Title
Isolation and characterization of transforming growth factors from human malignant gliomas

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ISOLATION AND CHARACTERIZATION OF TRANSFORMING GROWTH FACTORS FROM HUMAN MALIGNANT GLIOMAS: Possible Role for Transforming Growth Factors in the Pathogenesis of the Gliosarcoma

by

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DISSERTATION

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DOCTOR OF PHILOSOPHY

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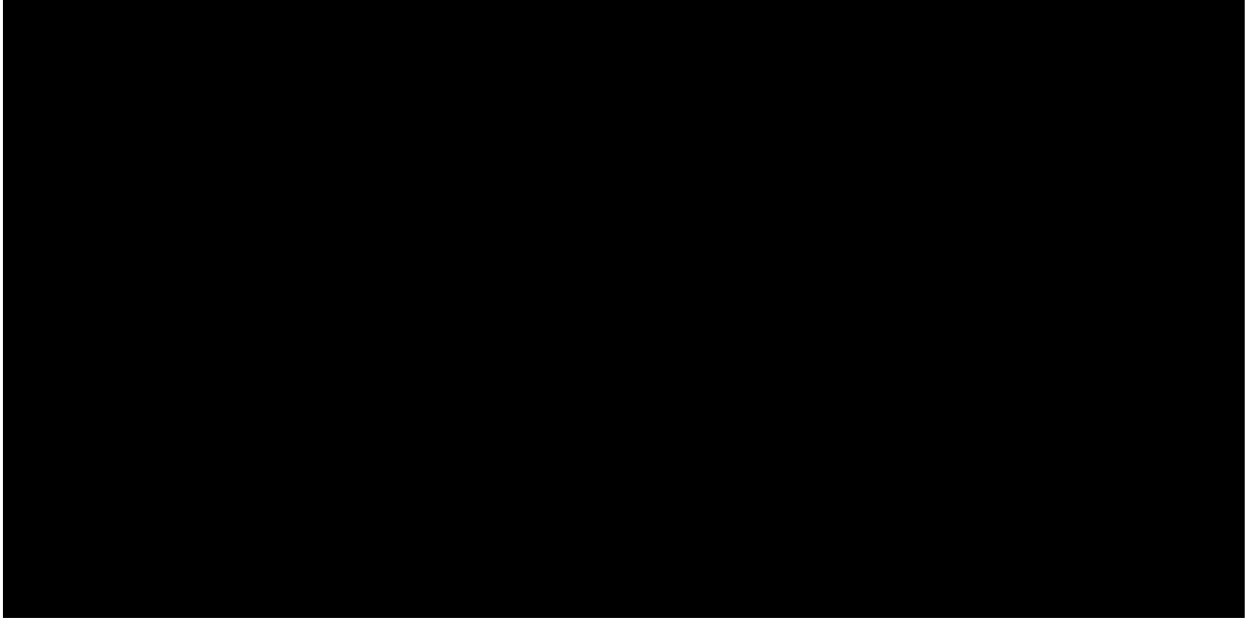

ABSTRACT:

This study provides the first direct experimental evidence that human malignant gliomas secrete soluble polypeptides with TGF-like activity. The conditioned medium from three well-characterized malignant glioma cell lines promote the growth of NRK indicator cells in soft agar. Following acid extraction and gel filtration, TGF-like activity was recovered from all three cell lines. Active fractions from gel filtration in two of the glioma cell lines (U 343 MG-A and SF-210) were further purified by reverse-phase HPLC. The TGF isolated from U 343 MG-A by HPLC is an acid- and heat-stable protein complex whose activity is destroyed by reducing agents and incubation with monospecific anti-TGF-alpha monoclonal antibodies. The NRK colony stimulating activity of this TGF is potentiated by the addition of TGF-beta. The partially purified U-343 MG-A TGF competes with radiolabeled [^{125}I]-EGF for the EGF-receptor on A431 epidermoid carcinoma cells. Finally, a total RNA preparation from U-343 MG-A contains a 4.8 kilobase mRNA for TGF-alpha. Therefore, U-343 MG-A secretes a soluble polypeptide with TGF-alpha-like activity. In contrast, the purified SF-210 malignant glioma cell line secretes an acid- and heat-stable TGF with neither TGF-alpha- nor TGF-beta-like activity. By HPLC, the purified SF-210 TGF is a single protein with a molecular weight of 20 kD whose migration is not altered by reducing agents. The activity in the purified SF-210 TGF is not eliminated by anti-TGF-alpha antibodies, by passage over a heparin-Sepharose column, or by incubation with anti-PDGF antibodies; in addition, it does not compete with radiolabeled EGF for the EGF receptor. The SF-210 TGF is capable of inducing NRK indicator cells and well-characterized human fetal leptomeningeal cells to grow anchorage independently. These data imply that human malignant gliomas secrete TGFs which can cause the phenotypic transformation of previously normal cells. The growth of human leptomeningeal cells in soft agar when exposed to the novel SF-210 TGF provides in vitro data to support the hypothesis that, by secreting TGFs, malignant glioma cells may induce peritumoral leptomeningeal cells to undergo phenotypic transformation. This phenomenon might explain the formation of complex mixed neoplasms in the CNS such as the gliosarcoma.

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The work in this thesis represents the final product of a three year fellowship that I completed at the Brain Tumor Research Center and in the Department of Pathology, School of Medicine, University of California San Francisco. For those three years, support from the Medical Research Council of Canada is gratefully acknowledged.

In many ways, I share this thesis and the occasion of my graduation with those that have helped me learn a variety of laboratory techniques, the scientific method, and the art of scientific writing. I thank Mark Rosenblum for introducing me to the science of experimental brain tumor research and his continuous support of my projects -- diverse as they were. I thank Dolores Dougherty and Jane Giblin for teaching me tissue culture technique and the characterization of cells in culture. I thank Jackson Hall for his never-faltering patience and for his instructions on the use of laboratory techniques for studying collagen biochemistry. I thank Gerard Apodaca and Linda Stowring for teaching me how to use molecular biological principles to answer certain questions posed in this thesis. I thank Ellen Levin for her kind and expert assistance with the HPLC-studies. And I thank Stephen Ordway for his invaluable lessons on manuscript writing and preparation.

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But above all, I share this thesis with my my wife, Mari, whose constant support, understanding and love, have truly provided the inspiration I needed for the design and completion of this work.

ABBREVIATIONS

BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
cDNA	Complementary DNA
CNS	Central nervous system
DTT	Dithiothreitol
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
GFAP	Glial fibrillary acidic protein
HBSS	Hank's balanced salt solution
HPLC	High performance liquid chromatography
IGF	Insulin-like growth factor
INT	2-(p-iodophenyl)-3-p-nitrophenyl-5-phenyl-2H-tetazolium chloride
MEM	Minimum essential medium
mRNA	Messenger RNA
NRK	Normal rat kidney
PAP	Peroxidase-anti-peroxidase
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
SDS-PAGE	SDS-polyacrylamide gel electrophoresis

SSC	Standard saline citrate
TFA	Trifluoracetic acid
TGF	Transforming growth factor

CHAPTER 1

GENERAL INTRODUCTION

1.1 Hypothesis

Of all the primary intracranial brain tumors, the glioblastoma multiforme is the most malignant and is associated with the worst prognosis. Despite all current forms of therapy, patients with glioblastoma multiforme usually die within 1 year of the diagnosis. Even though the glioblastoma multiforme is the most common glial neoplasm, little is known regarding its pathogenesis. One of the most conspicuous histopathological features of the glioblastoma multiforme is the marked proliferation of vascular mesenchymal cells (endothelial and arachnoid cells) within the tumor. The degree of vascular mesenchymal proliferation correlates positively with the degree of histological anaplasia and reduced patient survival. In a substantial subset of patients with glioblastoma multiforme, the tumor-associated vascular or perivascular mesenchymal cells become neoplastic and assume the morphology of a fibrosarcoma, thus creating a mixed mesenchymal and neuroepithelial tumor known as a gliosarcoma. Patients with glioasarcoma may have an even worse prognosis than those with glioblastoma multiforme alone. Determining the mechanism of the malignant transformation of the mesenchymal cells in a glioblastoma multiforme is the subject of this thesis. It is proposed that the malignant glial cells within a glioblastoma multiforme secrete a transforming growth factor which causes the malignant transformation of tumor-associated mesenchymal (leptomeningeal) cells.

1.2 Background Information

Each year, approximately 15,000 Americans are diagnosed as having primary brain tumors. At any given time, about 30,000 patients with primary brain tumors are alive which imparts a yearly cost of 150 million to the American health care system. Although occasional patients may recover completely after successfuly surgery and radiation therapy, more often after treatment there is an inexorable course towards profound neurological deficit

consisting of dementia, behavioural disturbance, language loss or paralysis. The burden placed on the families of such patients is incalculable.

The most common primary central nervous system (CNS) malignancy is the glioblastoma multiforme which accounts for approximately half of all gliomas (tumors of glial lineage). Despite all current forms of therapy -- surgery, cranial irradiation, and chemotherapy -- most patients with glioblastoma multiforme will succumb from their tumor about one year after treatment is initiated. Whereas the survival of patients with some leukemias and lymphomas, choriocarcinoma, and testicular cancer has improved dramatically with the advent of adjuvant chemotherapy and radiation therapy, the long term survival of patients with glioblastoma multiforme has not changed significantly in the last 3 decades (113). Despite the increased frequency with which the glioblastoma multiforme occurs relative to other primary brain tumors, little is known of its etiology, pathogenesis and tumor cell biology. It is hoped that an increased understanding of the glioblastoma multiforme tumor cell biology will improve efforts to treat this clinically devastating neoplasm.

Aside from the clinical signs referable to generalized increased intracranial pressure, specific focal signs attributed to a rapidly expanding glioblastoma multiforme are related to the localization of this neoplasm within the brain. Most frequently, the glioblastoma multiforme arises in the frontal lobes with the temporal lobes being the next most frequent site. A patient with a glioblastoma multiforme in the frontal lobes will often present with behavioural disturbances and dementia; a patient with a glioblastoma multiforme in the temporal lobes will often present with seizures. While a glioblastoma multiforme may be found in any part of the CNS, the cerebellum and spinal cord are exceedingly rare sites. Death results from localized, aggressive tumor growth and infiltration of surrounding normal brain. Extracranial metastases from a glioblastoma multiforme have only rarely been reported (252). Therefore, studies aimed at increasing our understanding of the interactions between glioblastoma multiforme tumor cells and normal brain cells (including perivascular and endothelial cells), and between glioblastoma multiforme tumor cells and the normal extracellular matrix (ECM) in the CNS may provide clues to more effective therapy which would then only have to be directed locally.

Grossly, the glioblastoma multiforme is usually infiltrative with poorly defined margins, but occasionally it may appear to be well circumscribed. The most characteristic macroscopic feature is the highly variegated cut surface of the neoplasm which demonstrates grayish or pinkish foci of moderately firm, viable growth alternating with extensive areas of creamy yellow necrosis and red or brown foci of recent or old hemorrhage (221). The marked increase in vascularization of the glioblastoma multiforme is sometimes manifest by the presence of small pinpoint petechiae which represent the cut surfaces of small enlarged blood vessels. The cut surface is usually soft, but where mesenchymal proliferation has occured, the tumor may demonstrate a firmer consistency.

Microscopically, the tumor is always highly cellular, and the cells demonstrate marked pleomorphism (104). The tumor cells are usually anaplastic of astrocytic lineage since glial specific intracytoplasmic intermediate filaments can often be demonstrated immunohistochemically (55,59,287). The cells frequently cluster around central areas of incipient necrosis, resulting in the classic appearance of pseudopalisades, a virtually diagnostic microscopic feature of the glioblastoma multiforme (104,221). Giant and multinucleated cells are common.

Vascular changes are invariably present in the glioblastoma multiforme and consist of proliferation of the endothelial and perivascular cells of arterioles, venules and capillaries (67,68,221). Although the regenerative potential of endothelial and perivascular cells can be observed in any organ during wound healing, it is in the CNS, under pathological conditions, and particularly with the glioblastoma multiforme, that this invoked vascular mesenchymal response is most marked. The reasons for this are poorly understood. Vascular mesenchymal proliferation is a frequent concomitant not only of the glioblastoma multiforme, but also, to lesser degrees, of oligodendrogliomas and metastatic carcinoma of the brain (66,69,194). Vascular mesenchymal proliferation also occurs in response to inflammatory and infectious processes within the brain (68). The recognition of vascular endothlial hyperplasia in malignant gliomas is important because of the direct correlation that exists between the degree of histological malignancy and the degree of vascular endothelial proliferation (68). Occasionally, the mesenchymal response may be so intense that the perivascular component assumes the morphology of a fibrosarcoma. In these

instances, a mixed tumor consisting of two distinct neoplasms -- glioblastoma multiforme and sarcoma -- is formed, and has been termed "gliosarcoma". Approximately 2-8% of all glioblastoma multiforme's are thought to progress to gliosarcoma formation (66,172,223). The prognosis of patients with gliosarcoma may be even more dismal than that of patients with glioblastoma multiforme alone (68).

