS-hCGF did not cross-react with the only bFGF antisera corresponding to the receptor activation site of the bFGF molecule. The reason for this result is unclear since human and bovine bFGF molecules are extremely well conserved throughout evolution, showing a 98.7% amino acid sequence homology (Abraham et al., 1986). A possible explanation may relate to a difference

TABLE 8: **Comparison of the functional domains of bFGF to the polyclonal antisera raised to synthetic fragments of bovine bFGF.**

bFGF residues	functional domain	bFGF antisera
18-22, 107-110	heparin-binding sites	1-24, 16-24
25-68	receptor activation site	30-50
37-40, 78-81	protein attachment to cell surface recognition sites	30-50, 69-87, 72-87

in the tertiary structure of the bovine and human mitogens which could cause a difference in their biological activity and/or antibody recognition. In fact, both the RFCs and the BCE cells showed a greater sensitivity to bovine pituitary bFGF (Collaborative Research) than to human pituitary hS-hCGF (Figures 4 and 9, respectively). This possibility is not without precedent since the potentiation of biological activity, increased receptor affinity and induction of epitope exposure of ECGF (aFGF) observed in the presence of heparin are all thought to be a result of conformational changes to the tertiary structure of the aFGF molecule by interaction with heparin (Schreiber et al., 1985). Since pure hPGF activity is also potentiated by heparin (Rowe et al., 1986b), the closely related, partially purified hS-hCGF may also interact with heparin and may even require heparin to alter immunological epitopes within its structure in order to recognize the bovine bFGF (30-50) antiserum.

5.3 Rat Pituitary Organ Culture Studies

Co-culture of chondrocytes with rat pituitary tissue caused a dose-dependent stimulation in RFC growth. The majority of the mitogenic activity could be localized to the anterior pituitary; corroborating the report by Baird et al., (1983) that cultured bovine adenohypophy-

seal cells release immunoreactive FGF into conditioned medium by some unknown mechanism. Recent studies have shown that the main cellular source of bFGF in the pituitary is the follicular cells (Gospodarowicz et al., 1987a, b). Follicular cells are prominent in the pars tuberalis region of the anterior pituitary. Since the pars tuberalis is the most highly vascularized region of the pituitary, a role of bFGF in growth and maintenance of the hypophyseal portal vessels has been suggested as the first known function of pituitary follicular cells.

Attempts to partially purify the activity in rat pituitary conditioned medium using heparin-Sepharose chromatography were not very successful since little activity could be recovered (data not shown). However, considering our previous report that ~98% of human pituitary-derived mitogenic activity for RFCs is heparin-binding and FGF-like, as well as the heparin-binding activity of cultured rat anterior pituitary cells mentioned above (Baird et al., 1985), it seems reasonable to assume that the mitogenic activity in the present studies is FGF-like.

Since bFGF lacks a signal peptide, the mechanism of release of this mitogen is uncertain. Capillary endothelial cells release bFGF (Schweiger et al., 1987c, Sato et al., 1988) into culture medium, as do anterior pituitary cells (Baird et al., 1985). In

these studies, the mechanism of release was either suggested to be cell lysis and/or leakage or was not mentioned. The immunoreactive FGF found in the rat anterior pituitary cultures was measured after only 3 days of culture in DMEM supplemented with 0.1% BSA. Likewise, the mitogenic activity detected in the rat pituitary conditioned medium in the present report could only be detected for a maximum of 4 to 6 days. These organ cultures were also maintained in medium containing 0.1% BSA. In both of these cases the pituitary cells had also suffered recent trauma, either collagenase treatment or mincing of the tissue, both of which would result in cell lysis or damage and could cause leakage of the mitogen into the culture medium.

In the rat pituitary/RFC co-cultured experiments, the pituitary-derived mitogenic activity was still detectable after prolonged maintenance of pituitary tissue in vitro. Since these studies were completed in media supplemented with 10% FBS, the lack of prolonged activity in the above studies could be the result of a lack of nutrients necessary to maintain tissue viability. Westall et al. (1983) reported the half-life of FGF in vitro to be 24 h. Slow leakage of mitogenic activity from rat pituitary cellular storage sites due to cell damage would therefore be detectable for only a few days, as was demonstrated in the sequential loss of activity in

the rat pituitary conditioned medium over 6 days of collection.

When the rat pituitary tissue was co-cultured with RFCs, mitogenicity was still apparent even after the tissue had been maintained in vitro for over 3 weeks. Considering the 24 h half-life of FGF as well as the slow rate of degradation of the internalized mitogen (Friesel and Maciag, 1988), the prolonged activity observed in the present experiments would suggest that the cellular stores of the mitogen would have been depleted long ago and that de novo synthesis and release of the mitogen was occurring. Since the activity present in damaged outer cells of the tissue fragments would have been released early in the assay, this would further suggest that the newly synthesized activity is being released from the cells by some other mechanism.

5.4 Human Pituitary Tumor Studies

The present study was completed to determine if the hCGF-like activity previously reported to be secreted from human pituitary fragments was a heparin-binding mitogen. In the present report the mitogenic effect of human pituitary conditioned medium on RFC proliferation descrbed by S. Kasper was not observed (Kasper, 1984; Kasper and Friesen, 1986). However, this study did

corroborate the previous work in that stimulation of DNA synthesis, an index of cell growth, by human pituitary conditioned medium, was demonstrated. The lack of effect on RFC growth may simply reflect sample variation in mitogenic activity, since the previous report was a observation of only one tumor (prolactinoma) as is the present study.

The heparin-binding ability of this pituitary tumor tissue activity suggests that it is similar to the FGFs. Whether this material is newly synthesized and released from the pituitary cells into the medium or whether its presence in the medium is simply the result of cell lysis or leakage is not known.

It should be noted that the serum samples from patients with pituitary adenomas utilized in the present experiments were 3-4 years old. Schreiber et al. (1985) reported that the mitogenic activity of aFGF decreased with prolonged storage, but at least some biologically active aFGF can be revitilized following binding to heparin. The heparin-binding activity found in these old serum samples could therefore be much more pro-nounced in fresh samples. Also as a consequence of sample age, no attempt was made to separate aFGF and bFGF-like activity (i.e. elution with 3M NaCl removes both mitogens). As such, repeat and elaboration of this investigation with 1M NaCl (aFGF) and 2-3M NaCl (bFGF) is warranted to

clearly establish the presence of aFGF and/or bFGF activity in human pituitary tumor conditioned medium and serum samples.

Mitogenic activity was detected in 3 of the 4 pituitary serum samples tested. Since the 3 mitogenic samples were taken from female patients with three different types of pituitary tumors (i.e. PRL or GH-secreting, and non-functional adenoma), this substantiates the report that pitutiary tumor type is not a significant aspect of growth factor activity (Kasper and Friesen, 1984). This result could relate to the probable association of the heparin-binding activity with the follicular cells of the pars tuberalis, rather than with the hormone-secreting lactotrophs or somatotrophs.

The results obtained with the one sample tested from a male patient (prolactinoma), was quite different from the female serum results. Although little activity could be detected in pituitary serum, the peripheral serum from this patient was quite mitogenic. Whether this result reflects an actual difference in growth factor activity associated with male versus female prolactinomas or is simply the result of sample variation would require further analysis with a large sample population to determine.

The presence of heparin-binding growth factor activity in the pituitary and peripheral serum of

pituitary tumor patients may correlate with a role in tumor angiogenesis and tumor growth -- mitogenic activity was not observed in male and female normal peripheral serum. This activity exhibits actions that are similar to the FGFs, namely, its affinity for heparin and stimulation of RFC growth in vitro.

Whether the human pituitary tumor-derived heparin-binding activity was actively secreted into the serum collected from the surgical site or into the conditioned medium is uncertain. In both cases, cell damage resulting from surgical removal of the tissue and from the mincing of the tissue prior to organ culture could have caused cellular leakage of the observed activity into the serum or medium.

The detection of growth factor activity in peripheral serum from pituitary tumor patients, and the ability of the activity in one sample to bind quite avidly to heparin-Sepharose indicates that this FGF-like activity is released from pituitary tissue (and possibly other sources) by some unknown mechanism.

Folkman's hypothesis that tumor angiogenesis must precede tumor growth may be manifested in pituitary tumorigenesis. bFGF has been localized to follicular cells in the highly vascular-ized pars tuberalis region of the anterior pituitary. A role for follicular bFGF in the normal growth and maintenance of hypophysial portal

vessels has been suggested. Capillary endothelial cells in the pituitary may also produce as well as release FGF to stimulate autocrine growth and neovascular-iz-ation and to support the growth of tumor cells. Invasion of the tumor site by mast cells and macrophages could activate ECM-associated FGF-heparin complexes allowing stimulation of adjacent cells via a paracrine mechanism. Other angiogenic factors such as TGF-alpha, TGF-B, and TNF-alpha either released by cells at the tumor site or chemoattracted to the area by the mast cells or macrophages, could further stimulate tumor growth. In the case of estrogen-dependent prolactin-secreting tumors, estrogen, which can directly stimulate the release of bFGF in pituitary cultures, (Baird et al., 1985) may also stimulate release of this mitogen in vivo. The bFGF gene is expressed in normal bovine pituitaries. (Abraham et al., 1986a) and in human primary intracranial tumors (Murphy et al., 1988b) where the level of gene expression is elevated. Elevated expression of bFGF in cultured rhabdomyo-sarcoma cells is associated with autocrine growth stimulation of these cells (Schweigerer et al., 1987). bFGF and TGF-B (also present in a variety of human tumors) gene expression should be examined in estrogen-dependent and independent pituitary tumors. The presence of the bFGF proto-oncogenes int-2 and hst/KS should also be evaluated.

5.5 Swarm Rat Chondrosarcoma *In Vivo* Study

A preliminary study utilizing the Swarm rat chondrosarcoma, has demonstrated a stimulatory effect of hCGF on the *in vivo* growth of this tumor. Several normal growth processes, including body weight gain, increased nose-to-tail length and testicular weight gain may also be responsive to pituitary-derived hCGF. Although no difference in these normal growth-related events were observed in normal rats, a trend towards stimulation of these events was observed in the hCGF-treated HPX animals. Use of a purified preparation of hCGF may establish a significant stimulation of normal growth processes.