Determining the mechanism(s) whereby such mixed mesenchymal and neuroepithelial tumors are formed may provide further insight into the growth, clinical behaviour, and response to therapy of the glioblastoma multiforme. Several theories have been proposed to explain the pathogenesis of these unusual mixed tumors: 1) The glioblastoma multiforme is the initial tumor which induces the proliferation of intrinsic blood vessels within it to undergo sarcomatous transformation (67,68,177,251). 2) The sarcoma is the initial tumor which induces glioma formation following intense glial hyperplasia in an area adjacent to the sarcoma (134,220). 3) The glioblastoma multiforme is the initial tumor, and the sarcoma is formed from infiltrative mesenchymal elements derived from the leptomeninges (101). 4) The glioblastoma multiforme and the sarcoma arise independently in different sites and "collide", resulting in the juxtaposition of two different tumors (165,182). 5) The glioblastoma multiforme and sarcoma occur simultaneously as a result of external influences such as radiation therapy (134,185). The first of these theories -- that the glioblastoma multiforme induces the proliferation and malignant transformation of the mesenchymal elements within the tumor -- is most widely accepted. However, direct experimental data supporting this hypothesis are lacking. Within the pages of this thesis, experiments are designed which support the view that the malignant astrocytes from a glioblastoma multiforme secrete soluble polypeptide factors which induce the proliferation and eventual malignant transformation of previously normal mesenchymal cells.

1.3 The Transforming Growth Factors

Five major types of tumor-derived endogenous growth factors have now been well described: TGF-alpha (154,155,270,271); TGF-beta (210-213); bombesin (175); monolayer mitogens such as PDGF and EGF (90,99,100); and insulin-like growth factors --IGF-I and IGF-II (205). Of these factors, the TGFs

(TGF-alpha and TGF-beta) may be the most likely mediators of the malignant transformation of previously normal vascular mesenchymal cells.

TGFs have been operationally defined as polypeptides which have the capacity to confer properties of the transformed phenotype on previously normal cells (212). There properties include the loss of density dependent inhibition of cell growth in monolayer culture, overgrowth in monolayer culture, characteristic alteration in cellular morphology, and acquisition of anchorage independent growth.

Two types of TGFs are produced by malignant cells in culture: Those which bind to the EGF-receptor (TGF-alpha) (270); and those which do not bind to the EGF-receptor but have their own receptors (TGF-beta) (212). TGF-alpha has been sequenced and found to have significant sequence homology with mouse and human EGF; it is released into the culture medium of a variety of malignant cells of both ectodermal and mesodermal origin (154,155). TGF-beta is produced by both malignant and non-malignant cells. This 25 kD polypeptide has been isolated from acid/ethanol extractions of whole tumor and normal cells, and in combination with TGF-alpha or EGF will stimulate NRK indicator cells to form colonies in soft agar (161,212). TGF-beta has been sequenced and cDNA clones encoding the protein have been purified (57).

There is evidence that growth factors may play a role in the pathogenesis of certain CNS malignancies. A number of human brain tumors have been reported to produce PDGF and EGF (99,100,183). Intriguingly, an increased expression of the erb-B oncogene which encodes for the EGF-receptor has been found in glioblastoma multiforme (28,140). However, there have been no previous attempts to use human glioblastoma multiformes as a source of TGFs. In this thesis, glioma cells and normal mesenchymal cells will be well characterized, physically separated and purified. The glioma cells and the normal cells will then be allowed to interact in a fashion which should permit testing of the original hypothesis. If a TGF is found in the glioblastoma multiforme which acts upon normal cells to induce malignant transformation, then one eventual outcome of this study would be the ability to antagonize the growth promoting effects of the tumor-derived growth factor. A summary scheme of the pathogenesis of the gliosarcoma is shown in Figure 1.

PATHOGENESIS OF THE GLIOSARCOMA

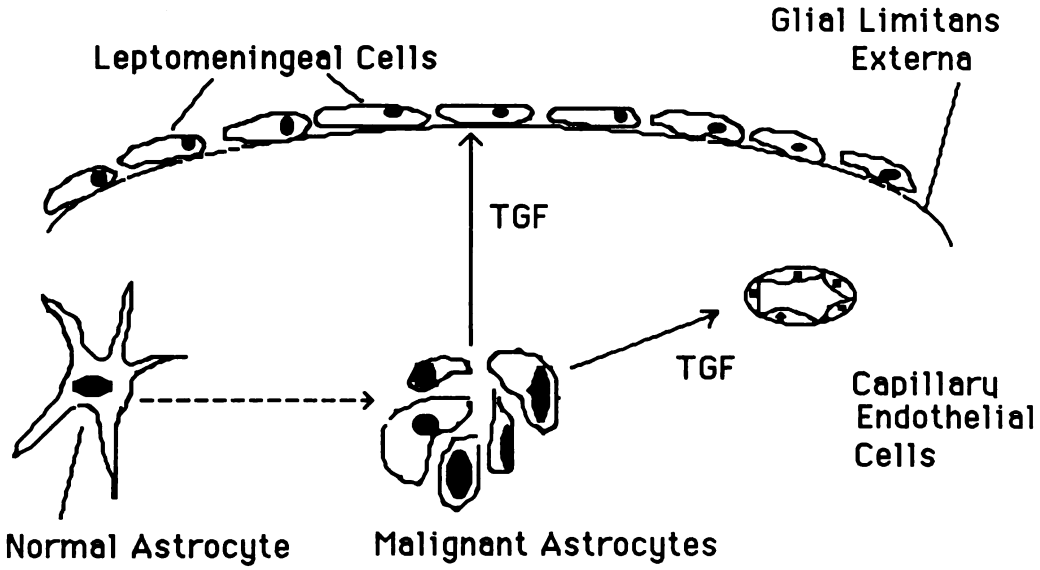

Figure 1

Scheme of the pathogenesis of the human gliosarcoma. It is proposed that malignant astrocytes secrete soluble polypeptides that cause previously normal leptomeningeal cells and capillary endothelial cells to undergo malignant transformation. Normally, astrocytes are separated form leptomeningeal cells by the glial limitans externa which functions as a supportive basal lamina. Similarly, endothelial cells are separated from astrocytes by a basement membrane. The malignant transformation of these normal cell elements may result in the creation of a "mixed" tumor and is a striking example of tumor:stromal cell interactions. This thesis sets out to prove the existence of TGFs in human malignant gliomas; and to show that these TGFs can influence the phenotype of normal leptomeningeal cells.

CHAPTER 2

AN ULTRASTRUCTURAL AND IMMUNOCYTOCHEMICAL ANALYSIS OF LEPTOMENINGEAL AND MENINGIOMA CULTURES

2.1 Introduction

It is clear that in any in vitro model system complete characterization of the cell types in culture is critical before statements concerning the relative importance and merit of the study are made. For this reason, a significant portion of this research (Chapters 2 - 6) was devoted to accurately identifying and characterizing the CNS cell types used to test the hypothesis of gliosarcoma formation (Chapters 9 and 10). The present chapter begins with the development of a model system to harvest and utilize normal adult leptomeningeal cells in culture.

The leptomeninges consist of the arachnoid mater and pia mater, which form a membranous bilayer connected by thin stands of cellular processes known as trabeculae (104,230). Between these two membranes lies the subarachnoid space, which is filled with cerebrospinal fluid. Together, these structures cushion, protect, and support the intrinsic neuronal and glial elements of the CNS. The germ cell layer of origin of the leptomeninges has been the subject of some controversy. Some authors believe they are derived from the neural crest (82), while others support a mesodermal origin (121,221,253); still others think the leptomeninges are formed from a mixture of mesodermal and neural crest elements (65,176,237).

The pia mater is separated from the astrocytic foot processes by a limiting basement membrane (glial limitans externa), and the arachnoid barrier cells are separated from the subarachnoid space by a thin basement membrane. We therefore sought to determine whether leptomeningeal cells in vitro synthesize any of the glycoproteins associated with either the formation of basement membranes or the adhesion of cells to basement membrane, that is, collagen type IV, laminin, and fibronectin. Because

arachnoid cells are found in a perivascular distribution for considerable distances along the length of penetrating cortical arteries (33,238), we also analyzed the cultures for the production of procollagen type III. Electron microscopy was performed on all cultures to help determine the purity of the leptomeningeal cells derived from biopsy samples. Finally, because there is evidence that meningiomas are derived from arachnoid cells (237,238,264), we studied a series of human meningiomas in vitro to ascertain whether the glycoprotein profile and the ultrastructure of these benign intracranial tumors are similar to those of the leptomeninges.

2.2 Materials and Methods:

2.2.1 Cell Cultures

A) Leptomeningeal Cells

Small samples (1-2 cm^2) of the leptomeninges were obtained from three patients without tumors by microdissection during routine neurosurgical procedures or at autopsy. The tissue fragments were rinsed thoroughly in HBSS and minced with scalpels into pieces smaller than 1 mm^3. These pieces of tissue were placed in 25-cm^2 flasks containing an enriched medium consisting of Eagle's MEM, nonessential amino acids, glutamine, gentamicin, and 10% fibronectin-free fetal calf serum at 37 C in a humidified atmosphere (95% air and 5% CO_2). Fibronectin-free serum was prepared by collecting the eluate of fetal calf serum passed over a column of gelatin-sepharose as described by Engvall and Ruoslahti (63). Gelatin-sepharose was a gift of Dr. Susan Fisher, Department of Anatomy, University of California, San Francisco (UCSF). Once colonies of cells had formed, the medium was changed twice each week (wk) until confluence was reached.

B) Meningiomas

Tumor specimens were obtained by biopsy from seven patients undergoing craniotomy for resection of meningioma at UCSF. A representative portion of each specimen was fixed in 10% buffered formalin, cut into thin sections, and stained with hematoxylin and eosin for histopathological analysis. All histological slides were reviewed by two of us (JTR and SJD) to confirm the diagnosis of meningioma. The remainder of each specimen was sterilely minced into pieces smaller than 1 mm^3 within 4 hours (h) after biopsy and disaggregated into single cells in an enzyme cocktail consisting of pronase (0.05% of PK units/mg; Calbiochem, San Diego, CA), collagenase (0.02% of 125 units/mg; US Biochemical Corp., Cleveland, OH) and DNAse (0.2% of 7 x 10^4) dornase units/mg; Calbiochem) at 37 C for 30 minutes (min). The cells were incubated in 75-cm^2 flasks under the same conditions used for leptomeningeal cells.

C) Neonatal Rat Astrocytes

Cerebral cells from five one- to two-day-old rats were cultured using a procedure based on the techniques of Fontana et al. (74) and Manthorpe et al. (151). Briefly, brain from the supratentorial compartment was microdissected from the brain stem, cleaned of meninges, and minced. The tissue fragments were placed in 10 ml of 0.25% trypsin (Gibco, Santa Clara, CA) in Ca^{++}- and Mg++ -free HBSS and incubated for 15 min at 37 C. The enzyme-dissociated mixture was centrifuged at 1000 g for two min, after which the trypsin was removed and replaced with 10 ml of MEM containing 10% fibronectin-free fetal calf serum. After a second centrifugation (1000 g for two min), the medium was removed and replaced with 5 ml of fresh medium. The tissue fragments so obtained were triturated by 30 up-and-down passages through a sterile Pasteur pipette (internal diameter 1 mm). The resultant cell suspension was filtered through a sterile 20-micron mesh. Approximately 1 x 10^6 cells were placed in 25-cm^2 flasks and allowed to incubate for two to three wk; the medium was changed every other day. The flasks were then vigorously shaken by hand, the nonadherent cells were discarded, and the remaining cell layer was washed three times with MEM. After trypsinization of the monolayer, the cells were seeded onto Lab-Tek chambers (Fisher, Santa Clara, CA) and allowed to adhere and spread for one to two days.

D) Endothelial Cells

Endothelial cell culture 929, from human umbilical vein, was obtained from Dr. J.M. Harlan, Harborview Medical Center, Seattle, Washington.

E) Fibroblasts

Two fibroblast cultures were obtained from the Cell Culture Facility at UCSF. HS 27 is a cell line cultured from adult human skin and HFF from human foreskin.

2.2.2 Monoclonal Antibodies and Antisera

Various monoclonal antibodies or antisera were used to localize glycoproteins in the cell cultures and to help rule out potentially contaminating cell types. Rabbit anti-human factor-VIII-related antigen antisera and rhodamine-conjugated swine anti-rabbit immunoglobulins were purchased from Dako, Westbury, NY; the specificity of the factor-VIII antisera was determined by crossed immunoelectrophoresis. Fluorescein-conjugated sheep anti-mouse IgG- and IgM- specific immunoglobulins and rabbit anti-human fibronectin antisera were purchased from Cappel, Cochraneville, PA. Rabbit anti-human antiserum to GFAP was provided by Dr. L. Eng (Stanford, CA); its specificity for human GFAP has been determined by Western blot analysis. Monoclonal antibodies to procollagen type III and collagen type IV (260,261) were donated by Dr. N. SundarRaj (University of Pittsburgh, PA); their specificities have been determined by ELISA and by Western blots (260,261). Lyophilized antibody against laminin and fibronectin, raised in rabbits, was a gift from Dr. H. Kleinman (National Institute of Dental Research, NIH, Bethesda, MD). The specificity of the the anti-laminin antisera was determined by ELISA (135) and immunoprecipitation (170); the specificity of the anti- fibronectin antisera was determined by ELISA, Western blots, and immunoprecipitation with radiolabeled human skin fibroblasts (Kleinman H: personal communication). The anti-fibronectin antibody reacts strongly with human cellular and plasma fibronectin, but reacts weakly with fibronectin from other species.

2.2.3 Electron Microscopy

Leptomeningeal cells and meningioma cells from all cultures were grown to confluence in 75-cm^2 flasks. The cells were then rinsed in PBS, (pH 7.4), gently scraped away from the plastic, placed in microcentrifuge tubes, and

centrifuged at 1000 g for two min. The resulting pellet was fixed in 2% glutaraldehyde in cacodylate buffer at 4 C for one to five h, post-fixed in 1% osmium tetroxide for one h, dehydrated, embedded in Epon, and cut into thin sections. The slides were stained first with uranyl acetate and then with lead citrate and examined in a JEOL 100S electron microscope.

2.2.4 Indirect Immunofluorescence

Leptomeningeal, meningioma, and neonatal rat astrocyte cultures were studied by indirect immunofluorescence. Endothelial cell and fibroblast cultures were used as internal controls. Between 5×10^3 and 1×10^4 cells from a single-cell suspension were planted in Lab-Tek eight-well chambers and allowed to attach for 24 to 48 h. Tris buffer (0.5 M, pH 7.6) diluted 1:10 in PBS was used for all subsequent dilutions and rinses. The medium was aspirated from each well and the cell layer was rinsed in Tris-PBS for five to ten min. The cells were fixed in a solution of methanol and ethanol (1:1) for ten min. Nonspecific background staining was eliminated by incubating the cells for 20 min with nonimmune serum (diluted 1:5) from the same animal species that produced the second antibody (normal swine and sheep sera, Dako). The primary antibody was applied directly and the cells were incubated for 30 min. All primary antibodies except laminin were diluted 1:100. Lyophilized anti-laminin (10 ug/ul) was diluted 1:10 and then applied. Normal rabbit immunoglobulins (Dako) and normal ascites fluid (Cappel) were used in corresponding 1:100 dilutions in all cases to serve as negative controls. The cell layer was rinsed with Tris-PBS for ten min with five changes of equal volume. The secondary antibody (diluted 1:32) was then applied for 30 min. After a final rinse in Tris-PBS for 15 min, the Lab-Tek carriage and plastic grid were removed and the slides were mounted with a coverslip and Aquamount.

Fluorescence microscopy was performed with an Olympus BHS system microscope equipped with an Olympus BH-RFL-W reflected-light fluorescence attachment and an HBO 100w/2 high-pressure mercury lamp. FITC fluorescence was detected with blue emission light (490 nm); the filters used to detect specific FITC fluorescence were excitation filters IF- 490, dichroic mirror DM-500 + D-515, and barrier filter 0.530. Rhodamine-labeled antibodies were detected with green emission light (546 nm); the specific filters used were excitation filter IF-545 + BG-36, dichroic mirror DMO-580 + 0-

590, and barrier filter R-610. The intensity of staining was rated as negative (-), low (+), moderate (++) or high (+++). The percentage of cells staining positively was calculated in each case. Negative controls were examined for each stain in all cell lines studied.

2.2.5 Isolation and Characterization of the Extracellular Matrix

Cells from all leptomeningeal and meningioma cultures were grown to confluence in Lab-Tek chambers in the presence of 10% fibronectin-free fetal calf serum. The cells were then maintained at confluence for seven days. The cultures were treated with 25 mM freshly prepared ammonium hydroxide (Mallinckrodt, KY) for one to two minutes to remove the cells, leaving the extracellular matrix (114); the treatment was monitored with an inverted-phase microscope. The prepared matrix was thoroughly rinsed twice in PBS and immediately analyzed by indirect immunofluorescence, as described above, for the presence of laminin, fibronectin, collagen type IV, and procollagen type III.

2.2.6 SDS-Polyacrylamide Gel Electrophoresis

Leptomeningeal cultures HuA1 P4 and 514P P3 and meningioma cultures 528 P2, 526 P2, 524 P1, and 545 P4 were studied by SDS-PAGE. Briefly, the cells were incubated in 75-cm^2 flasks containing 10 ml of proline- and glutamine-free RPMI 1640 medium supplemented with ascorbate (25 ug/ml, Calbiochem) and beta aminopropionitrile (50 ug/ml, Sigma) for four h. After the volume had been reduced to 6 ml, radiolabeled proline (L-2,3,4,5-^{3}[H] proline, 102 Ci/mmole; Amersham, Arlington Heights, IL), 40 uCi/ml, was added to the medium and the cells were allowed to incubate for 24 h. The medium was withdrawn and saved for analysis by DEAE-cellulose chromatography. The cell layer was rinsed in 0.1% Nonidet P40 for 100 min, scraped off the flask, and homogenized. A 4.5% stacking gel and a 5.0% separating gel were used to separate the radiolabeled proteins into bands (133). Vitrogen (Flow Labs, Inglewood, CA) served as an internal standard for the collagen subtypes. The samples were dissolved in sample buffer and heated at 60 C for 10 min before being applied to the gel. To reduce, the samples were treated with 2 mercaptoethanol (10% v/v, Sigma) at 60 C for 10 min. The gels were stained with 0.15% Coomassie blue (in acetic acid, 10% v/v, and methanol, 30% v/v) for 30 min and destained for 30 min in a solution of

methanol (50% v/v) and acetic acid (10% v/v), which also served as a protein fixative. The gels were then impregnated with En^{3}Hance (New England Nuclear, Boston, MA) for fluorography on x-ray film.

2.2.7 DEAE-Cellulose Chromatography

The procollagens secreted into the medium reserved from the preceding experiment were separated and quantitated as described by Tseng et al (277). The proline-labeled medium was dialyzed at 4 C against a solution of 0.15 M NaCl and 50 mM Tris-HCl (pH 7.5) containing 20 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM p-hydroxymercuribenzoate, 1 mM benzamidine, and 0.5 mM N- ethylmaleimide (all Sigma) to remove traces of free radiolabeled amino acid. Saturated ammonium sulfate (100% v/v) was added to achieve a final concentration of 30% (v/v).

The ammonium sulfate suspension was then stirred gently overnight at 4 C in the presence of 3 mg of unlabeled Vitrogen. The precipitates were collected by centrifugation (20,000 g for 30 min at 4 C), dissolved in the initial buffer (2 mM urea and 50 mM Tris-HCl, pH 7.5), and dialyzed overnight (4 C) against the same solution. Before analysis, any insoluble material was removed by filtering through cotton. Samples were applied to a 1.5 x 10 cm column (DE 52; Whatman Inc., Clifton, NJ) of DEAE-cellulose and washed with 25 ml of the initial buffer. A gradient of NaCl (0-0.02 M, total volume 160 ml) was applied at a flow rate of 7.5 ml/h. Fractions of 2.45 ml were collected, of which a 0.3-ml aliquot was removed for counting. Three milliliters of Aquasol were added and the samples were counted in a scintillation counter (LS 8000; Beckman, San Jose, CA) with a counting efficiency of 28% for the tritiated material.

2.3 Results

2.3.1 Culture Morphology

A) Leptomeningeal Cultures

Leptomeningeal cells grew readily from the mechanically dissociated fragments, forming colonies within a few days and reaching confluence by 14-21 days. In the exponential phase of growth (subconfluence), the cells were broadly bipolar with relatively large, rounded central nuclei and generous cytoplasm. The cytoplasmic edges tended to be curvilinear (Fig. 1a). Coarse streaming of cells in gentle arcs from colonies was apparent. At confluence the cells ceased to be bipolar and became homogeneously flat and polygonal. The terminal cell density in early passages was 3-5 x 10^4 cells/cm^2, and there was little or no cell crowding compared with the fibroblast cultures, which had a terminal cell density of 1-2 x 10^5 cells/cm^2. Leptomeningeal cells grew vigorously in early passage (doubling time 24 h), were passed 1:2 after trypsinization, and maintained the typical morphological features described above for the duration of the culture's life. By passages five to seven, however, signs of senescence--an increased cytoplasmic:nuclear ratio (Fig. 1b), cytoplasmic vesiculations, and giant, often multinucleated, cells--were present. Although no cell culture was viable after 15 passages, the culture time could be substantially augmented by increasing the interval between passages.

B) Meningiomas

The <u>in vitro</u> morphological and growth characteristics of meningiomas from the histologically different subtypes were identical to those of leptomeningeal cells (Fig. 2). In early passages, arc development in all meningioma cultures was accentuated to the point of whorl formation; other features of meningiomas in vitro were early senescence and prolonged contact inhibition.

C) Fibroblasts, Endothelial Cells, and Neonatal Rat Astrocytes

Fibroblasts could be distinguished morphologically from leptomeningeal cells and meningiomas in vitro by their spindle shape and a

tendency to crowd at confluence. Endothelial cells did not survive beyond passages two to three. Neonatal rat astrocytes had typical stellate processes with scanty cytoplasm and limited ability to form colonies.

2.3.2 Immunocytochemical Characterization of Cells

The results of the immunocytochemical characterization studies are summarized in Table 1. The leptomeningeal cells stained uniformly and intensely for fibronectin, laminin, collagen type IV, and procollagen type III. All meningiomas also stained uniformly for these proteins, but the intensity of staining was highly variable. There was no appreciable difference in staining characteristics between the subtypes of meningiomas examined. Neither leptomeningeal cells nor meningiomas stained positively for GFAP or Factor VIII. Endothelial cell and newborn rat astrocyte cultures could be distinguished immunocytochemically from leptomeningeal cells and meningiomas by the presence of Factor VIII and GFAP, respectively. Fibroblasts showed variable uniformity and low intensity of staining for all proteins analyzed.

The staining patterns for fibronectin and procollagen type III appeared to be very similar in all cultures. Perinuclear granular cytoplasmic localization, as described previously for other cell types (214,269), and considerable extracellular fiber formation were observed (Fig. 3). Immunocytochemical stains for laminin and collagen type IV also revealed a perinuclear granular halo (Fig. 4), and a modest amount of extracellular staining. However, the amount of extracellular staining for laminin and collagen type IV was increased by maintaining the cells at confluence for one to two wk.

Staining for GFAP was positive in neonatal rat astrocytes and showed the long intracytoplasmic fibrils characteristic of this intermediate filament. Factor- VIII-related antigen was localized to the cytoplasm in a perinuclear, granular pattern in the endothelial cell culture. All negative controls were appropriately nonstaining.

2.3.3 Characterization of the Extracellular Matrix

In all leptomeningeal and miningioma cultures, the matrix stained positively for fibronectin, laminin, collagen type IV, and procollagen type III. All negative controls were appropriately nonstaining. Fibronectin and

procollagen type III were deposited as dense, granular focal plaques, with long thick fibrils forming a loose meshwork thoughout. Laminin and collagen type IV were deposited as much finer focal plaques (Fig. 5); short fibers were also seen. There appeared to be no difference in the matrices laid down by leptomeningeal cells and meningiomas or in those produced by the different subtypes of meningiomas.

2.3.4 Electron Microscopy

Electron microscopic studies of all cultures showed marked ultrastructural similarity between leptomeningeal and meningioma cells in vitro. Interdigitation of cell membranes and invagination of cytoplasm into the nucleus were prominent features. Specialized intercellular junctions such as gap junctions and desmosomes were also identified (Figs. 6 and 7). The cytoskeleton was formed by intermediate filaments (tonofilaments) and monofilaments. In all cultures, fine granular and filamentous basement membrane-like material was found in the extracellular spaces between cells. These features have been described previously in arachnoid cells and meningiomas in vitro (76,121,250).

2.3.5 SDS-PAGE Fluorography

When fluorographs of the samples were compared with the Coomassie blue-stained standards, the cell layers of leptomeningeal cultures HuA1 P4 and 514P P3 and meningioma cultures 528 P2, 526 P2, 542 P1, and 545 P4 contained bands that were identified as alpha1 and alpha2 collagen type I chains (Fig. 8). When the samples were reduced, additional bands representing procollagens type I and type III were observed near the alpha1 band of collagen type I.

2.3.6DEAE Cellulose Chromatography

The medium of leptomeningeal cultures HuA1 P4 and 514P P3 and meningioma cultures 528 P2, 526 P2, 545 P1, and 542 P1 showed tall peaks for procollagen type I and much smaller peaks for procollagen type III (Fig. 9).