The inhibition of tumor growth observed in the animals administered growth hormone may be the result of down regulation of receptors and decreased responsiveness of the GH-dependent chondrosarcoma to circulating GH and somatomedins.

In addition to promoting chondrosarcoma growth, hCGF also affected the morphological appearance of this tumor in both normal and HPX animals. The well-defined pink nodular configuration and peripheral vascularization described as the typical tumor morphology (Breitkrutz *et al.*, 1979) were apparent in hCGF-treated rats but not in

the hGH-treated animals. The hCGF effects may reflect FGF-like regulatory role(s) for this mitogen on the endothelial cells and chondrocytes present in this tumor. Pituitary-derived hCGF may have promoted the tumor angiogenesis required for maintenance and growth of the chondrosarcoma *in vivo*.

6. SUMMARY

The human pituitary-derived mitogen, hCGF is similar if not identical to bovine pituitary bFGF based on several criteria. Biochemically, both mitogens are similar in MW and isoelectric points and are acid, heat and trypsin sensitive. The mitogens share a strong affinity for heparin, requiring 1.5 M NaCl for elution from heparin-Sepharose. Mitogens sharing the above characteristics have been termed heparin-binding growth factors. Pure hCGF, designated hPGF, is identical to bFGF at its amino-terminus.

Human CGF cross-reacts with bovine bFGF antisera raised to specific regions of the synthetic bFGF molecule. The lack of cross-reactivity of hCGF to antisera against residues 30-50 of bFGF may reflect a difference in the tertiary arrangement of the human and bovine mitogens. Addition of heparin to the incubation may enhance epitope recognition as is observed in the case of aFGF.

The biological activity of hCGF in vitro was examined using several mesodermal cell types. As summarized in Table 7, hCGF was a potent mitogen for RFCs causing a 6-fold increase in cell number. The increase in cell number observed with hCGF addition on BCE, T-47D and fibroblast cell growth, although not as dramatic as for the RFCs was significant. The MCH6 and Balb/c3T3 cells were particularly sensitive to hCGF, requiring < 1ng/ml to

elicit a mitogenic response.

Addition of EGF or TGF-alpha to RFCs did not stimulate cell growth in the presence of 10% serum, but did promote thymidine uptake by the cells. Similar to previous reports comparing the effects of FGF and EGF in BCE cells, the cells were more sensitive to EGF than to hCGF. The effects of hCGF on the morphology and doubling time of RFC and BCE cells was also similar to FGF addition. The stimulatory effects of hCGF on cells of mesodermal origin demonstrates the similar biological activities of hCGF and FGF in vitro.

The mechanism of release of pituitary-derived FGF-like activity was examined using a rat pituitary organ culture system. Conditioned medium from the pituitaries stimulated RFC proliferaton, demonstrating the release of activity from the tissue. Activity was localized to the anterior pituitary. Long-term rat pituitary/RFC co-culture experiments suggested that FGF-like activity was being produced and released from the tissue to stimulate RFC growth directly. The release of the activity from the pituitary tissue was probably initially the result of cell lysis and leakage, although the continued release detected in sequential assays, may suggest a second unknown mechanism of release was also occurring.

Heparin-binding mitogenic activity was partially purified from human pituitary tumor fragments as well as from peripheral serum and serum removed from the site of

adenomectomy in several patients. The type of pituitary adenoma had no effect on the FGF-like activity.

The in vivo effects of hCGF were examined using the transplantable rat chondrosarcoma tumor model. A trend towards stimulation of overall body growth as well as testicular growth was observed in hypophysectomized animals. In normal animals, an increase in the weight of the chondrosarcoma in animals injected with hCGF was apparent. Since anti-FGF antibodies have recently been reported to inhibit the growth of a transplantable mouse chondrosarcoma, these results indicate that hCGF and FGF have similar in vivo actions as well.

The extreme interest in growth factor research, is indicated by the wide variety of mitogens which were purified from a multitude of sources and eventually shown to be similar or identical to the FGFs. Present research emphasis in this area suggests that the the FGF family of mitogens regulate normal and neoplastic growth using paracrine and/or autocrine mechanisms. The primary function of FGF is thought to be as an angiogenic agent stimulating production of the blood vessels required for the growth of normal and tumor cells. Although the bFGF and aFGF do not contain classical secretory signals, the recent findings of oncogenes that code for FGF-like proteins and carry signal sequences suggests a strong influence of this (almost) ubiquitous family of growth factors on growth processes.

7. REFERENCES

Abraham, J.A., Whang, J.L., Tumolo, A., Mergia, A., Friedman, J., Gospodarowicz, D., and Fiddes, J.C. (1986b) Human basic fibroblast growth factor: nucleotide sequence and genomic organization. EMBO J. 5: 2523-2528.

Abraham, J.A., Mergia, A., Whang, J.L., Tumolo, A., Friedman, J., Hjerrild, K.A., Gospodarowicz, D., and Fiddes, J.C. (1986a) Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. Science 233: 545-548.

Abraham, J.A., Whang, J.L., Tumolo, A., Mergia, A., and Fiddes, J.C. (1986c) Human basic fibroblast growth factor: nucleotide sequence, genomic organization, and expression in mammalian cells. Cold Spring Harbor Symp. Quant. Biol. 51: 657-668.

Albini, A., Mitchell, C.D., Thompson, E.W., Seeman, R., Martin, G.R., Wittek, A.E., and Quinnan, G.V. (1988) Invasive activity and chemotactic response to growth factors by Kaposi's sarcoma cells. J. Cell. Biochem. 36: 369-376.

Armelin, H.A., Armelin, M.C.S., Kelly, K., Stewart, T., Leder, P., Cochran, B.H., and Stiles, C.D. (1984) Functional role for c-myc in mitogenic response to platelet-derived growth factor. Nature 310: 655-660.

Armelin, H.A. (1973) Pituitary extracts and steroid hormones in the control of 3T3 cell growth. Proc. Natl. Acad. Sci. USA. 70: 2702-2706.

Assoian, R.K. and Sporn, M.B. (1986) Type B transforming growth factor in human platelets: release during platelet degranulation and action on vascular smooth muscle cells. J. Cell. Biol. 102: 1217-1223.

Auron, P.E., Webb, A.C., Rosenwasser, L.J., Mucci, S.F., Rich, A., Wolff, S.M., and Dinarello, C.A. (1984) Nucleotide sequence of human monocyte interleukin 1 precursor cDNA. Proc. Natl. Acad. Sci. USA. 81: 7907-7911.

Baird, A., Mormede, P., Ying, S-Y., Wehrenberg, W.B., Ueno, N., Ling, N., and Guillemin, R. (1985d) A nonmitogenic pituitary function of fibroblast growth factor: regulation of thyrotropin and prolactin secretion. Proc. Natl. Acad. Sci. USA. 82: 5545-5549.

Baird, A. and Ling, N. (1987) Fibroblast growth factors are present in the extracellular matrix produced by endothelial cells in vitro: implications for a role of heparinase-like enzymes in the neovascular response. Biochem. Biophys. Res. Commun. 142: 428-435.

Baird, A. and Durkin, T. (1986) Inhibition of endothelial cell proliferation by type B-transforming growth factor: interactions with acidic and basic fibroblast growth factors. Biochem. Biophys. Res. Commun. 138: 476-482.

Baird, A., Esch, F., Gospodarowicz, D., and Guillemin, R. (1985b) Retina- and eye-derived endothelial cell growth factors: partial molecular characterization and identity with acidic and basic fibroblast growth factors. Biochemistry 24: 7855-7860.

Baird, A., Mormede, P.,and Bohlen, P. (1986) Immunoreactive fibroblast growth factor (FGF) in a transplantable chondrosarcoma: inhibition of tumor growth by antibodies to FGF. J. Cell. Biochem. 30: 79-85.

Baird, A., Esch, F., Bohlen, P., Ling, N., and Gospodarowicz, D. (1985a) Isolation and partial characterization of an endothelial cell growth factor from the bovine kidney: homology with basic fibroblast growth factor. Regul. Peptides. 12: 201-213.

Baird, A., Esch, F., Mormede, P., Ueno, N., Ling, N., Bohlen, P., Ying, S-Y., Wehrenberg, W.B., and Guillemin, R. (1986) Molecular characterization of fibroblast growth factor: distribution and biological activities in various tissues. Rec. Prog. in Horm. Res. 42: 143-205.

Baird, A., Mormede, P., and Bohlen, P. (1985c) Immunoreactive fibroblast growth factor in cells of peritoneal exudate suggests its identity with macrophage-derived growth factor. Biochem. Biophys. Res. Commun. 126: 358-364.

Barritault, D., Plouet, J., Courty, J., and Courtois, Y. (1982) Purification, characterization, and biological properties of the eye-derived growth factor from retina: analogies with brain-derived growth factor. J. Neurosci. Res. 8: 477-490.

Betsholtz, C., Westermark, B., Ek, B., and Heldin, C-H. (1984) Coexpression of a PDGF-like growth factor and PDGF receptors in a human osteosarcoma cell line: implications for autocrine receptor activation. Cell 39: 447-457.

Bohlen, P., Esch, F., Baird, A., Jones, K.L., and Gospodarowicz, D. (1985) Human brain fibroblast growth factor. Isolation and partial chemical characterization. FEBS Lett. 185: 177-181.

Bohlen, P., Baird, A., Esch, F., Ling, N., and Gospodarowicz, D. (1984) Isolation and partial molecular characterization of pituitary fibroblast growth. Proc. Natl. Acad. Sci. USA. 81: 5364-5368.

Bradshaw, R.A. and Sporn, M.B. (1983) Polypeptide growth factors and the regulation of cell growth and differentiation. FASEB (Symp.): 2590-2591.

Breakefield, X.O. and Stern, D.F. (1986) Oncogenes in neural tumors. Trends in Neurosci. pp. 150-185.

Breitkrutz, D., Diaz de Leon, L., Paglia, L., Gay, S., Swarm, R., and Stern, R. (1979) Histological and biochemical studies of a transplantable rat chondrosarcoma. Cancer Res. 39: 5093-5100.

Brigstock, D.R., Laurie, M.S., Heap, R.B., Brown, K.D. (1986) Characterization of an acidic, heparin-binding growth factor from pig uterus. Biol. Reprod. (Suppl., Abstr.) 34: 167.

Broad, T.E. and Itam, R.G. (1983) Growth and adipose differentiation of sheep preadipocytes fibroblasts in serum free medium. Eur. J. Biochem. 135: 33-39.

Burgess, W.H., Mehlman, T., Friesel, R., Johnson, W.V., and Maciag, T. (1985) Multiple forms of endothelial cell growth factor: rapid isolation and biological characterization. J. Biol. Chem. 260: 11389-11392.

Burnette, W.N. (1981) "Western Blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. 112: 195-203.

Carpenter, G., Stoscheck, C.M., Preston, Y.A., and DeLarco, J.E. (1983) Antibodies to the epidermal growth factor receptor block the biological activities of sarcoma growth factor. Proc. Natl. Acad. Sci. USA. 80: 5627-5630.

Carpenter, G. and Cohen, S. (1979) Epidermal growth factor. Ann. Rev. Biochem. 48: 193-216.

Carswell, E.A. Old, L.J. Kassel, R.L. Green, S. Fiore, N., and Williamson, B. (1975) Proc. Natl. Acad. Sci. USA. 72: 3666-3670.

Castellot, J.J., Addinizio, M.L., Rosenberg, R.D., and Karnovsky, M.J. (1981) Cultured endothelial cells produce a heparin-like inhibitor of smooth muscle cell growth. J. Cell Biol. 90: 372-379.

Castellot, J.J., Hoover, R.L., Harper, P.A., and Karnovsky, M.J. (1985) Heparin and glomerular epithelial cell-secreted heparin-like species inhibit mesanglial-cell proliferation. Am. J. Pathol. 120: 427-435.

Chiang, C.P. and Nilsen-Hamilton, M. (1986) Opposite and selective effects of epidermal growth factor and human platelet transforming growth factor-beta on the production of secreted proteins by murine 3T3 cells and human fibroblasts. J. Biol. Chem. 261: 10478-10481.

Chodak, G.W., Hospelhorn, V., Judge, S.M., Mayforth, R., Koeppen, H., Sasse, J. (1988) Increased levels of fibroblast growth factor-like activity in urine from patients with bladder or kidney cancer. Cancer Res. 48: 2083-2088.

Chodak, G.W., Shing, Y., Borge, M., Judge, S.M., and Klagsbrun, M. (1986) Presence of heparin binding growth factor in mouse bladder tumors and urine from mice with bladder cancer. Cancer Res. 46: 5507-5510.

Clark, J.L., Jones, K.L., Gospodarowicz, D., and Sato, G.H. (1972) Growth response to hormones by a new rat ovary cell line. Nature 236: 180-181.

Clegg, C.H., Linkhart, T.A., Olwin, B.B., Hauschka, S.D. (1987) Growth factor control of skeletal muscle differentiation: commitment to terminal differentiation occurs in G1 phase and is repressed by fibroblast growth factor. J. Cell Biol. 105: 949-956.

Clowes, A.W. and Karnovsky, M.J. (1977) Suppression by heparin of smooth muscle cell proliferation in injured arteries. Nature 265: 625-626.

Cochet, C., Gill, G.N., Meisenhelder, J., Cooper, J.A., and Hunter, T. (1984) C-Kinase phosphorylates the epidermal growth factor receptor and reduces its EGF-stimulated tyrosine protein kinase activity. J. Biol. Chem. 259: 2553-2558.

Cohen, S., Ushiro, H., Stoscheck, C., and Chinkers, M. (1982) A native 170,000 epidermal growth factor receptor-kinase complex from shed plasma membrane vesicles. J. Biol. Chem. 257: 1523-1531.

Cohen, S., Carpenter, G., and King, L., Jr. (1980) Epidermal growth factor-receptor-protein kinase interactions: co-purification of receptor and epidermal growth factor-enhanced phosphorylation activity. J. Biol. Chem. 255: 4834-4842.

Corvol, M-T., Malemud, C.J., and Sokoloff, L. (1972) A pituitary growth-promoting factor for articular chondrocytes in monolayer culture. Endocrinology 90: 262-271.

Coughlin, S.R., Barr, P.J., Cousens, L.S., Fretto, L.J., and Williams, L.S. (1988) Acidic and basic fibroblast growth factors stimulate tyrosine kinase activity in vivo. J. Biol. Chem. 263: 988-993.

Courty, J., Loret, C., Moenner, M., Chevallier, B., Lagente, O., Courtois, Y., and Bamtault, D. (1985) Bovine retina contains 3 growth factors with different affinities for heparin: eye-derived growth factor I, II, III. Biochemie 67: 265-269.

Crabb, J.W., Armes, L.G., Johnson, C.M., and McKeehan, W.L. (1986) Characterization of multiple forms of prostatropin (prostate epithelial cell growth factor) from bovine brain. Biochem. Biophys. Res. Commun. 136: 1155-1161.

Croze, F., Walker, A., and Friesen, H.G. (1988) Stimulation of growth of Nb2 lymphoma cells by interleukin-2 in serum-free and serum-containing media. Mol. Cell. Endocrinol. 55: 253-259.

Crum, R., Szabo, S., and Folkman, J. (1985) A new class of steroids inhibits angiogenesis in the presence of heparin or a heparin fragment. Science 230: 1375-1378.

D'Amore, P.A. and Klagsbrun, M. (1984) Endothelial cell mitogens derived from retina and hypothalamus: biochemical and biological similarities. J. Cell Biol. 99: 1545-1549.

DeLarco, J.E. and Todaro, G.J. (1980) Sarcoma growth factor (SGF): specific binding to epidermal growth factor (EGF) membrane receptors. J. Cell. Physiol. 102: 267-277.

Delli Bovi, P., Curatola, A.M., Kern, F.G., Greco, A., Ittman, M., and Basilico, C. (1987) An oncogene isolated by transfection of Kaposi's sarcoma DNA encodes a growth factor that is a member of the FGF family. Cell 50: 729-737.

Delli Bovi, P., and Basilico, C. (1987) Isolation of a rearranged human transforming gene following transfection of Kaposi's sarcoma DNA. Proc. Natl. Acad. Sci. USA. 84: 5660-5664.

Dembinski, T.C., Leung, C.K.H, and Shiu, R.P.C. (1985) Evidence for a novel pituitary factor that potentiates the mitogenic effect of estrogen in human breast cancer cells. Cancer Res. 45: 3083-3089.

Denhardt, D.T., Hamilton, R.T., Parfett, C.L., Edwards, D.R., St. Pierre, R., Waterhouse, P., and Nilsen-Hamilton, M. (1986) Close relationship of the major excreted protein of transformed murine fibroblasts to thiol-dependent cathepsins. Cancer Res. 46: 4590-4593.

Dernyk, R., Jarrett, J.A., Chen, E.Y., Eaton, D.H., Bell, J.R., Assoian, R.K., Roberts, A.B., Sporn, M.B., and Goeddel, D.V. (1985) Human transforming growth factor-beta cDNA sequence and expression in tumour cell lines. Nature 316: 701-705.

Derynck, R., Roberts, A.B., Winkler, M.E., Chen, E.W., and Goeddel, D.V. (1984) Human transforming growth factor-alpha: precursor structure and expression in E. coli. Cell 38: 287-297.

Deuel, T.F., Huang, J.S., Huang, S.S., Stroobant, P., and Waterfield, M.D. (1983) Expression of a platelet-derived growth factor-like protein in simian sarcoma virus transformed cells. Science 221: 1348-1350.

Dickson, C., Smith, R., Brookes, S., and Peters, G. (1984) Tumorigenesis by mouse mammary tumor virus: proviral activation of a cellular gene in the common integration region int-2. Cell 37: 529-536.

Dickson, C. and Peters, G. (1987) Potential oncogene product related to growth factors. Nature 326: 833.

Dodge, W.H. (1985) Avian monocytic leukemia cells release fibroblast growth factor: implications to associated myelofibrosis. Leukemia Res. 9: 1559-1564.

Doolittle, R.F., Hunkapiller, M.W., Devare, S.G., Robbins, K.C., Aaronson, S.A., and Antoniades, H.N. (1983) Simian sarcoma virus onc. gene, v-sis, is derived from the gene (or genes) encoding a platelet-derived growth factor. Science 221: 275-277.

Downward, J., Yarden, Y., Mayes, E., Scrace, E., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J., and Waterfield, M.D. (1984) Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. Nature 307: 521-527.

Dryja, T.P., Cavenee, W., White, R., Rapaport, J.M., Petersen, R., Albert, D.M., and Bruns, G.A.P. (1984) Homozygosity of chromosome 13 in retinoblastoma. N. Engl. J. Med. 310: 550-553.

Ek, B. and Heldin, C-H. (1982) Characterization of a tryosine-specific kinase activity in human fibroblast membranes stimulated by platelet-derived growth factor. J. Biol. Chem. 257: 10486-10492.

Esch, F., Baird, A., Ling, N., Ueno, N., Hill, F., Denoroy, L., Klepper, R., Gospodarowicz, D., Bohlen, P., and Guillemin, R. (1985b) Primary structure of bovine pituitary basic fibroblast growth factor (FGF) and comparison with the amino terminal sequence of bovine brain acidic FGF. Proc. Natl. Acad. Sci. USA. 82: 6507-6511.

Esch, F., Ueno, N., Baird, A., Hill, F., Denoroy, L., Ling, N., Gospodarowicz, D., and Guillemin, R. (1985a) Primary structure of bovine brain acidic fibroblast growth factor. Biochem. Biophys. Res. Commun. 133: 554-562.

Fanger, B.D., Wakefield, C.M., and Sporn, M.B. (1986) Structure and properties of the cellular receptor for TGFb. Biochemistry 25: 3083-3091.

Fearon, E.R., Vogelstein, B., and Feinberg, A.P. (1984) Somatic deletion and duplication of genes on chromosome 11 in Wilm's tumours. Nature 309: 176-178.

Fett, J.W., Strydom, D.J., Lobb, R.R., Alderman, E.M., Bethune, J.L., Riordan, J.F., and Vallee, B.L. (1985) Isolation and characterization of angiogenin, an angiogenic protein from human carcinoma cells. Biochemistry 24: 5480-5486.