2.4 Discussion

There have been few reports on the growth characteristics of leptomeningeal cells in vitro (250,255). Polygonal cells showing marked contact inhibition and early senescence were found consistently from explants of arachnoid membranes in culture (76). Ultrastructurally, these cells, like ours, contained desmosomes and tonofilaments similar to those seen in vivo. Because cells of the pia mater are indistinguishable morphologically and ultrastructurally from cells of the arachnoid mater (264), and because of the possible difficulty in culturing these cells, our practice in initiating cell cultures was to carefully peel the leptomeninges as a unit from the brain so as to effectively double the number of cells going into culture. We quickly realized that leptomeningeal cells were extremely well suited to the present culture conditions and grew vigorously in early passages.

In contrast to the relatively recent discovery that normal leptomeningeal cell cultures are easily initiated, meningiomas were first cultured in the 1920's in Cushing's laboratory (121). Subsequently, it was observed repeatedly, as pointed out by Lumsden (149), that meningiomas grow more easily and more quickly in vitro than any other brain tumor, and that their cultural behavior has little bearing on their histological structure. Bland and Russell (25) were the first to report that fibroblastic, syncytial, and meningothelial meningiomas were all morphologically identical in vitro. This finding gave rise to the hypothesis that all meningiomas, regardless of their histological appearance, are ultimately derived from the same cell type. This "unitarian" hypothesis has been corroborated several times (237,262,264). By light microscopy, we and others (122,149,178,250) have observed that meningiomas in early passages grow vigorously and tend to form whorls. Meningiomas in vitro retain many of the ultrastructural features seen in vivo (118,250). Examination of all our meningiomas in early passage culture by electron microscopy revealed characteristic specialized intercellular junctions in the form of gap junctions and desmosomes, as well as pronounced interdigitation of the plasma membrane. These findings suggest that the cells in our cultures were histologically representative of those found in vivo.

Although it appeared that our leptomeningeal and meningioma cultures were predominantly homogeneous in cell type, it is conceivable that

the cultures might have been contaminated and overgrown by highly selected, undesirable cell types. Despite the predominance of normal pial and arachnoid cells in leptomeningeal biopsy specimens, macrophages, fibroblasts, endothelial cells, and perivascular supporting cells (smooth muscle cells and pericytes) are also found in the leptomeninges. Fibroblasts can be eliminated as possible contaminants because they lack the specialized intercellular junctions found in our cultures. Additionally, fibroblasts have much higher terminal cell densities and retain their spindle shape after repeated passages. Finally, the glycoprotein profile of the fibroblast cultures resembled that described by others (131) and showed variable uniformity of staining not seen with either meningioma or leptomeningeal cultures.

It is also unlikely that our cultures were contaminated by macrophages or endothelial cells, which do not survive in monolayer culture beyond passages two to three without attention to their special metabolic requirements (79). Moreover, macrophages lack desmosomes, which were found in our leptomeningeal and meningioma cultures. Further evidence that our cultures were free of endothelial cells is the absence of Factor-VIII-related antigen in all meningioma and leptomeningeal culture tested immunocytochemically. Non-neoplastic leptomeningeal muscle cells have been reported rarely in the literature (181) and in any case should retain ultrastructural features of muscle cells in vitro. Examination of our cultures by electron microscopy showed no evidence of myofibril or myofiber formation.

We have shown that leptomeningeal cells and meningiomas in vitro can synthesize and secrete several collagenous and noncollagenous glycoproteins found in the normal extracellular matrix. Although the CNS is unusual in its relative paucity of recognizable extracellular collagens and fibrous proteins (32), basement membranes are found perivascularly, between pial and endothelial cells and astrocytic foot processes, and beneath ependymal linings (42,191). The major constituents of normal basement membrane that have been identified are laminin, collagen type IV, and heparin sulfate proteoglycan (98,109,123,135,268).

Laminin, an 800,000 dalton, noncollagenous glycoprotein, is an important attachment factor to basement membrane between epithelial cells and collagen type IV (268). Synthesis of laminin by Schwann cells (170), fetal rat astrocytes (143), neuroblastoma cells (4), and a malignant human glioma

cell line U251MG (5) has been demonstrated in vitro. McComb et al (167) recently reported on the immunohistochemical localization of laminin in a series of human brain tumors. In 29 meningiomas, laminin expression was related to histological subtype; fibroblastic meningiomas demonstrated intense laminin immunostaining in a fibrillary pattern. In all meningiomas staining positively for laminin, the localization was completely extracellular. No intracytoplasmic staining was demonstrated.

Collagen type IV, the major structural component of the lamina densa of the basement membrane, provides a collagenous scaffolding to which laminin, fibronectin, and other glycoproteins bind cells (98,206). Poor in glucose and rich in hydroxylysine and cysteine, collagen type IV does not form fibrils and probably retains its amino and carboxy precursor extensions in mature form (123). Recently, Bellon et al (13) performed an immunofluorescence analysis of human gliomas and meningiomas using a variety of collagen antibodies, including antisera to collagen type IV. Capillary vessels, arterioles, and venules were markedly stained; meningioma cells were not stained.

Fibronectin, a large, highly asymmetric glycoprotein of the extracellular matrix, is thought to promote adhesion and spreading of cells by linking them to collagen substrates or plastic. Fibronectin may mediate cell adhesion by binding to other matrix components such as proteoglycans and glycosaminoglycans (42,268). In the CNS, fibronectin has been localized immunohistochemically to blood vessel walls and to the leptomeninges, but not to cells of glial or neuronal origin (79,131), and may help to promote the growth of neurites (65). In meningiomas, fibronectin has been found within and between cells in whorl formations and in psammoma bodies (13,125).

To our knowledge, laminin and collagen type IV have not been previous localized to leptomeningeal cells <u>in vitro</u>. Not only were we able to demonstrate immunoreactivity to collagen type IV and laminin intracytoplasmically in all cell cultures, we we also able to document the deposition of these glycoproteins in the extracellular matrix. In addition, electron microscopy of all leptomeningeal cultures revealed granular and filamentous extracellular basement membrane-like materal. It appears that, at least <u>in vitro</u>, leptomeningeal cells can synthesize and secrete basement membrane components. However, a true basement membrane was not identified by electron microscopy. Recently, Kusaka et al. (132) demonstrated

the formation of a basal lamina between astrocytes and pia-arachnoid cells in organotypic cultures of mouse spinal cord. If pial cells in vivo contribute to the formation of basal lamina, then perhaps a specific glial message (soluble polypeptide) or direct contact with glial elements at the glial-mesenchymal junction is needed to trigger the pia to form such an organized basement membrane.

In this study we have also shown that procollagen III, the collagen precursor that has retained its aminopropeptide, can be localized immunohistochemically to leptomeningeal cells and meningiomas in vitro. Collagen type III is composed of three alpha$_1$ (III) chains and has been found in varying amounts in fibroblasts and myoblasts in blood vessels, skin, and the parenchyma of internal organs such as the lungs and the intestines (114). Although there is still considerable debate over which compounds are positively identified by reticulin staining (94), some authors believe that collagen type III is the preponderant protein (15,126). In the CNS, reticulin staining is normally limited to the leptomeninges and perivascular spaces (1). In pathological alterations such as tumors (lymphoma, sarcoma, hemangioblastoma) and infection (abscess, meningitis), reticulin staining may be exuberant (221,223).

In addition to having stained the cytoplasm in a granular perinuclear fashion, the monoclonal antibody to procollagen III also recognized extracellular focal plaques and an impressive meshwork of fibers. Although ultrastructural studies of the leptomeninges and meningiomas have repeatedly demonstrated collagen fibers in the extracellular space (109,264), the ability of pial cells, arachnoid cells, or meningiomas to synthesize collagen has not been clearly proven. Using SDS-PAGE and DEAE-cellulose chromatography, we were able to show that both leptomeningeal cells and meningiomas synthesize type I and type III procollagens. Procollagen types I and III are largely secreted into the medium, whereas collagen type I remains on the cell layer. Depletion of elements essential for collagen synthesis, such as ascorbate and iron (123), from the medium or inadequate oxygenation may therefore explain the relatively early senescence of leptomeningeal cells in vitro

The bulk of evidence suggests that most meningiomas derive from arachnoid cells. Ultrastructural studies have shown that arachnoid cells and meningiomas in vitro are nearly identical (118). Clusters of normal

arachnoid cells closely resembling miniature meningiomas are often found incidentally at autopsy (223). Our study, which includes ultrastructural, biochemical, and immunocytochemical data, shows little difference in the chacteristics of three leptomeningeal and seven meningioma cultures. The intensity of staining for all glycoproteins varied among meningiomas but not in arachnoid cells. The ability of arachnoid and meningioma cells in culture to behave like stromal cells (synthesis of interstitial collagens) and like epithelial cells (formation of specialized epithelial cytoskeletal junctions) implies to us that these cells have a mixed mesodermal-epithelial phenotype. This may provide new insight into the embrylogical origin of the leptomeninges.

In summary, we have developed a model system in which adult human leptomeningeal cells have been cultivated and characterized. The leptomeningeal cells in culture retained many of the phenotypic characteristics of leptomeningeal cells in situ. These well characterized adult leptomeningeal cells can now serve as the target cells for the growth factors present in the conditioned medium harvested from human malignant gliomas (Chapter IX). The alternative target system, brain capillary endothelial cells and pericytes, does not lend itself readily to analysis at the present time as these cell types do not grow well in monolayer culture.

(Part of the material in this chapter has been previously published in the Journal of Neuropathology and Experimental Neurology 45: 285-303, 1986. Permission to reproduce this material was granted by the American Association of Neuropathologists - see Appendix)

2.5 Figure Legends and Tables

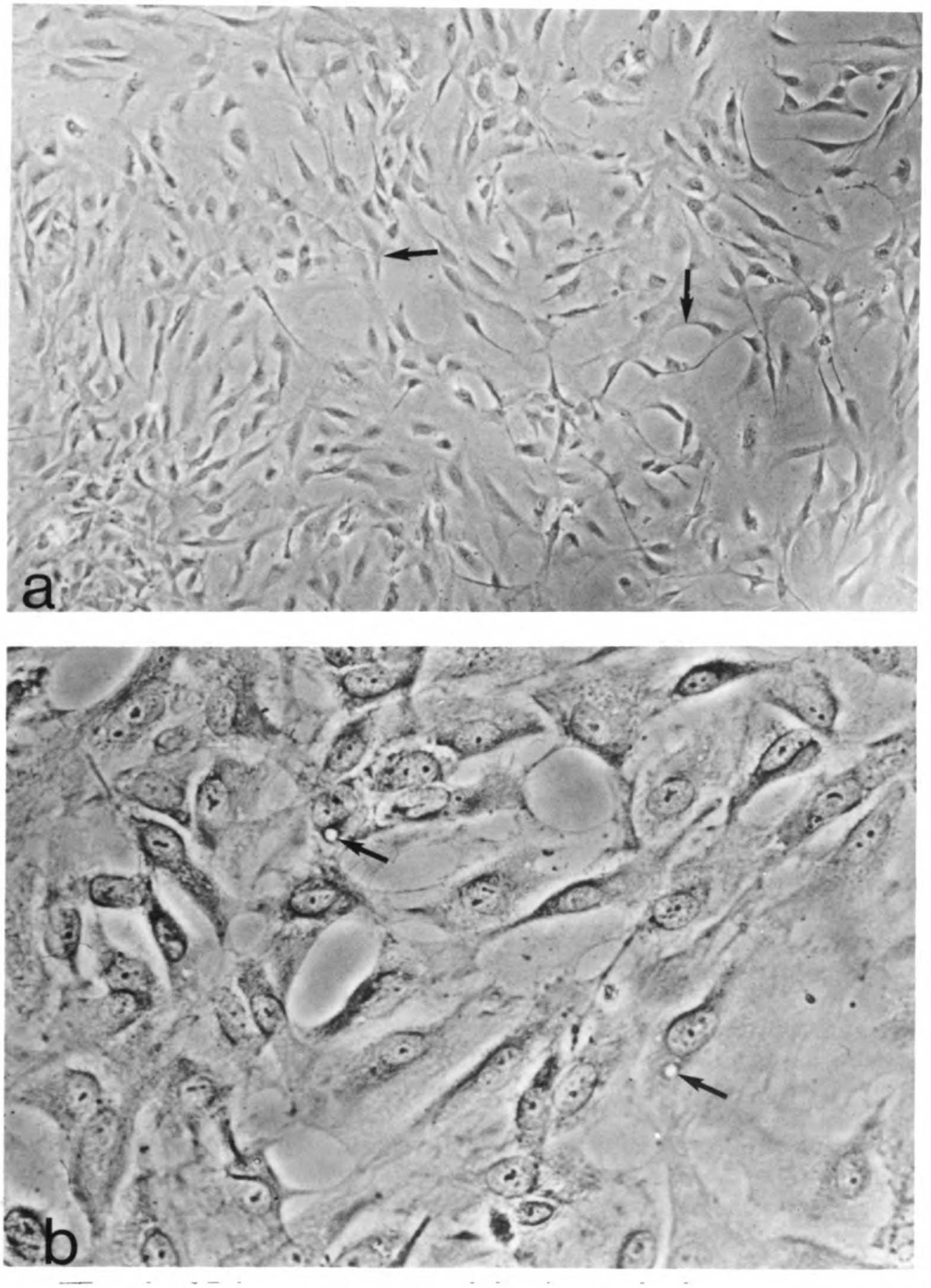

Fig. 1. a: Leptomeningeal culture 514P P3 approaching confluence. Broadly bipolar cells with curvilinear cyplasmic edges (arrows) are present. Phase microscopy, 125X. b: Leptomeningeal culture HuA1 P9 at confluence. Polygonal cells with well-rounded nuclei and well-defined nucleoli are present. Senescence of the culture is suggested by an increased cytoplasmic:nuclear ratio and by several cytoplasmic vesiculations (arrows). Phase microscopy, X500.