Folkman, J. (1986) How is blood vessel growth regulated in normal and neoplastic tissue? G.H.A. Clowes memorial award lecture. Cancer Res. 46: 467-473.

Folkman, J. and Klagsbrun, M. (1987b) A family of angiogenic peptides. Nature 329: 671-672.

Folkman, J. and Klagsbrun, M. (1987a) Angiogenic factors. Science 235: 442-447.

Folkman, J. and Klagsbrun, M. (1987) A family of angiogenic peptides. Nature 329: 671-672.

Folkman, J., Langer, R., Linhardt, R.J., Haudenschild, C., and Taylor, S. (1983) Angiogenesis inhibition and tumor regression caused by heparin fragment in the presence of cortisone. Science 221: 719-725.

Frater-Schroder, M., Muller, G., Birchmeier, M., and Bohlen, P. (1986) Transforming growth factor-beta inhibits endothelial cell proliferation. Biochem. Biophys. Res. Commun. 137: 295-302.

Friesel, R., Burgess, W.H., Mehlman, T., and Maciag, T. (1986) The characterization of the receptor for endothelial cell growth factor by covalent ligand attachment. J. Biol. Chem. 261: 7581-7584.

Friesel, R. and Maciag, T. (1988) Internalization and degradation of heparin binding growth factor-1 by endothelial cells. Biochem. Biophys. Res. Commun. 151: 957-964.

Frolik, C.A., Dart, L.L., Meyers, C.A., Smith, D.M., and Sporn, M.B. (1983) Purification and initial characterization of a type-beta transforming growth factor from human placenta. Proc. Natl. Acad. Sci. USA. 80: 3676-3680.

Gambarini, A.G. and Armelin, H.A. (1982) Purification and partial characterization of an acidic fibroblast growth factor from bovine pituitary. J. Biol. Chem. 257: 9692-9697.

Gautschi-Sova, P., Jiang, Z., Frater-Schroder, M., and Bohlen, P. (1987) Acidic fibroblast growth factor is present in nonneural tissue: isolation and chemical characterization from bovine kidney. Biochemistry 26: 5844-5847.

Gertler, A., Walker, A., and Friesen, H.G. (1985) Enhancement of human growth hormone-stimulated mitogenesis of Nb2 node lymphoma cells by 12-0-tetradecanoyl-phorbol-13-acetate. Endocrinology 116: 1636-1644.

Gimenez-Gallego, G., Conn, G., Hatcher, V.B., and Thomas, K.A. (1986b) The complete amino acid sequence of human brain-derived acidic fibroblast growth factor. Biochem. Biophys. Res. Commun. 138: 611-617.

Gimenez-Gallego, G., Conn, G., Hatcher, V.B., and Thomas, K.A. (1986a) Human brain-derived acidic and basic fibroblast growth factors: amino terminal sequences and specific mitogenic activities. Biochem. Biophys. Res. Commun. 135: 541-548.

Gimenez-Gallego, G., Rodkey, J., Bennett, C., Rios-Candelore, M., DiSalvo, J., Thomas, K. (1985) Brain-derived acidic fibroblast growth factor: complete amino acid sequence and homologies. Science 230: 1385-1388.

Gospodarowicz, D. and Moran, J.S. (1975) Mitogenic effect of fibroblast growth factor on early passage cultures of human and murine fibroblasts. J. Cell Biol. 66: 451-456.

Gospodarowicz, D., Cheng, J., Lui, G-M., Baird, A., Esch, F., and Bohlen, P. (1985b) Corpus luteum angiogenic factor is related to fibroblast growth factor. Endocrinology 117: 2283-2291.

Gospodarowicz, D., Vlodavsky, I., and Savion, N. (1980) The extracellular matrix and the control of proliferation of vascular endothelial and vascular smooth muscle cells. J. Supramol. Struct. 13: 339.

Gospodarowicz, D. and Mescher, A.L. (1981) Fibroblast growth factor and vertebrate regeneration. In: Riccardi, V.M., Mulvihill, J.J. (eds.) Advances in Neurology: Neurofibromatosis. Raven Press, New York, vol. 29: 149-171.

Gospodarowicz, D. and Mescher, A.L. (1977) A comparison of the responses of cultured myoblasts and chondrocytes to fibroblast and epidermal growth factors. J. Cell. Physiol. 93: 117-128.

Gospodarowicz, D., Vlodavsky, I., and Savion, N. (1981) The role of fibroblast growth factor and the extracellular matrix in the control of proliferation and differentiation of corneal endothelial cells. Vision Res. 21: 87-103.

Gospodarowicz, D., Moran, J.S., Braun, D.L., and Birdwell, C.R. (1976) Clonal growth of bovine vascular endothelial cells: fibroblast growth factor as a survival agent. Proc. Natl. Acad. Sci. USA. 73: 4120-4124.

Gospodarowicz, D., Hirabayashi, L., Giguere, L., and Tauber, J-P. (1981) Factors controlling the proliferative rate, final cell density, and life span of bovine vascular smooth muscle cells in culture. J. Cell Biol. 89: 568-578.

Gospodarowicz, D. (1983) Growth factors and their action in vivo and in vitro. J. Pathol. 141: 201-233.

Gospodarowicz, D., Cheng, J., Lui, G-M., Fujii, D.K., Baird, A., and Bohlen, P. (1985c) Fibroblast growth factor in the human placenta. Biochem. Biophys. Res. Commun. 128: 554-562.

Gospodarowicz, D. (1974) Localization of a fibroblast growth factor and its effect alone and with hydrocortisone on 3T3 cell growth. Nature 249: 123-127.

Gospodarowicz, D. and Moran, J. (1974a) Effect of a fibroblast growth factor, insulin, dexamethasone, and serum on the morphology of Balb/c3T3 cells. Proc. Natl. Acad. Sci. USA. 71: 4648-4672.

Gospodarowicz, D., Cheng, J., Lui, G-M., Baird, A., and Bohlen, P. (1984) Isolation of brain fibroblast growth by heparin-sepharose affinity chromatography: identity with pituitary fibroblast growth factor. Proc. Natl. Acad. Sci. USA. 81: 6963-6967.

Gospodarowicz, D., Ferrara, N., Schweigerer, L., and Neufeld, G. (1987) Structural characterization and biological functions of fibroblast growth factor. Endocrine Rev. 8: 95-114.

Gospodarowicz, D. (1975) Purification of a fibroblast growth factor from bovine pituitary. J. Biol. Chem. 250: 2515-2520.

Gospodarowicz, D., Greenberg, G., Bialecki, H., and Zeller, B.R. (1978) Factors involved in the modulation of cell proliferation in vivo and in vitro: the role of fibroblast and epidermal growth factors in the proliferative response of mammalian cells. In Vitro 14: 85-118.

Gospodarowicz, D. and Moran, J.S. (1974b) Stimulation of division of sparse and confluent 3T3 cell populations by a fibroblast growth factor, dexamethasone, and insulin. Proc. Natl. Acad. Sci. USA. 71: 4584-4588.

Gospodarowicz, D., Bialecki, H., and Greenburg, G. (1978a) Purification of the fibroblast growth factor activity from bovine brain. J. Biol. Chem. 253: 3736-3743.

Gospodarowicz, D., Lui, G-M., and Cheng, J. (1982) Purification in high yield of brain fibroblast growth factor by preparative isoelectric focusing at pH 9.6. J. Biol. Chem. 257: 12266-12276.

Gospodarowicz, D., Ill, C.R., and Birdwell, C.R. (1977b) Effects of fibroblast and epidermal growth factors on ovarian cell proliferation in vitro. II. Proliferative response of luteal cells to FGF but not EGF. Endocrinology 100: 1121-1128.

Gospodarowicz, D., Moran, J.S., and Braun, D.L. (1977) Control of proliferation of bovine vascular endothelial cells. J. Cell. Phys. 91: 377-386.

Gospodarowicz, D., Mescher, A.L., and Birdwell, C.R. (1978b) Control of cellular proliferation by the fibroblast and epidermal growth factors. Natl. Cancer Inst. Monogr. 48: 109-130.

Gospodarowicz, D., and Moran, J.S. (1976) Growth factors in mammalian cell culture. Ann. Rev. Biochem. 45: 531-558.

Gospodarowicz, D., Weseman, J., Moran, J., and Linstrom, J. (1976) Effect of fibroblast growth factor on the division and fusion of bovine myoblasts. J. Cell Biol. 70: 395-405.

Gospodarowicz, D., Baird, A., Cheng, J., Lui, G-M., Esch, F., and Bohlen, P. (1986a) Isolation of a fibroblast growth factor from bovine adrenal gland: physiochemical and biological characterization. Endocrinology 118: 82-90.

Gospodarowicz, D. (1979) Fibroblast and epidermal growth factors: their uses in vivo and in vitro in studies on cell functions and cell transplantation. Mol. Cell. Biochem. 25: 79-110.

Gospodarowicz, D., Massoglia, S., Cheng, J., Lui, G-M., and Bohlen, P. (1985a) Isolation of pituitary fibroblast growth factor by fast protein liquid chromatography (FPLC): partial chemical and biological characterization. J. Cell. Phys. 122: 323-332.

Gospodarowicz, D., Neufeld, G., and Schweigerer, L. (1986b) Fibroblast growth factor. Mol. Cell. Endocrinol. 46: 187-204.

Gospodarowicz, D., Jones, K.L., and Sato, G. (1974) Purification of a growth factor for ovarian cells from bovine pituitary glands. Proc. Natl. Acad. Sci. USA. 71: 2295-2299.

Gout, P.W. Beer, C.T., and Noble, R.L. (1980) Cancer Res. 40: 2433-2436.

Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J-M., Argos, P., and Chambon, P. (1986a) human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. Nature 320: 134-139.

Green, G.L., Gilna, P., Waterfield, M., Baker, A., Hort, Y., and Shine, J. (1986b) Sequence and expression of human estrogen receptor complementary DNA. Science 231: 1150-1154.

Greenburg, G., Vlodavsky, I., Foidart, J.M., and Gospodarowicz, D. (1980) Conditioned medium from endothelial cell cultures can restore the normal phenotypic expression of vascular endothelium maintained in vitro in the absence of fibroblast growth factor. J. Cell. Phys. 103: 333-347.

Gross, J.L., Moscatelli, D., Jaffe, E.A., and Rifkin, D.B. (1982) Plasminogen activator and collagenase production by cultured capillary endothelial cells. J. Cell Biol. 95: 974-981.

Halaban, R., Ghosh, S., and Baird, A. (1987) bFGF is the putative natural growth factor for human melanocytes. In Vitro Cell. and Devl. Biol. 23: 47-52.

Harper, J.W., Auld, D.S., Riordan, J.F., and Vallee, B.L. (1988) Enzymatically active angiogenin/ribonuclease A hybrids formed by peptide interchange. Biochemistry 27: 219-226.

Harper, J.W., Strydom, D.J., and Lobb, R.R. (1986) Human class 1 heparin-binding growth factor: structure and homology to bovine acidic brain fibroblast growth factor. Biochemistry 25: 4097-4103.

Hatten, M.E., Lynch, M., Rydel, R.E., Sanchez, J., Joseph-Silverstein, J., Moscatelli, D., and Rifkin, D.B. (1988) In Vitro neurite extension by granule neurons is dependent upon astroglial-derived fibroblast growth factor. Devl. Biol. 125: 280-289.

Hoffman, R.S. (1940) The growth-activating effect of extracts of adult and embryonic tissues of rat on fibroblast colonies in vitro. Growth 4: 361-376.

Holley, R.W. and Kiernan, J.A. (1968) "Contact inhibition" of cell division in 3T3 cells. Proc. Natl. Acad. Sci. USA. 60: 300-304.

Huang, S.S. and Huang, J.S. (1986) Association of bovine brain-derived growth factor receptor with protein tyrosine kinase activity. J. Biol. Chem. 261: 9568-9571.

Jacobs, S., Kull, F.C., Jr., Earp, H.S., Svoboda, M.E., Van Wyk, J.J., and Cuatrecasas, P. (1983) Somatomedin-C stimulates the phosphorylation of the B-subunit of its own receptor. J. Biol. Chem. 258: 9581-9584.

Jahn, R., Schiebler, W., and Greengard, P. (1984) A quantitative dot-immunobinding assay for proteins using nitrocellulose membrane filters. Proc. Natl. Acad. Sci. USA. 81: 1684-1687.

Jakobovits, A., Shackleford, G.M., Varmus, H.E., and Martin, G.R. (1986) Two proto-oncogenes implicated in mammary carcinogenesis, int-1 and int-2, are independently regulated during mouse developement. Proc. Natl. Acad. Sci. USA. 83: 7806-7810.

Jaye, M., Howk, R., Burgess, W., Ricca, G.A., Chiu, I-M., Ravera, M.W., O'Brien, S.J., Modi, W.S., Maciag, T., and Drohan, W. (1986) Human endothelial cell growth factor: cloning, nucleotide sequence and chromosome localization. Science 233: 541-545.

Jaye, M., Lyall, R.M., Mudd, R., Schlessinger, J., and Sarver, N. (1988) Expression of acidic fibroblast growth factor cDNA confers growth advantage and tumorigenesis to Swiss 3T3 cells. EMBO J. 7: 963-969.

Jeanny, J.C., Fayein, N., Moenner, M., Chevallier, B., Barritault, D., and Courtois, Y. (1987) Specific fixation of bovine brain and retinal acidic and basic fibroblast growth factors to mouse embryonic eye basement membranes. Expl. Cell. Res. 171: 63-75.

Kahn, C.R. (1985) The molecular mechanism of insulin action. Ann. Rev. Med. 36: 429-451.

Kaibuchi, K., Tsuda, T., Kikuchi, A., Tanimoto, T., Yamashita, T., and Takai, Y. (1986) Possible involvement of protein kinase C and calcium ions in growth factor induced expression of c-myc oncogene in Swiss 3T3 cells. J. Biol. Chem. 261: 1187-1192.

Kamata, T. and Feramisco, J.R. (1984) Epidermal growth factor stimulates guanine nucleotide binding activity and phosphorylation of ras oncogene proteins. Nature 310: 147-150.

Kardami, E., Spector, D., and Strohman, R.C. (1985) Myogenic growth factor present in skeletal muscle is purified by heparin-affinity chromatography. Proc. Natl. Acad. Sci. USA. 82: 8044-8047.

Kasper, S., Worsley, I.G., Rowe, J.M., Shiu, R.P.C., and Friesen, H.G. (1982) Chondrocyte growth factor from the human pituitary gland. J. Biol. Chem. 257: 5226-5230.

Kasper, S. (1984) Ph.D. Thesis, University of Manitoba, Winnipeg, Manitoba, Canada.

Kasper, S. and Friesen, H.G. (1986) Human pituitary tissue secretes a potent growth factor for chondrocyte proliferation. J. Clin. Endocrinol. and Metab. 62: 70-76.

Kato, Y. and Gospodarowicz, D. (1985) Sulfated proteoglycan synthesis by confluent cultures of rabbit costal chondrocytes grown in the presence of fibroblast growth factor. J. Cell Biol. 100: 477-485.

Kellet, J.G., Tanaka, T., Rowe, J.M., Shiu, R.P.C., and Friesen, H.G. (1981) The characterization of growth factor activity in human brain. J. Biol. Chem. 256: 54-58.

Keski-Oja, J., Leof, E.B., Lyons, R.M., Coffey, R.J., and Moses, H.L. (1987) Transforming growth factors and control of neoplastic cell growth. J. Cell. Biochem. 33: 95-107.

Klagsbrun, M., Sasse, J., Sullivan, R., and Smith, J.A. (1986) Human tumor cells synthesize an endothelial cell growth factor that is structurally related to basic fibroblast growth factor. Proc. Natl. Acad. Sci. USA. 83: 2448-2452.

Klagsbrun, M., Smith, S., Sullivan, R., Shing, Y., Davidson, S., Smith, J.A., and Sasse, J. (1987) Multiple forms of basic fibroblast growth factor: amino-terminal cleavages by tumor cell- and brain cell-derived acid proteinases. Proc. Natl. Acad. Sci. USA. 84: 1839-1843.

Klagsbrun, M. and Shing, Y. (1985) Heparin affinity of anionic and cationic capillary endothelial cell growth factors: analysis of hypothalamus-derived growth factors and fibroblast growth factors. Proc. Natl. Acad. Sci. USA. 82: 805-809.

Koufos, A., Hansen, M.F., Copeland, N.G., Jenkins, N.A., Lampkin, B.C., and Cavenee, W.K. (1985) Loss of heterozygosity in three embryonal tumours suggests a common pathogenic mechanism. Nature 316: 330-334.

Kurachi, K., Davie, E.W., Strydom, D.J., Riordan, J.F., and Vallee, B.L. (1985) Sequence of the cDNA and gene for angiogenin, a human angiogenesis factor. Biochemistry 24: 5494-5499.

Kurokawa, T., Sasada, R., Iwane, M., and Igarski, K. (1987) Cloning and expression of cDNA encoding human basic fibroblast growth factor. FEBS Lett. 213: 189-194.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophages. Nature 227: 680-685.

Lagente, O., Diry, M., and Courtois, Y. (1986) Isolation of heparin-binding growth factors from dogfish (Mustela canis) brain and retina. FEBS Lett. 202: 207-210.

Lathrop, B., Thomas, K., and Glaser, L. (1985) Control of myogenic differentiation by fibroblast growth factor is mediated by position in the G1 phase of the cell cycle. J. Cell Biol. 101: 2194-2198.

Lau, D.C.W., Roncari, D.A.K., Yip, D.K., Kindler, S., and Nilsen, S.G.E. (1983) Purification of a pituitary polypeptide that stimulates the replication of adipocyte precursors in culture. FEBS Lett. 153: 395-398.

Leibovich, S.J., Polverini, P.J., Shepard, H.M., Wiseman, D.M., Shively, V., and Nuseir, N. (1987) Macrophage-induced angiogenesis is mediated by tumour necrosis factor-alpha. Nature 329: 630-632.

Lemke, G.E. and Brockes, J.P. (1983) Glial growth factor: a mitogenic polypeptide of the brain and pituitary. Fed. Proc. 42: 2627-2629.

Lemmon, S.K., Riley, M.C., Thomas, K.A., Hoover, G.A., Maciag, T., and Bradshaw, R.A. (1982) Bovine fibroblast growth factor: comparison of brain and pituitary preparations. J. Cell Biol. 95: 162-169.

Leof, E.B., Proper, J.A., Goustin, A.S., Shipley, G.D., DiCorleto, P.E., and Moses, H.L. (1986) Induction of c-sis mRNA and activity similar to platelet-derived growth factor by transforming growth factor beta: a proposed model for indirect mitogenesis involving autocrine activity. Proc. Natl. Acad. Sci. USA. 83: 2453-2457.

Leung, C.K.H. and Shiu, R.P.C. (1981) Required presence of both estrogen and pituitary factors for the growth of human breast cancer cells in athymic nude mice. Cancer Res. 41: 546-551.

Libermann, T.A., Friesel, R., Jaye, M., Lyall, R.M., Westermarck, B., Drohan, W., Schmidt, A., Maciag, T., and Schlessinger, J. (1987) An angiogenic factor is expressed in human glioma cells. EMBO J. 6: 1627-1632.

Linzer, D.I. and Nathans, D. (1984) Nucleotide sequence of a growth-related mRNA encoding a member of the prolactin-growth hormone family. Proc. Natl. Acad. Sci. USA. 81: 4255-4259.

Lobb, R.R. and Fett, J.W. (1984) Purification of two distinct growth factors from bovine neural tissue by heparin affinity chromatography. Biochemistry 23: 6295-6299.

Lobb, R.R., Strydom, D.J., and Fett, J.W. (1985) Comparison of human and bovine brain derived heparin-binding growth factors. Biochem. Biophys. Res. Commun. 131: 586-592.

Lobb, R.R., Alderman, E.M., and Fett, J.W. (1985) Induction of angiogenesis by bovine brain derived class 1 heparin-binding growth factor. Biochemistry 24: 4969-4973.

Lopez-Pousa, S., Ferrier, I., Vich, J.M., and Domenech-Mateau, J. (1981) Angiogenic activity in the CSF in human malignancies. Experientia 4: 413-414.