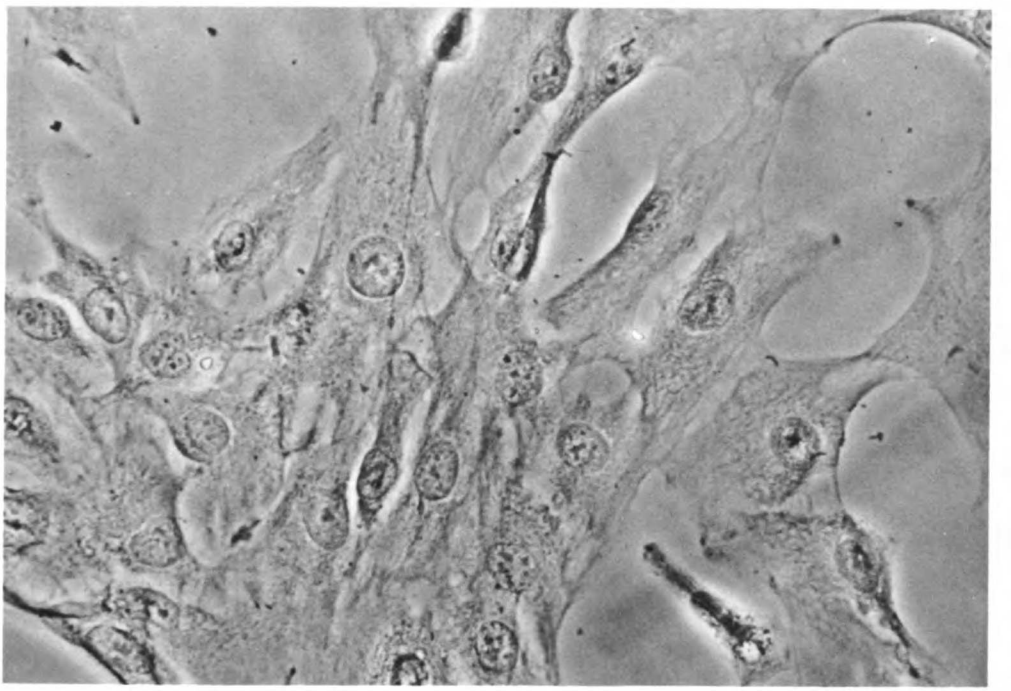

Fig. 2 Meningioma culture 528 P2 at confluence. Flat polygonal cells with round central nuclei and generous cytoplasm are present. Their appearance is similar to that of the leptomeningeal cells in Fig. 1b. Phase microscopy, X500.

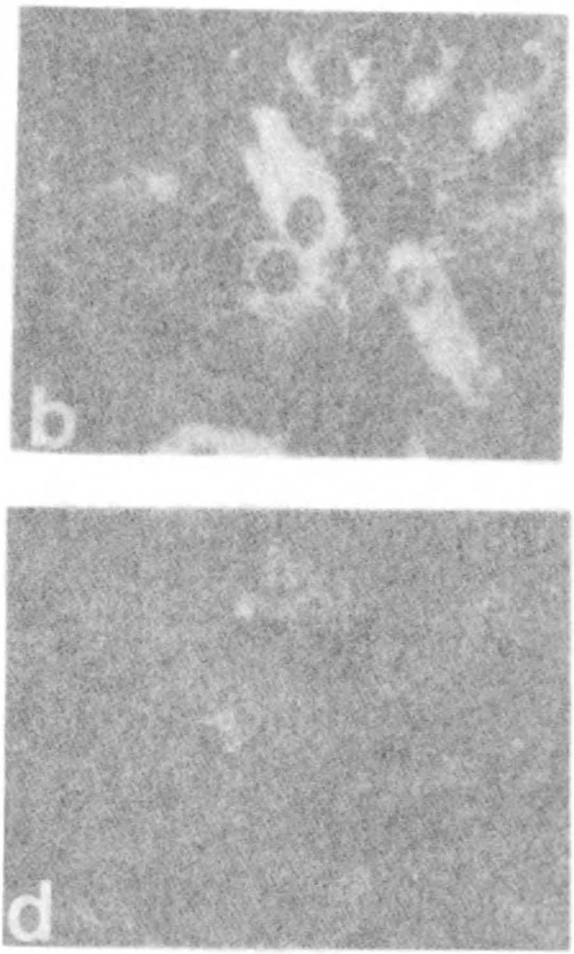

Fig. 3 Phase microscopy (a, c) and immunofluorescence staining (b, d) for fibronectin and procollagen type III. In meningioma cell line (MM), perinuclear granular intracytoplasmic staining for fibronectin is evident in cytoplasmic extensions and extracellular fibers also stain positively. In leptomeningeal culture 514P P2 (c, d), staining for procollagen type III revealed a similar localization within the cytoplasm and extracellular space. ×

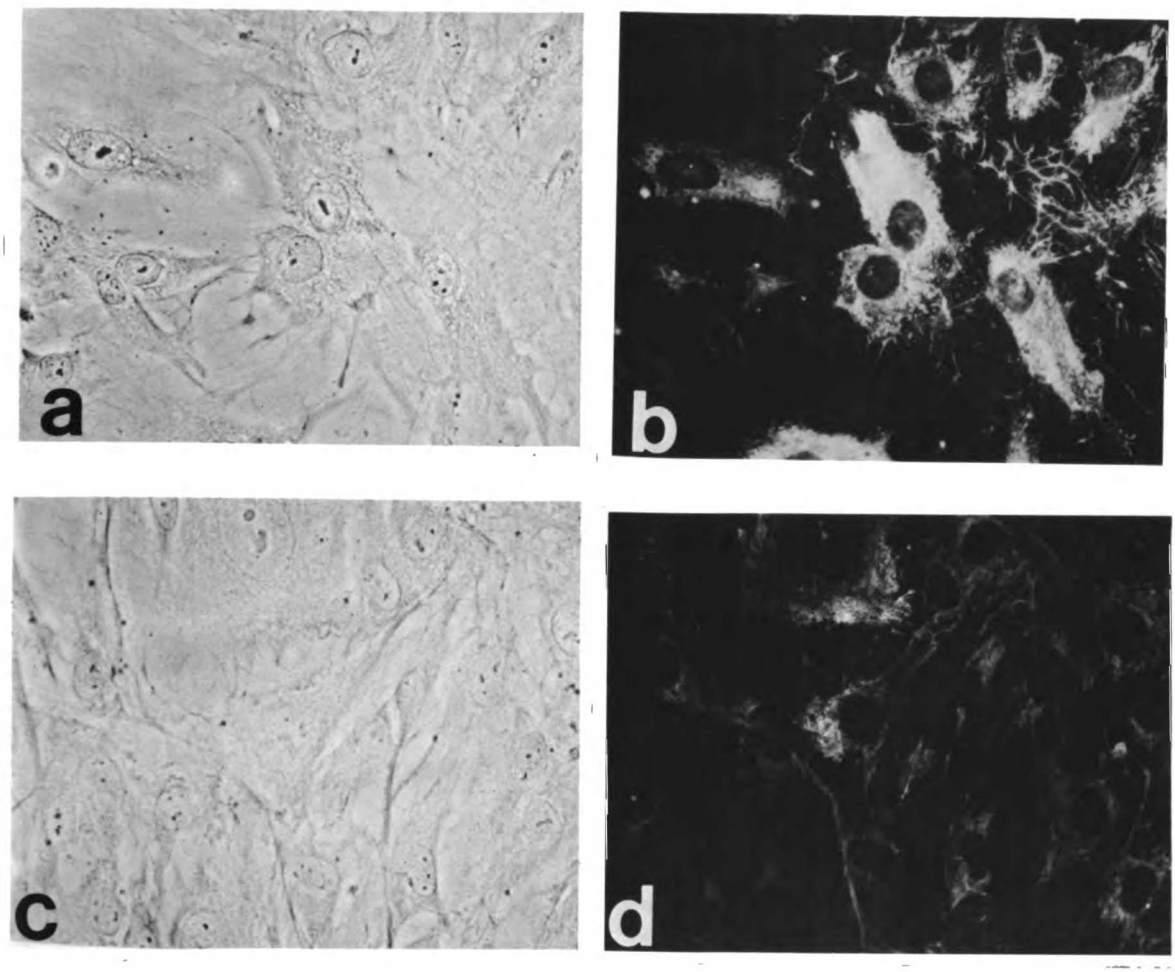

Fig. 3. Phase microscopy (a, c) and immunofluorescence staining (b, d) for fibronectin and procollagen type III. In meningioma culture 528 P3 (a, b), perinuclear granular intracytoplasmic staining for fibronectin is evident; cytoplasmic extensions and extracellular fibers also stain positively. In leptomeningeal culture 514P P2 (c, d), stains for procollagen type III reveal a similar localization within the cytoplasm and extracellular space. X350.