Maciag, T., Cerundolo, J., Ilsley, S., Kelley, P.R., and Forand, R. (1979) An endothelial cell growth factor from bovine hypothalamus: identification and partial characterization. Proc. Natl. Acad. Sci. USA. 76: 5674-5678.

Maciag, T., Hoover, G.A., and Weinstein, R. (1982) High and low molecular weight forms of endothelial cell growth factor. J. Biol. Chem. 257: 5333-5336.

Marquardt, H., Hunkapiller, M.W., Hood, L.E., and Todaro, G.J. (1984) Rat transforming growth factor type I: structure and relation to epidermal growth factor. Science 223: 1079-1082.

Marx, J.L. (1987a) Oncogene action probed. Science 237: 602-603.

Marx, J.L. (1987b) Angiogenesis research comes of age. Science 237: 23-24.

Massague, J. (1983) Epidermal growth factor-like transforming growth factor. II. Interaction with epidermal growth factor receptors in human placenta membranes and A431 cells. J. Biol. Chem. 258: 13614-13620.

Massague, J. and Like, B. (1985) Cellular receptors for type B transforming growth factor: ligand binding and affinity labeling in human and rodent cell lines. J. Biol. Chem. 260: 2636-2645.

Matuo, Y., Nishi, N., Matsui, S-I., Sandberg, A.A., Isaacs, J.T., and Wada, F. (1987) Heparin binding affinity of rat prostatic growth factor in normal and cancerous prostates: partial purification and characterization of rat prostatic growth factor in the Dunning tumor. Cancer Res. 47: 188-192.

McCumbee, W.D. and Lebovitz, H.E. (1980a) Hormone responsiveness of a transplantable rat chondrosarcoma. I. In vitro effects of growth hormone-dependent serum factors and insulin. Endrocrinology 106: 905-910.

McCumbee, W.D., McCarty, K.S., and Lebovitz, H.E. (1980b) Hormone responsiveness of a transplantable rat chondrosarcoma. II. Evidence for in vivo hormone dependence. Endocrinology 106: 1930-1940.

McKeehan, W.L. and Adams, P.S. (1988) Heparin-binding growth factor/ prostatropin attenuates inhibition of rat prostrate tumor epithelial cell growth by transforming growth factor type beta. In Vitro Cell. Devl. Biol. 24: 243-246.

Mergia, A., Eddy, R., Abraham, J.A., Fiddes, J.C., and Shows, T.B. (1986) The genes for basic and acidic fibroblast growth factors are on different human chromosomes. Biochem. Biophys. Res. Commun. 138: 644-651.

Mescher, A.L. and Gospodarowicz, D. (1979) Mitogenic effect of a growth factor derived from myelin on denervated regenerates of newt forelimbs. J. Exp. Zool. 207: 497-503.

Mescher, A.L. and Loh, J.J. (1980) Newt forelimb regeneration blastemas in explant and effects of various growth-promoting substances. J. Exp. Zool. 216: 235-245.

Mizel, S.B., Kilian, P.L., Lewis, J.C., Paganelli, K.A., and Chizzonite, R.A. (1987) J. Immunol. 138: 2906-2912.

Moenner, B., Chevallier, B., Badet, J., and Barritault, D. (1986) Evidence and characterization of the receptor to eye derived growth factor I, the retinal form of basic fibroblast growth factor on bovine epithelial cells. Proc. Natl. Acad. Sci. USA. 83: 5024-5028.

Moore, R., Casey, G., Brookes, S., Dixon, M., Peters, G., and Dickson, C. (1986) Sequence, topography and protein coding potential of mouse int-2: a putative oncogene activated by mouse mammary tumor virus. EMBO J. 5: 919-924.

Mormede, P., Baird, A., and Pigeon, P. (1985) Immunoreactive fibroblast growth factor (FGF) in rat tissues: molecular weight forms and the effects of hypophysectomy. Biochem. Biophys. Res. Commun. 128: 1108-1113.

Morrison, R.S., De Veillis, J., Lee, Y.L., Bradshaw, R.A., and Eng, L.F. (1985) Hormones and growth factors induced the synthesis of glial fibrillary acidic protein in rat brain adipocytes. J. Neurosci. Res. 14: 167-176.

Morrison, R.S., Sharma, A., De Veillis, J., and Bradshaw, R.A. (1986) Basic fibroblast growth factor supports the survival of cerebral cortical neurons in primary culture. Proc. Natl. Acad. Sci. USA. 83: 7537-7541.

Morrison, R.S. (1987) Fibroblast growth factors: potential neurotrophic agents in the central nervous system. J. Neurosci. Res. 17: 99-101.

Moscatelli, D., Presta, M., and Rifkin, D.B. (1986a) Purification of a factor from human placenta that stimulates capillary endothelial cell protease production, DNA synthesis and migration. Proc. Natl. Acad. Sci. USA. 83: 2091-2095.

Moscatelli, D., Presta, M., Joseph-Silverstein, J., and Rifkin, D. (1986b) Both normal and tumor cells produce basic fibroblast growth factor. J. Cell. Phys. 129: 273-276.

Moscatelli, D., Joseph-Silverstein, J., Manejias, R., and Rifkin, D.B. (1987) Mr 25,000 heparin-binding protein from guinea pig brain is a high molecular weight form of basic fibroblast growth factor. Proc. Natl. Acad. Sci. USA. 84: 5778-5782.

Moscatelli, D. (1987) High and low affinity binding sites for basic fibroblast growth factor on cultured cells: absence of a role for low affinity binding in the stimulation of plasminogen activator production by bovine capillary endothelial cells. J. Cell. Phys. 131: 123-130.

Moses, H.L., Branum, E.L., Proper, J.A., and Robinson, R.A. (1981) Transforming growth factor production by chemically transformed cells. Cancer Res. 41: 2842-2848.

Muller, R. and Wagner, E.F. (1984) Differentiation of F9 teratocarcinoma stem cells after transfer of c-fos proto-oncogenes. Nature 311: 438-442.

Murphy, P.R., Sato, R., Sato, Y., and Friesen, H.G. (1988a) Fibroblast growth factor messenger ribonucleic acid expression in a human astrocytoma cell line: regulation by serum and cell density. Mol. Endocr. 2: 591-598.

Murphy, P.R., Myal, Y., Sato, Y., Sato, R., West, M., and Friesen, H.G. (1988b) Elevated expression of basic fibroblast growth factor messenger RNA in acoustic neuromas. (submitted)

Neufeld, G., Ferrara, N., Schweigerer, L., Mitchell, R., and Gospodarowicz, D. (1987) Bovine granulosa cells produce basic fibroblast growth factor. Endocrinology 121: 597-603.

Neufeld, G. and Gospodarowicz, D. (1985) The identification and partial characterization of the fibroblast growth factor receptor of baby hamster kidney cells. J. Biol. Chem. 260: 13860-13868.

Neufeld, G. and Gospodarowicz, D. (1987) Protamine sulfate inhibits the mitogenic activities of the extracellular matrix and FGF, but potentiates that of epidermal growth factor. J. Cell. Phys. 132: 287-294.

Neufeld, G., Mitchell, R., Ponte, P., and Gospodarowicz, D. (1988) Expression of human basic fibroblast growth factor cDNA in baby hamster kidney-derived cells results in autonomous cell growth. J. Cell Biol. 106: 1385-1394.

Neufeld, G. and Gospodarowicz, D. (1986) Basic and acidic fibroblast growth factor interact with the same cell surface receptor. J. Biol. Chem. 261: 5631-5637.

Neufeld, G. and Gospodarowicz, D. Identification of the FGF in human endothelial cells. J. Clin. Invest. (in press).

Neufeld, G., Gospodarowicz, D., Dodge, L., and Fujii, D.K. (1987) Heparin modulation of the neurotropic effects of acidic and basic fibroblast growth factors and nerve growth factors on PC-12. J. Cell. Physiol. 131: 131-140.

Nicolson, G.L. (1987) Tumor cell instability, diversification, and progression to the metastatic phenotype: from oncogene to oncofetal expression. Cancer Res. 47: 1473-1487.

Nilsen-Hamilton, S.L. (1981) Stimulation of the release of two glycoproteins from mouse 3T3 cells by growth factors and by agents that increase intralysosomal pH. Biochem. Biophys. Res. Commun. 101: 411-417.

Nishi, N., Matuo, Y., Muguruma, Y., Yoshitake, Y., Nishikawa, K., and Wada, F. (1985) A human prostatic growth factor (hPGF) : partial purification and characterization. Biochem. Biophys. Res. Commun. 132: 1103-1109.

O'Keefe, E.J., Chiu, M.L., Payne, R.E. (1988) Stimulation of growth of Keratinocytes by basic fibroblast growth factor. J. Invest. Dermatol. 90: 767-769.

O'Keefe, E.J. and Pledger, W.J. (1983) Review: a model of cell cycle control: sequential events regulated by growth factors. Mol. Endocrinol. 31: 167-186.

Olwin, B.B. and Hauscka, S.D. (1986) Identification of the fibroblast growth factor receptor of Swiss 3T3 cells and mouse skeletal muscle myoblasts. Biochemistry 25: 3487-3492.

Orlidge, A. and D'Amore, P.A. (1987) Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells. J. Cell. Biol. 105: 1455-1462.

Pardee, A.B. (1987) The Yang and Yin of cell proliferation: an overview. J. Cell. Physiol. (Supp. 5): 107-110.

Peters, G., Brookes, S., Smith, R., and Dickson, C. (1983) Tumorigenesis by mouse mammary tumor virus: evidence for a common region for provirus integration in mammary tumors. Cell 33: 369-377.

Pettman, B., Weibel, M., Sensenbrenner, M., and Labourdette, G. (1985) Purification of two astroglial growth factors from bovine brain. FEBS Lett. 189: 102-108.

Pledger, W.J., Stiles, C.D., Antoniades, H.N., and Scher, C.D. (1977) Induction of DNA synthesis in Balb/c3T3 cells by serum components: reevaluation of the commitment process. Proc. Natl. Acad. Sci. USA. 74: 4481-4485.

Plouet, J., Mascarelli, F., Loret, M.D., Faure, J.P., and Courtois, Y. (1988) Regulation of eye derived growth factor binding to membranes by light, ATP or GTP in photoreceptor outer segments. EMBO J. 7: 373-376.