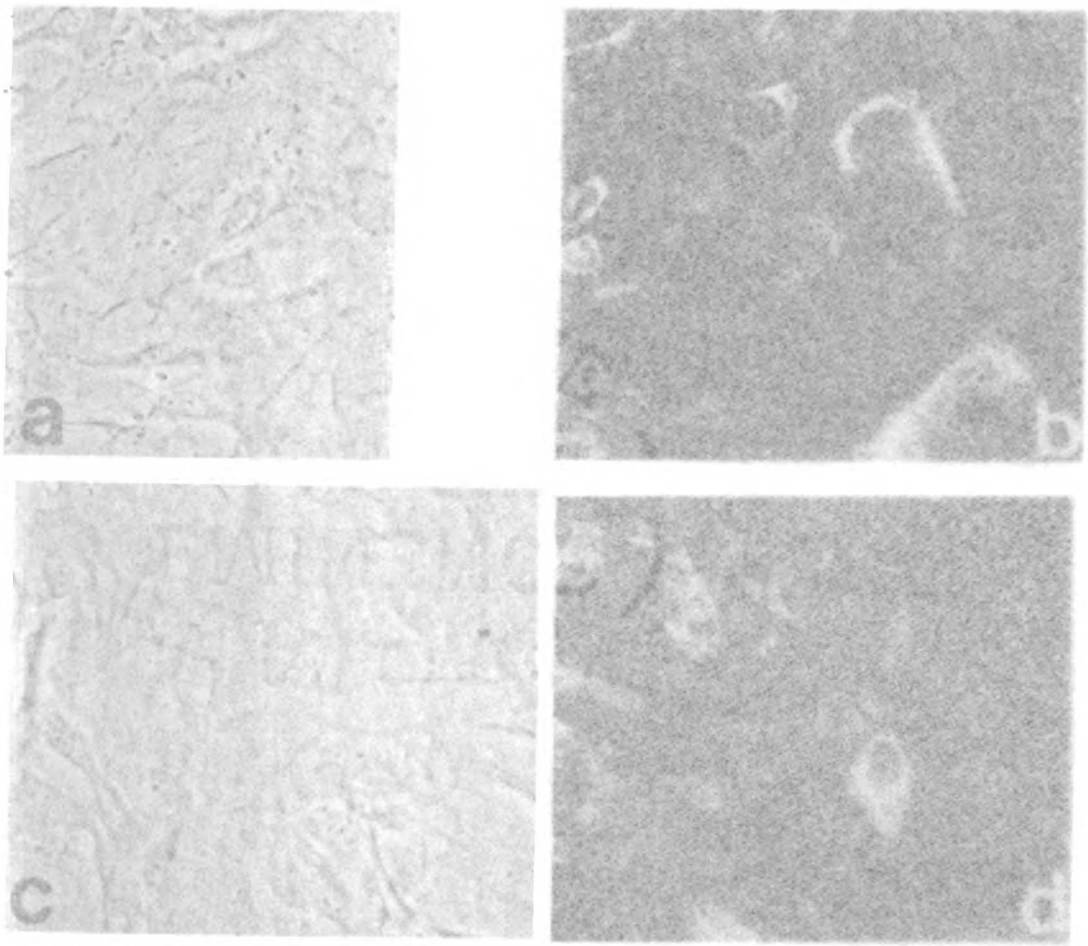

Fig. 4 [illegible]
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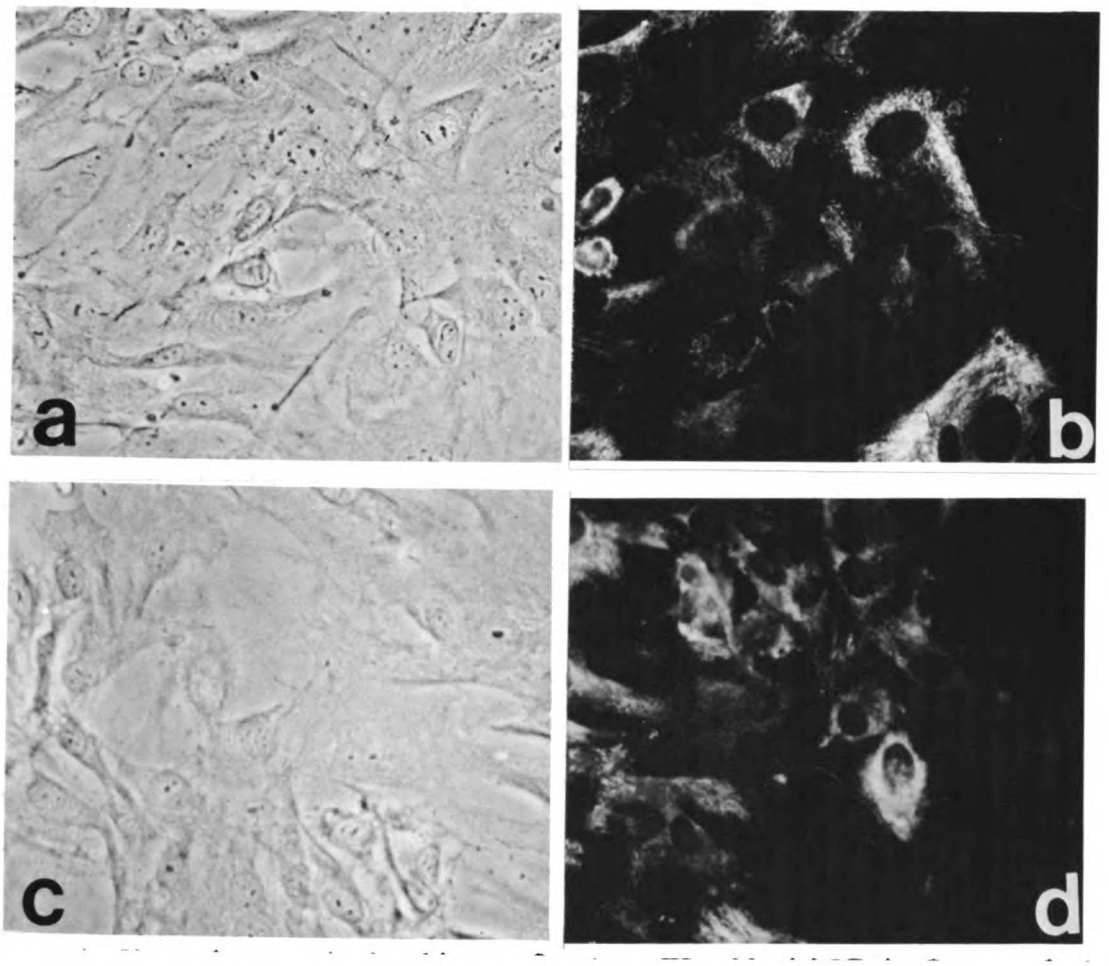

Fig. 4. Phase microscopy (a, c) and immunofluorescence staining (b, d) for collagen type IV and laminin. Perinuclear, granular immunostaining for collagen type IV in leptomeningeal culture HuA1 P1 (a, b) is similar to staining for laminin in meningioma culture 526 P2. X350.

Fig. 5 L[illegible] [illegible] [illegible] [illegible]
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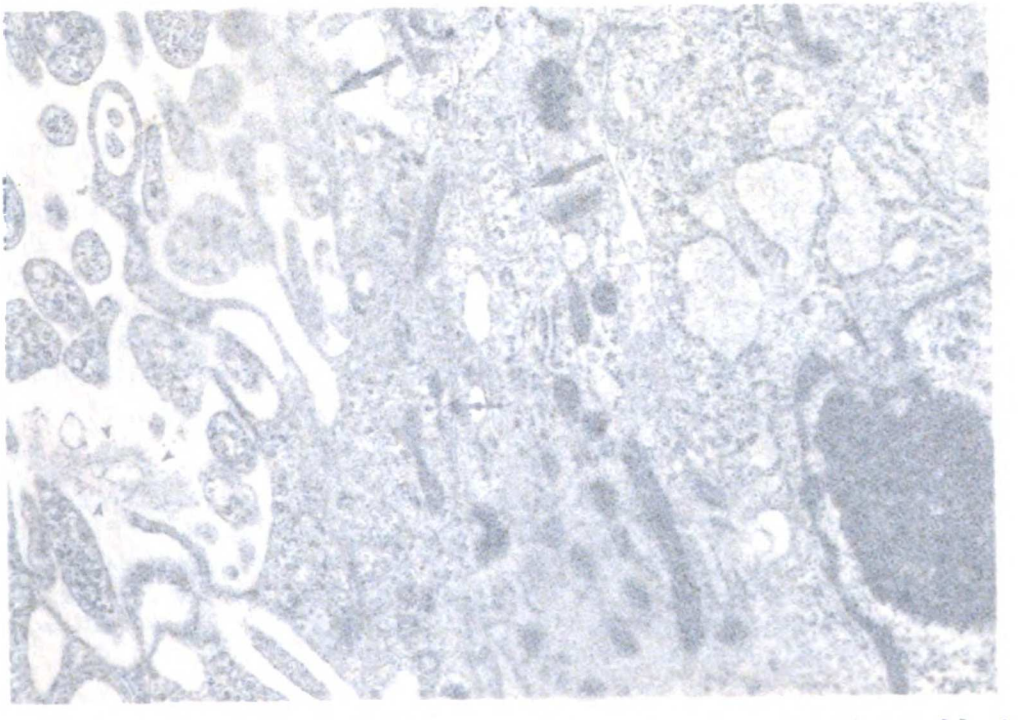

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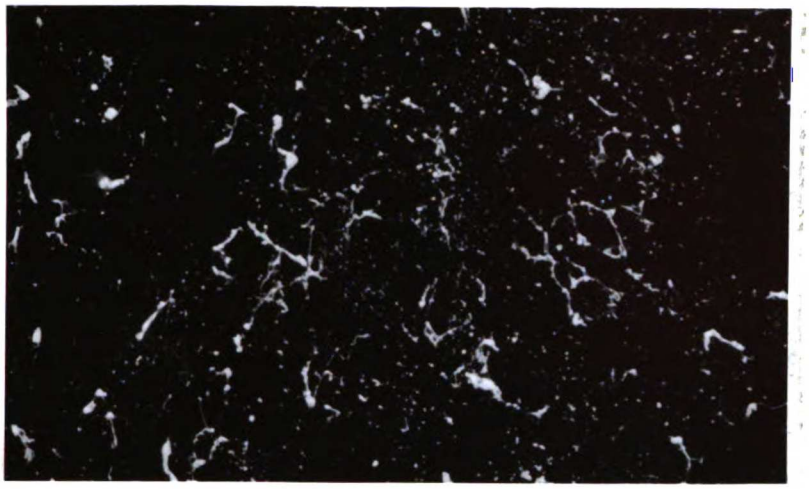


Fig. 5 Extracellular matrix as determined by indirect immunofluorescence of leptomeningeal culture 514P P2. Laminin is identified as dense focal plaque deposits and short fibers. X350.

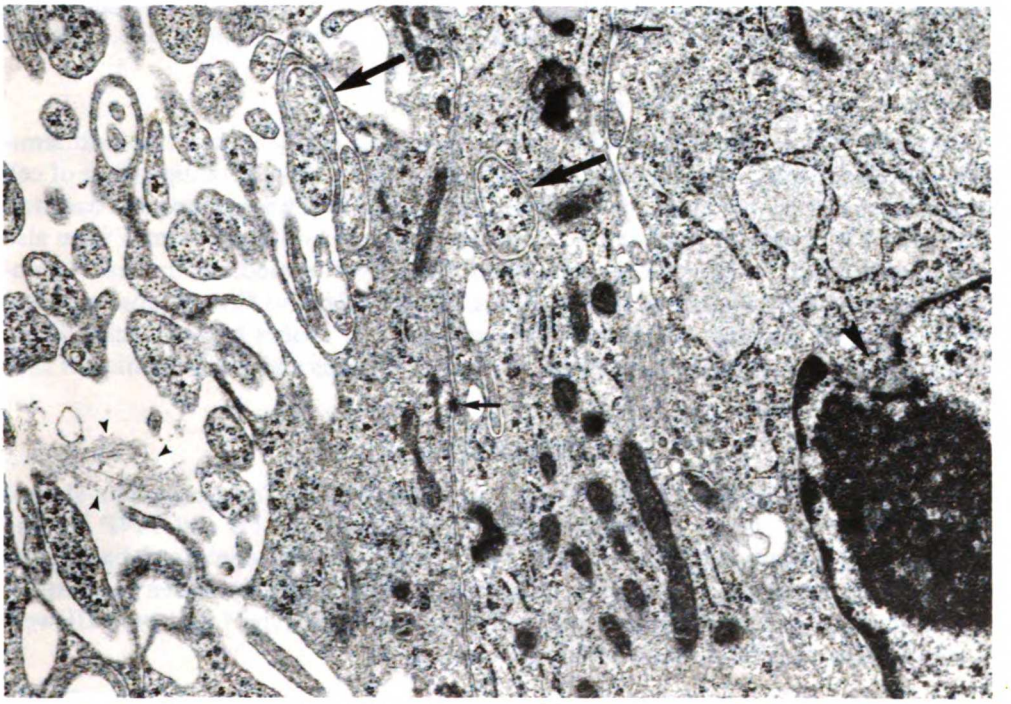


Fig. 6. Ultrastructural detail of leptomeningeal culture HuA1 P4. Desmosomes (small arrows) and interdigitations of the plasma membranes (large arrows) are present. Invagination of the cytoplasm into the nucleus is evident (large arrowhead). Filamentous and granular material is seen in the extracellular space (small arrowheads) Electron microscopy, X25,000.

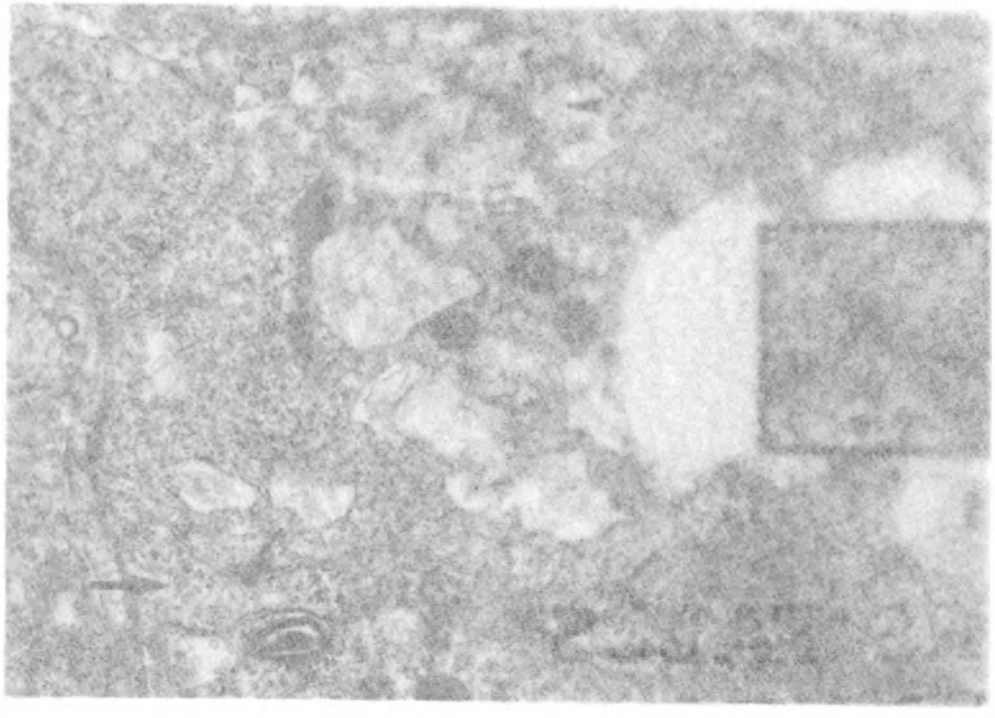


Fig. 7. Ultrastructural [illegible] invagination of the plasma membrane [illegible] arrows), microfilaments (small arrowhead[illegible] filamentous basement membrane-like material [illegible] Inset shows detail of desmosome at [illegible] microscopy, X20,000; inset, X60,000.

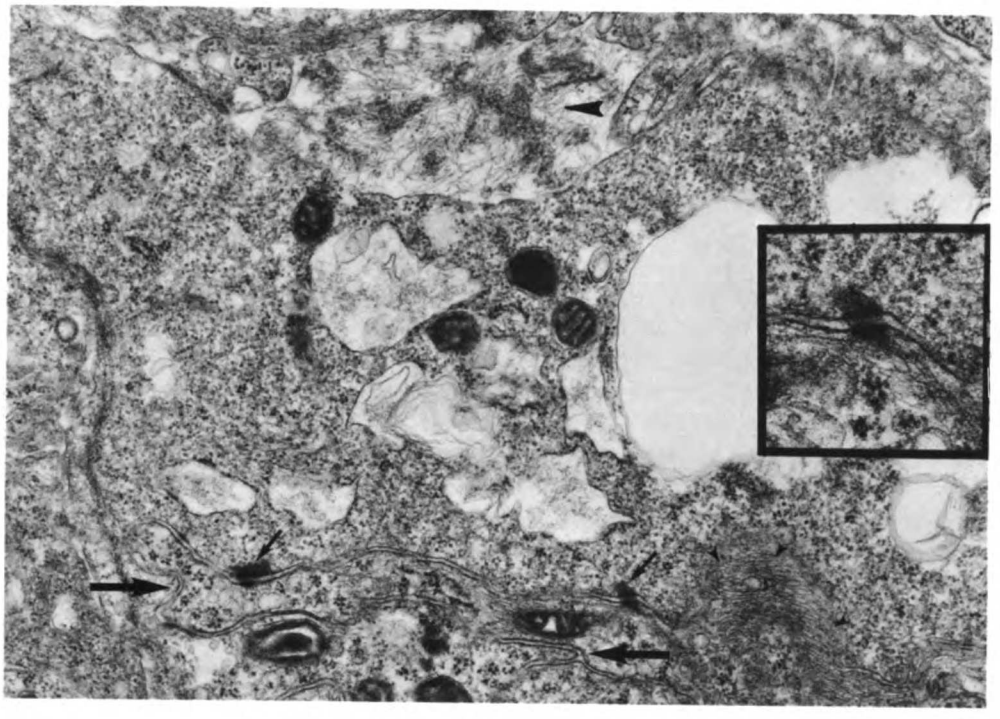


Fig. 7. Ultrastructural detail of meningioma culture 526 P2. Interdigitation of the plasma membrane (large arrow), desmosomes (small arrows), microfilaments (small arrowheads), and abundant extracellular filamentous basement membrane-like material (large arrowhead) are present. Inset shows detail of desmosome at higher magnification. Electron microscopy, X30,000; inset, X60,000.

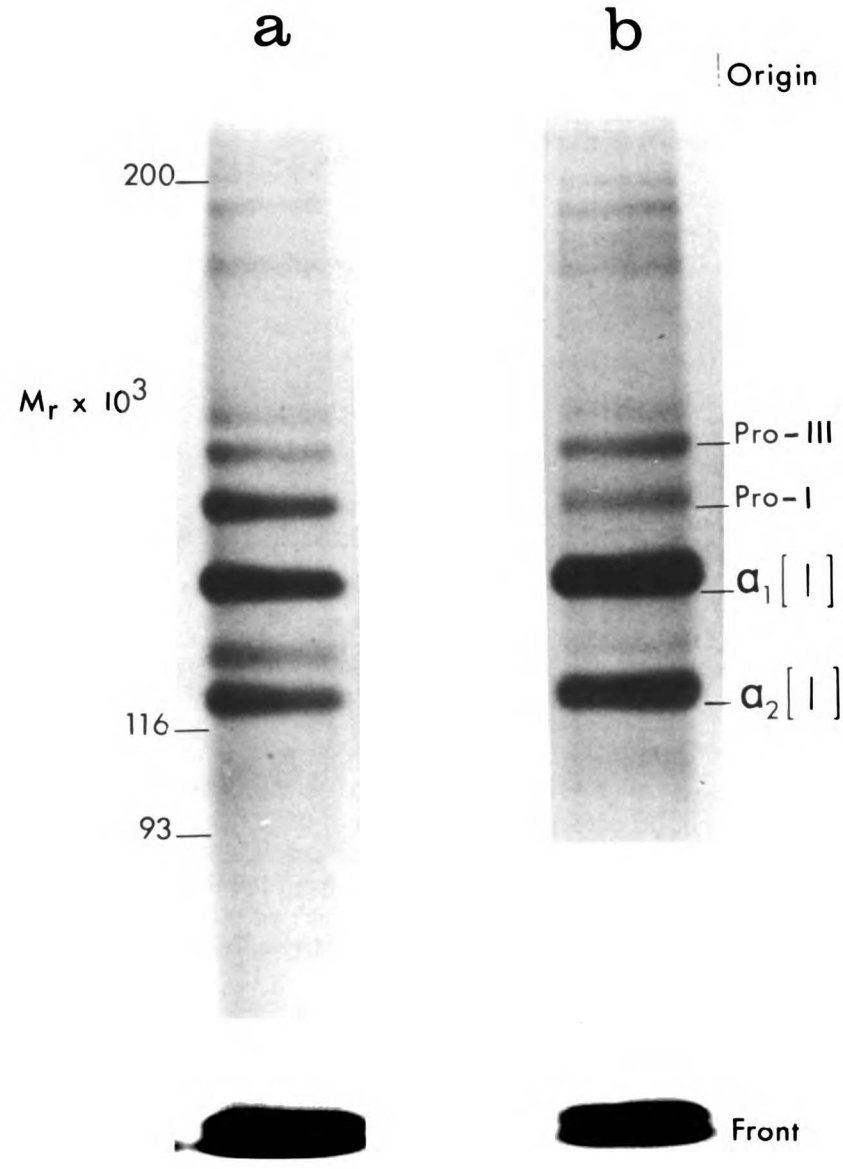
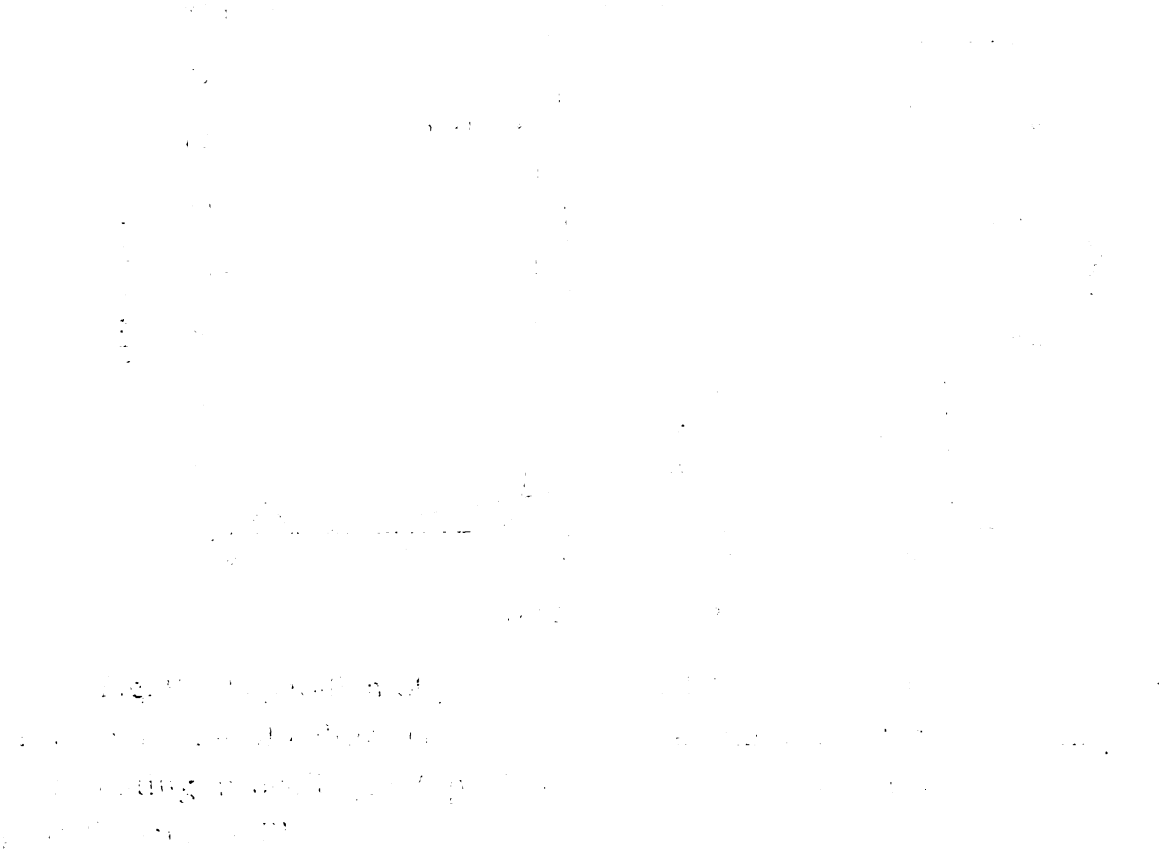


Fig. 8. SDS-polyacrylamide gel electrophoresis analysis of ^{3}[H] proline-labeled and reduced proteins within the cell layer of meningioma (528) and leptomeningeal (HuA1) cultures. Molecular weight (M_r standards (BioRad) were run concurrently and stained with Coomassie blue: myosin, 200,000;] galactosidase, 116,000; phosphorylase B, 93,000. Vitrogen was also loaded into a lane in the adjacent gel (not shown). Lane a (leptomeningeal culture) shows bands representing collagen type I (alpha$_1$ (I) and alpha$_2$ (I)). The alpha$_1$ (I) band is darker than the alpha$_2$ (I) band. Procollagen I and III bands are also present. The meningioma culture (lane b) shows a banding pattern nearly identical to that of the leptomeningeal culture (lane a).



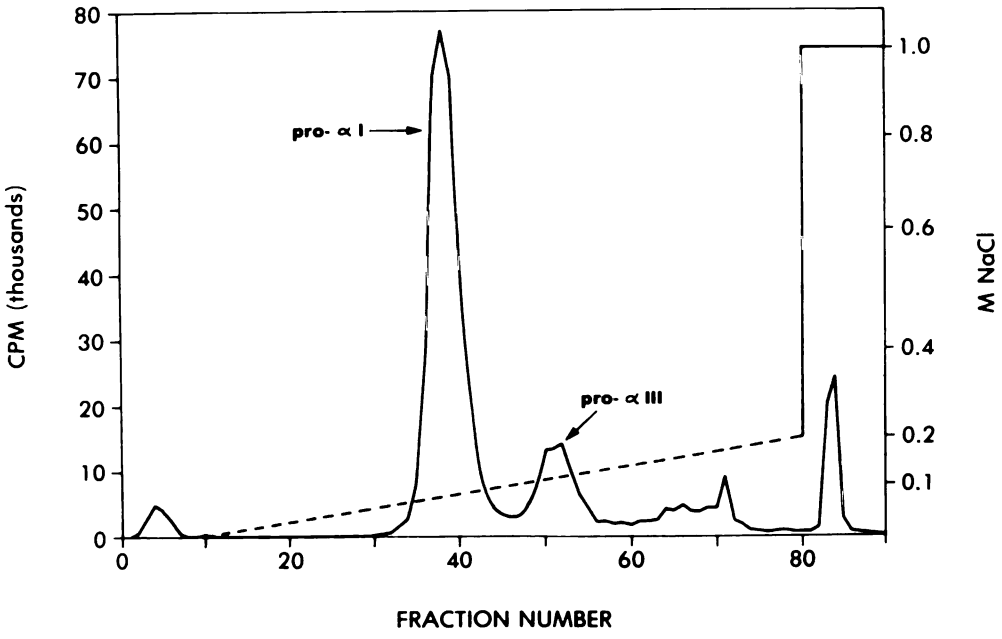


Fig. 9. Separation of procollagens by DEAE cellulose chromatography of leptomeningeal culture HuA1 P4. The medium separated into a tall peak representing procollagen type I and a much smaller peak representing procollagen type III.