Ratner, N., Bunge, R.P., and Glaser, L. (1985) A neuronal cell surface heparan sulfate proteoglycan is required for dorsal root ganglion neuron stimulation of Schwann cell proliferation. J. Cell. Biol. 101: 744-754.

Redding, T.W. and Schally, A.V. (1983) Inhibition of growth of the transplantable rat chondrosarcoma by analogs of hypothalamic hormones. Proc. Natl. Acad. Sci. USA. 80: 1078-1082.

Reilly, C.F., Fritze, L.M.S., and Rosenberg, R.D. (1987) Antiproliferative effects of heparin on vascular smooth muscle cells are reversed by epidermal growth factor. J. Cell. Physiol. 131: 149-157.

Risau, W., Gautschi-Sova, P., and Bohlen, P. (1988) Endothelial cell growth factors in embryonic and adult chick brain are related to human acidic fibroblast growth factor. EMBO J. 7: 959-962.

Rizzino, A. and Ruff, E. (1986) Fibroblast growth factor induces the soft agar growth of two non-transformed cell lines. In Vitro Cell and Devl. Biol. 22: 749-755.

Rizzino, A., Ruff, E., and Rizzino, H. (1986) Induction and modulation of anchorage-independent growth by PDGF, FGF and TGFb. Cancer Res. 46: 2816-2820.

Robbins, K.C., Leal, F., Pierce, J.H., and Aaronson, S.A. (1985) The v-sis/PDGF-2 transforming gene product localizes to cell membranes but is not a secretory protein. EMBO J. 4: 1783-1792.

Roberts, A.B. and Sporn, M.B. (1985) Transforming growth factors. Cancer Surveys 4: 683-705.

Roberts, A.B., Anzano, M.A., Wakefield, L.M., Roche, N.S., Stern, D.F., and Sporn, M.B. (1985) Type beta transforming growth factor: a bifunctional regulator of cellular growth. Proc. Natl. Acad. Sci. USA. 82: 119-123.

Roberts, A.B. and Sporn, M.B. (1986) Growth factors and transformation. Cancer Surveys 5: 405-412.

Roberts, A.B., Anzano, M.A., Meyers, C.A., Wideman, J., Blacher, R., Pan, Y-CE., Stein, S., Lehrman, S.R., Smith, L.C., Lamb, L.C., and Sporn, M.B. (1983) Purification and properties of a type beta transforming growth factor from bovine kidney. Biochemistry 22: 5692-5698.

Roberts, A.B., Sporn, M.B., Assoian, R.K., Smith, J.M., Roche, N.S., Wakefield, L.M., Heine, U.I., Liotta, L.A., Falanga, V., Kehrl, J.H., and Fauci, A.S. (1986) Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis is in vivo and stimulation of collagen formation in vitro. Proc. Natl. Acad. Sci. USA. 83: 4167-4171.

Robinson, J. and Gospodarowicz, D. (1983) Glycosaminoglycans synthesized by culture bovine corneal endothelial cells. J. Cell. Phys. 117: 368-376.

Rogelj, S., Weinberg, R.A., Fanning, P., and Klagsbrun, M. (1988) Basic fibroblast growth factor fused to a signal peptide transforms cells. Nature 331: 173-175.

Rowe, J.M., Kasper, S., Shiu, R.P.C., and Friesen, H.G. (1986a) Purification and characterization of a human mammary tumor-derived growth factor. Cancer Res. 46: 1408-1412.

Rowe, J.M., Henry, S.F., Friesen, H.G. (1986b) Purification and characterization of a human pituitary growth factor. Biochem. 25: 6421-6425.

Rudland, P.S., Seifert, W., and Gospodarowicz, D. (1974) Growth control in culture mouse fibroblasts: induction of the pleiotypic and mitogenic responses by a purified growth factor. Proc. Natl. Acad. Sci. USA. 71: 2600-2604.

Sakamoto, H., Mori, M., Taira, M., Yoshida, T., Matsukawa, S., Shimizu, K., Sekiguchi, M., Terada, M., and Sugimura, T. (1986) Transforming gene from human stomach cancers and a noncancerous portion of stomach mucosae. Proc. Natl. Acad. Sci. USA. 83: 3997-4001.

Sakamoto, H., Yoshida, T., Nakakuki, M., Odagiri, H., Miyagawa, K., Sugimura, T., and Terada, M. (1988) Cloned hst gene from normal human leukocyte DNA transforms NIH 3T3 cells. Biochem Biophys. Res. Commun. 151: 965-972.

Saksela, O., Moscatelli, D., and Rifkin, D. (1987) The opposing effects of basic fibroblast growth factor and transforming growth factor beta on the regulation of plasminogen activator activity in capillary endothelial cells. J. Cell. Biol. 105: 957-963.

Samaan, N.A. Leavens, M.E. (1981) Calcitonin-like immunoreactivity in the pituitary vascular bed of man. J. Clin. Endocrinol. Metab. 53: 985.

Sasada, R., Kurokawa, T., Iwane, M., and Igarashi, K. (1988) Transformation of mouse Balb/c3T3 cells with human basic fibroblast growth factor cDNA. Mol. and Cell. Biol. 8: 588-594.

Sato, Y. and Sato, R. (1988) Basic FGF (bFGF) in the cultured medium of bovine corneal endothelial cells (BCE) can be detected by a sensitive 2-site immunoradiometric assay. in Proceedings 70th annual meeting Endocrine Society, New Orleans, LO, Abstract 1239.

Schecter, A.L., Stern, D.F., Vaidyanathan, L., Decker, S.J., Drebin, J.A., Greene, M.I., and Weinberg, R.A. (1984) The neu oncogene: an erb-B related gene encoding a 185,000-Mr tumour antigen. Nature 312: 513-516.

Scher, C.D., Shepard, R.C., Antoniades, H.N., and Stiles, C.D. (1979b) Platelet-derived growth factor and the mammalian fibroblast cell cycle. Biochim. Biophys. Acta. 560: 217-241.

Scher, C.D., Stone, M.E., and Stiles, C.D. (1979a) Platelet-derived growth factor prevents G0 growth arrest. Nature 281: 390-392.

Schreiber, A.B., Kenney, J., Kowalski, W.J., Friesel, R., Mehlman, T., and Maciag, T. (1985a) Interaction of endothelial cell growth factor with heparin: characterization by receptor and antibody recognition. Proc. Natl. Acad. Sci. USA. 82: 6138-6142.

Schreiber, A.B., Winkler, M.E., and Derynck, R. (1986) Transforming growth factor-alpha: a more potent angiogenic mediator than epidermal growth factor. Science 232: 1250-1253.

Schreiber, A.B., Kenny, J., Kowalski, J., Thomas, K.A., Gimenez-Gallego, G., Rios-Candelore, M., DiSalvo, J., Barritault, D., Courty, J., Courtois, Y., Moenner, M., Loret, C., Burgess, W.H., Mehlman, T., Friesel, R., Johnson, W., and Maciag, T. (1985b) A unique family of endothelial cell polypeptide mitogens: the antigenic and receptor cross-reactivity of bovine endothelial cell growth factor, brain-derived acidic fibroblast growth factor, and eye-derived growth factor-II. J. Cell Biol. 101: 1623-1626.

Schubert, D., Ling, N., and Baird, A. (1987) Multiple influences of a heparin-binding growth factor on neuronal development. J. Cell. Biol. 104: 635-643.

Schultz, G.S., White, M., Mitchell, R., Brown, G., Lynch, J., Twardzik, D.R., and Todaro, G.J. (1987) Epithelial wound healing enhanced by transforming growth factor-alpha and vaccinia growth factor. Science 235: 350-352.

Schwarzbauer, J.E., Tamkun, J.W., Lemischka, I.R., and Hynes, R.O. (1983) Three different fibronectin mRNAs arise by alternative splicing within the coding region. Cell 35: 421-431.

Schweigerer, L., Neufeld, G., Friedman, J., Abraham, J.A., Fiddes, J.C., and Gospodarowicz, D. (1987c) Capillary endothelial cells express basic fibroblast growth factor, a mitogen that promotes their own growth. Nature 325: 257-259.

Schweigerer, L., Neufeld, G., Mergia, A., Abraham, J.A., Fiddes, J.C., and Gospodarowicz, D. (1987b) Basic fibroblast growth factor in human rhabdomyosarcoma cells: implications for the proliferation and neovascularization of myoblast-derived tumors. Proc. Natl. Acad. Sci. USA. 84: 842-846.

Schweigerer, L., Neufeld, G., Friedman, J., Abraham, J.A., Fiddes, J.C., and Gospodarowicz, D. (1987a) Basic fibroblast growth factor: production and growth stimulated in cultured adrenal cortex cells. Endocrinology 120: 796-800.

Schweigerer, L., Malerstein, B., Neufeld, G., and Gospodarowicz, D. (1987d) Basic fibroblast growth factor is synthesized in cultured retinal pigment epithelial cells. Biochem. Biophys. Res. Commun. 143: 934-940.

Seifert, R.A. Schwartz, S.M., and Bowen-Pope, D.F. (1984) Developmentally regulated production of platelet-derived growth factor-like molecules. Nature 311: 669-671.

Seizinger, B.R., Martuza, R.L., and Gusella, J.F. (1986) Loss of genes on chromosome 22 in tumorigenesis of human acoustic neuroma. Nature 322: 644-647.

Serrero, G. and Khoo, J.C. (1982) An in vitro model to study adipocyte differentiation in serum free medium. Anal. Biochem. 120: 351-359.

Shapiro, R., Riordan, J.F., and Vallee, B.L. (1986) Characteristic ribonucleolytic activity of human angiogenin. Biochemistry 25: 3527-3532.

Sherr, C.J., Rettenmier, C.W., Sacca, R., Roussel, M.F., Look, A.T., and Stanley, E.R. (1985) The c-fms proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF -1. Cell 41: 665-679.

Shiu, R.P.C. (1981) Prolactin, pituitary hormones and breast cancer. In: M.C. Pike, P.K. Sitteri, and C.W. Welsch (eds.) Hormones and Breast Cancer. Banbury Report No. 8. pp. 185-197. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Slack, J.M.W., Darlington, B.G., Heath, J.K., and Godsave, S.F. (1987) Mesoderm induction in early Xenopus embryos by heparin-binding growth factors. Nature 326: 197-200.

Smith, R., Peters, G., and Dickson, C. (1988) Multiple RNAs expressed from the int-2 gene in mouse embryonal carcinoma cell lines encode a protein with homology to fibroblast growth factors. EMBO J. 7: 1013-1022.

Snover, D.C. and Rosai, J. (1985) Vascular sarcomas of the skin. In Clinical and Biochemical Analysis. vol. 20. M.R. Wick, ed. (New York: Marcel Dekker, Inc.) pp. 181-209.

Sommer, A., Brewer, M., Thompson, R., Moscatelli, D., Presta, M., and Rifkin, D.B. (1987) A form of human basic fibroblast growth factor with an extended amino terminus. Biochem. Biophys. Res. Commun. 144: 543-550.

Sporn, M.B. and Roberts, A.B. (1988) Peptide growth factors are multifunctional. Nature 332: 217-219.

Sporn, M.B. and Todaro, G.J. (1980) Autocrine secretion and malignant transformation of cells. N. Engl. J. Med. 303: 878-880.

Stiles, C.D. (1984) Autocrine control of growth. Nature 311: 604-605.

Stiles, C.D., Capone, G.T., Scher, C.D., Antoniades, H.N., Van Wyk, J.J., and Pledger, W.J. (1979) Dual control of cell growth by somatomedins and platelet-derived growth factor. Proc. Natl. Acad. Sci. USA. 76: 1279-1283.

Story, M.T., Esch, F., Shimasaki, S., Sasse, J., Jacobs, S.C., and Lawson, R.K. (1987) Amino-terminal sequence of a large form of basic fibroblast growth factor isolated from human benign prostatic hyperplastic tissue. Biochem. Biophys. Res. Commun. 142: 702-709.

Strydom, D.J., Harper, J.W., and Lobb, R.R. (1986) Amino acid sequence of bovine brain derived class 1 heparin- binding growth factor. Biochemistry 25: 945-951.

Strydom, D.J., Fett, J.W., Lobb, R.R., Alderman, E.M., Bethune, J.L., Riordan, J.F., Vallee, B.L. (1985) Amino acid sequence of human tumor derived angiogenin. Biochemistry 24: 5486-5494.

Taira, M., Yoshida, T., Miyagawa, K., Sakamoto, H., Terada, M., and Sugimura, T. (1987) cDNA sequence of human transforming gene hst and identification of the coding sequence required for transforming activity. Proc. Natl. Acad. Sci. USA. 84: 2980-2984.

Tanaka, T., Shiu, R.P.C., Gout, P.W., Beer, C.T., Noble, R.L., and Friesen, H.G. (1980) A new sensitive and specific bioassay for lactogenic hormones: measurement of prolactin and growth hormone in human serum. J. Clin. Endocrinol. Metab. 51: 1058-1063.

Tapper, D., Klagsbrun, M., and Neumann, J. (1979) The identification and clinical implications of human breast milk mitogen. J. Pediatr. Surg. 14: 803-808.

Taylor, S. and Folkman, J. (1982) Protamine is an inhibitor of angiogenesis. Nature 297: 307-312.

Thomas, K.A., Rios-Candelore, M., and Fitzpatrick, S. (1984) Purification and characterization of acidic fibroblast growth factor from bovine brain. Proc. Natl. Acad. Sci. USA. 81: 357-361.

Thomas, K.A., Rios-Candelore, M., Gimenez-Gallego, G., DiSalvo, J., Bennett, C., Rodkey, J., and Fitzpatrick, S. (1985) Pure brain-derived acidic fibroblast growth factor is a potent angiogenic vascular endothelial mitogen with sequence homology to interleukin 1. Proc. Natl. Acad. Sci. USA. 82: 6209-6413.

Thomas, K.A. and Gimenez-Gallego, G. (1986) Fibroblast growth factors: broad spectrum mitogens with potent angiogenic activity. Trends in Biochem. Sci. 11: 81-84.

Thomas, K.A., Riley, M.C., Lemmon, S.C., Baglan, N.C., and Bradshaw, R.A. (1980) Brain fibroblast growth factor: nonidentity with myelin basic protein fragments. J. Biol. Chem. 255: 5517-5520.

Togari, A., Baker, D.G., Dickens, G., and Guroff, G. (1983) The neurite-promoting effect of fibroblast growth factor on PC12 cells. Biochem. Biophys. Res. Commun. 114: 1189-1193.

Togari, A., Dickens, G., Ku-Zyua, H., and Guroff, G. (1985) The effect of fibroblast growth factor on PC12 cells. J. Neurosci. 5: 307-316.

Too, C.K.L., Murphy, P.R., Hamel, A-M., and Friesen, H.G. (1987) Further purification of human pituitary-derived chondrocyte growth factor: heparin-binding and cross-reactivity with antiserum to basic FGF. Biochem. Biophys. Res. Commun. 1448: 1128-1134.

Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA. 76: 4350-4354.

Trowell, O.A., Chir, B., and Willmer, E.N. (1939) J. Expl. Biol. 16: 60-70.

Tseng, S., Savion, N., Stern, R., and Gospodarowicz, D. (1982) Fibroblast growth factor modulates synthesis of collagen in cultured vascular endothelial cells. Euro. J. Biochem. 122: 355-360.

Tsuda, T., Kaibuchi, K., Kawahara, Y., Fukuzaki, H., and Takai, Y. (1985) Induction of protein kinase C and calcium ion mobilization by fibroblast growth factor in Swiss 3T3 cells. FEBS Lett. 191: 205-210.

Ueno, N., Baird, A., Esch, F., Ling, N., and Guillemin, R. (1986) Isolation of an amino terminal extended form of basic fibroblast growth factor. Biochem. Biophys. Res. Commun. 138: 580-588.

Uhlrich, S., Lagente, O., Lenfant, M., and Courtois, Y. (1986) Effect of heparin on the stimulation of non-vascular cells by human acidic and basic FGF. Biochem. Biophys. Res. Commun. 137: 1205-1215.

van Veggel, J.H., van Oostwaard, T.M., de Laat, S.W., and van Zoelen, E.J. (1987) PC13 embryonal carcinoma cells produce a heparin-binding growth factor. Exp. Cell Res. 169: 280-286.

Vlodavsky, I., Fridman, R., Sullivan, R., Sasse, J., and Klagsbrun, M. (1987) Aortic endothelial cells synthesize basic fibroblast growth factor which remains cell associated and platelet-derived growth factor-like protein which is secreted. J. Cell. Phys. 131: 402-408.

Vlodavsky, I. and Gospodarowicz, D. (1979) Structural and functional alterations in the surface of vascular endothelial cells associated with the formation of a confluent cell monolayer and with the withdrawal of fibroblast growth factor. J. Supramol. Struct. 12: 73-114.

Vlodavsky, I., Johnson, L.K., Greenburg, G., and Gospodarowicz, D. (1979) Vascular endothelial cells maintained in the absence of fibroblast growth factor undergo structural and functional alterations that are incompatible with their in vivo differentiated properties. J. Cell. Biol. 83: 468-486.

Vlodavsky, I., Folkman, J., Sullivan, R., Friedman, R., Ishai-Michaeli, R., Sasse, J., and Klagsbrun, M. (1987) Endothelial cell derived basic fibroblast growth factor: synthesis and deposition into subendothelial extracellular matrix. Proc. Natl. Acad. Sci. USA. 84: 2292-2296.

Vogel, A., Raines, E., Kariya, B., Rivest, M-J., and Ross, R. (1978) Coordinate control of 3T3 cell proliferation by platelet-derived growth factor and plasma components. Proc. Natl. Acad. Sci. USA. 75: 2810-2814.

Walicke, P., Cowan, W.M., Ueno, N., Baird, A., and Guillemin, R. (1986) Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension. Proc. Natl. Acad. Sci. USA. 83: 3012-3016.

Waterfield, M.D., Scrace, G.T., Whittle, N., Stroobant, P., Johnsson, A., Wasteson, A., Westermark, B., Heldin, C-H., Huang, and deuel, T.F. (1983) Platelet-derived growth factor is structurally related to the putative transforming protein $p28^{sis}$ of simian sarcoma virus. Nature 304: 35-39.

Weinberg, R.A. (1985) The action of oncogenes in the cytoplasm and nucleus. Science 230: 770-776.

Weinberger, C., Hollenberg, S.M., Rosenfeld, M.G., and Evans, R.M. (1985) Domain structure of human glucocorticoid receptor and its relationship to the v-erb-A oncogene product. Nature 318: 670-672.

Weinstein, I.B. (1987) Growth factors, oncogenes, and multistage carcinogenesis. J. Cell. Biochem. 33: 213-224.

Westall, F.C., Lennon, V.A., and Gospodarowicz, D. (1978) Brain-derived fibroblast growth factor: identity with a fragment of the basic protein of myelin. Proc. Natl. Acad. Sci. USA. 75: 4675-4678.

Westall, F.C. Rubin, R., and Gospodarowicz, D. (1983) Brain-derived growth factor: a study of its inactivation. Life Sci. 33: 2425.

Wilkinson, D.G., Peters, G., Dickson, C., and McMahon, A.P. (1988) Expression of the FGF-related proto-oncogene int-2 during gastrulation and neurulation in the mouse. EMBO J. 7: 691-695.

Winkles, J., Friesel, R., Burgess, W.H., Howk, R., Mehlman, T., Weinstein, R., and Maciag, T. (1987) Proc. Natl. Acad. Sci. USA. 84: 7124-7128.

Yamada, T. (1982) Transdifferentiation of lens cells and its regulation. In: McDevitt, D.S. (ed.) Cell Biology of the Eye. Academic Press, New York, pp. 193-234.

Yee, C. and Shiu, R.P.C. (1986) Degradation of endothelial basement membrane by human breast cancer cell lines. Cancer Res. 46:1835-1839.

Yoshida, T., Miyagawa, K., Odagiri, H., Sakamoto, H., Little, P.F.R., Terada, M., and Sugimura, T. (1987) Genomic sequence of hst, a transforming gene encoding a protein homologous to fibroblast growth factors and the int-2 encoded protein. Proc. Natl. Acad. Sci. USA. 84: 7305-7309.