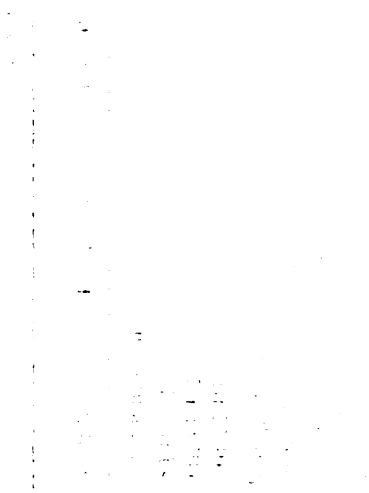


TABLE 1
Summary of *In Vitro* Immunocytochemical Characterization Studies

Cell line	Histology	GFAP	Factor VIII	Fibronectin	Laminin	Collagen type IV	Procollagen III
Leptomeningeal							
514P P2		–	–	+++ (100%)	+++ (100%)	+++ (100%)	+++ (100%)
HuA1 P2		–	–	+++ (100%)	+++ (100%)	+++ (100%)	+++ (100%)
HuA2 P1		–	–	+++ (100%)	+++ (100%)	+++ (100%)	+++ (100%)
Meningiomas							
406 P3	Transitional	–	–	+++ (100%)	+ (100%)	++ (100%)	++ (100%)
489 P4	Syncytial	–	–	+++ (100%)	+ (100%)	++ (100%)	+ (100%)
495 P6	Syncytial	–	–	++ (100%)	+ (100%)	+ (100%)	+++ (100%)
526 P1	Syncytial	–	–	+++ (100%)	+++ (100%)	+ (100%)	++ (100%)
528 P1	Syncytial	–	–	+++ (100%)	+++ (100%)	++ (100%)	++ (100%)
542 P1	Fibroblastic	–	–	++ (100%)	++ (100%)	+ (100%)	+ (100%)
545 P2	Malignant	–	–	++ (100%)	++ (100%)	++ (100%)	++ (100%)
Fetal rat astrocytes							
SDNR P1		+++ (90%)	–	++ (10%)	–	–	–
Endothelial							
929 P1		–	+++ (100%)	+ (100%)	+ (100%)	+ (50%)	–
Fibroblasts							
HS27 P20		–	–	++ (100%)	+++ (100%)	+ (50%)	++ (70%)
HFF P10		–	–	+++ (100%)	++ (100%)	+ (90%)	+ (5%)

Key: Staining intensity: –, negative; +, low; ++, moderate; +++, high.
Numbers in parentheses are percentages of cells staining positively.
GFAP = glial fibrillary acidic protein.

CHAPTER 3

Characterization of Normal Adult Human Brain Cultures: Evidence for the Outgrowth of Leptomeningeal Cells

3.1 Introduction

The autocrine hypothesis of CNS carcinogenesis could be tested in vitro if a model system existed in which well-characterized normal human glial cells were available for monolayer culture studies. Normal human glial cells could then serve as the target for glioma-derived growth factors. Although there have been several reports on the in vitro growth of human "glia-like" cells, at present a monolayer system with well-characterized and indisputable glial cells does not exist. In the present chapter, we have made an attempt to cultivate normal adult human glial cells dissociated from normal human brain specimens. Through our efforts, we have identified the histogenetic origin of the cell type previously reported as "glia-like" by several authors (83,84,196-198).

In general, human CNS tissue is particularly well suited for in vitro growth. The lack of inherent stromal elements, the paucity of interstitial collagens (except in perivascular locations), and the gelatinous consistency of normal gray and white matter are histological properties unique to the CNS, which probably contribute to the high rate of success in initiating cultures from normal human brain (83,196). Because brain tissue has a greater proportion of glial cells than of other cell types, excluding neurons in the gray matter, which do not proliferate in vitro, the cell type most likely to grow in vitro from dissociated brain specimens should be the astrocyte. For this reason, and because of the morphological and behavioral similarities to astrocytes in vivo, cells in cultures derived from human brain have been referred to as "glia-like" (199). The histogenetic origin of the "glia-like" cell has never been fully delineated (198).

If human brain cultures are to serve as models for studying the role of astrocytes in chronic neurological diseases (46,301) or for predicting the effects of various agents on the function and growth regulation of astrocytes in vivo (144), the predominant cell type in these cultures must be fully characterized. In this study, we provide evidence that cultures derived from adult human brain are of leptomeningeal rather than astrocytic origin.

3.2 Materials and Methods

3.2.1 CELL CULTURE

A) Normal brain

Normal brain specimens were obtained from eight patients undergoing routine craniotomy for trauma, arteriovenous malformations, or intractable seizure disorders at the University of California, San Francisco (UCSF). A representative portion of each specimen was fixed in 10% buffered formalin, embedded in paraffin, cut into thin sections, and stained with hematoxylin and eosin for histological examination. All histological slides were reviewed to confirm the absence of pathologic abnormalities. The remainder of each specimen was processed within 4 hours after biopsy as previously described (Section 2.2.1B). The cells were incubated in 75-cm^2 flasks at 37 C in a humidified atmosphere (95% air and 5% CO_2) in an enriched medium containing Eagle's MEM, nonessential amino acids, glutamine, gentamicin, and 10% fibronectin-free fetal calf serum. The medium was changed twice each week until the cells had reached confluence. At confluence, the cultures were split 1:2.

B) Leptomeningeal.

Small samples 1-2 cm^2 of the leptomeninges were obtained by microdissection from three patients without tumors during routine neurosurgical procedures or at autopsy under sterile conditions. The tissue specimens were rinsed thoroughly in HBBS, diced with scapels into fragment smaller than 1 mm^3, placed in 25-cm^2 flasks, and incubated under the same culture conditions used for the normal brain cultures.

C) Neonatal Rat Brain.

Cerebral cells from five 1-2-day-old rats were combined and cultured using a procedure based on the techniques of Manthorpe et al. (151) and Fontana et al. (74), and as described in Section 2.2.1C

D) Endothelial Cells.

Endothelial cell culture 929, from human umbilical vein, was obtained from Dr. J.M. Harlan, Harborview Medical Center, Seattle, Washington.

E) Fibroblasts.

Fibroblast cultures HS 27, a cell line initiated from adult human skin, and HFF, intiated from human foreskin, were obtained from the Cell Culture Facility at UCSF.

2.2 Monoclonal Antibodies and Antisera

The monoclonal antibody and antisera panel used in the antigenic characterization of the cultures in this study is the same as that described in detail in Section 2.2.2. In addition, in the present study, a mouse monoclonal antibody to vimentin was purchased from Monosan (Sanbio, The Netherlands); its specificity for the 58 Kd cytoskeletal component has been determined by Western blots.

3.2.3 Characterization of Cells in culture

Cultures of the different cell types were characterized by several techniques.

A) Cell Morphology, Karyotype, and Cell Kinetics.

In all cultures, the morphology of cells during exponential and plateau phases of growth was analyzed and recorded with an Olympus PM-10 phase microscope and camera. Morphologic changes occurring after repeated cell passages were also recorded. Karyotyping by Giemsa banding was performed on four normal human brain cultures (183G, 185G, 233, and 319W) and two leptomeningeal cultures (514P and HuA1). To exclude anchorage-independent cell growth, cells from two human normal brain and two leptomeningeal cultures were placed into a soft agar system, as described by Courtenay and Mills (45). The life span of cell cultures was determined by maintaining six cultures of normal human brain (183G, 185G, 319G, 514W,

514G, and 418) and three leptomeningeal cell lines (514P, HuA1, and HuA2) continuously and splitting them 1:2 at confluence. The terminal cell density was calculated for cultures of each cell type in the late plateau phase using a hemocytometer and trypan blue dye exclusion. In one normal human brain, one leptomeningeal, and one fibroblast culture, the labeling index and growth fraction were estimated (52,179) by incubating the cells with 10 uM bromodeoxyuridine (Sigma) for 1 hour and 48 hours, respectively, during the exponential and plateau phases of growth. The cells were then stained with FITC-conjugated anti-bromodeoxyuridine monoclonal antibody (Becton Dickinson, Mt. View, California) and scored using an Olympus BHS System fluorescence microscope as described below.

B) Indirect Immunofluorescence.

All normal human brain cultures were studied by indirect immunofluorescence to determine whether GFAP, vimentin, fibronectin, procollagen type III, laminin, and collagen type IV could be localized to cells derived from normal brain. Neonatal rat astrocyte, endothelial cell, and fibroblast cultures were similarly tested and served as internal controls. The technique of indirect immunofluroescence used in this study has been described in detail in Section 2.2.4.

C) Double Immunofluorescence.

Double immunofluorescence staining was also used to study the neonatal rat brain cultures. The cells were first incubated for 30 minutes with antisera to fibronectin or laminin (1:100 dilution), washed in Tris-PBS, and stained with rhodamine-conjugated swine anti-rabbit immunoglobulins for 30 minutes. The cells were then washed in Tris-PBS, incubated with the monoclonal antibodies to procollagen III or collagen type IV for 30 minutes, rinsed in Tris-PBS, and stained with the appropriate fluorescein-conjugated sheep anti-mouse immunoglobulins for 30 minutes. The cell layers were rinsed, mounted with Aquamount and coverslips, and viewed with the Olympus fluorescent microscope. In control experiments, the fluorescein- and rhodamine-conjugated antisera did not react with each other.

D) Characterization of the Extracellular Matrix.

Cells from all human brain cultures were grown to confluence in Lab-Tek chambers in the presence of 10% fibronectin-free fetal calf serum. The cells were maintained at confluence for 7 days. The normal cell matrix was then isolated and characterized as described previously (Section 2.2.5).

E) Electron Microscopy

The ultrastructural features of cells in all normal human brain cultures were studied by electron microscopy.using the procedure described in Section 2.2.3.

F) SDS Polyacrylamide Gel Electrophoresis.

Cells from three normal human brain cultures were prepared as described in Section 2.2.6 for gel electrophoresis to determine whether they had produced any of the collagen subtypes.

G) DEAE Cellulose Chromatography

To separate the procollagens secreted into the medium of normal human brain cell cultures, the labeled medium reserved from the SDS-PAGE experiment was processed according to the procedure described in Section 2.2.7.

H) Addition of Proline Analogue to Normal Brain Cultures.

cis-Hydroxyproline (cis-4-hydroxy-L-proline, Sigma) has been shown to decrease normal cellular proliferation in fibroblast cultures by inhibiting collagen synthesis (116). To determine the effect of this proline analogue on leptomeningeal cultures, which synthesize collagen types I, III, and IV (225), and on normal human brain cultures, 5×10^4 cells of each type were initially placed in two groups of 25-cm2 flasks containing an enriched medium that consisted of MEM, glutamine, gentamicin, and 10% fibronectin-free fetal calf serum, and incubated under the same culture conditions described previously. During the exponential phase of growth, cis-hydroxyproline (200 ug/ml) was added to cells in each group on days 0, l, or 3. New culture medium with fresh cis-hydroxyproline was added every other day after incubation. Growth curves were generated for control and treated flasks of normal human brain and leptomeningeal cultures by counting cells in a hemocytometer (trypan blue exclusion).

3.3 Results

3.3.1 Cell morphology, karyotype and cell kinetcs

All eight specimens of normal human brain grew in vitro. The primary cultures usually reached confluence within 2 to 3 weeks. During the

first few passages, cells of several types--polygonal, elongate, stellate, giant, and spindle--were easily identified in each culture. This heterogeneous population was succeeded in later passages by the predominance of cells that were broadly bipolar with rounded central nuclei and generous cytoplasm during the exponential phase (Fig. 1). At confluence, these cells ceased to be bipolar and became homogenously flat and polygonal (Fig. 2A). While it appeared that the plasma membranes of some cells were in close contact, in other regions the cells had areas of pericellular clearing bridged only by fine intercellular processes. The morphological appearance of leptomeningeal cell cultures was identical to that of normal human brain cultures in later passages (Fig. 3).

The karyotypes of four normal human brain cultures (183G, 185G, 233, and 319W) and two leptomeningeal cultures (514P and HuA1) were normal without evidence of transformation. Leptomeningeal and normal human brain cells had similar lifespans in vitro. The interval between passages (approximately the doubling time) gradually increased, and the cells entered a senescent phase, followed by degeneration of the culture (Fig. 4). Normal human brain cultures placed in a soft agar system did not achieve anchorage-independent growth. The growth characteristics of normal human brain and leptomeningeal cell lines at exponential and plateau phase in culture also appeared identical. The labeling index of a normal human brain culture (183G passage 7) in the exponential phase was 5%, decreasing to 0.5% at confluence. The growth fraction of this culture was 50% in the exponential phase and 1% at plateau. Similarly, leptomeningeal cell culture (514P passage 6) had a labeling index of 7% in the exponential phase and 1% at plateau; the growth fraction was 60% in the exponential phase and 3% at plateau. The doubling times and terminal cell densities of normal human brain culture 183G, leptomeningeal culture 514P, and fibroblast culture HS27 are shown in Table 1.

3.3.2 Characterization of Cells

The results of indirect immunofluorescence studies of normal human brain cultures are shown in Table 2. All cells from all normal human brain cultures stained positively for fibronectin, laminin, and collagen type IV; stains for procollagen type III were positive in a variable percentage of cells. All negative controls were appropriately nonstaining. The leptomeningeal

cell cultures previous studied stained intensely and uniformly for all four glycoproteins (225, Section 2.3.2). GFAP was identified in two normal human brain cultures in early passages. In culture 514G (primary), 0.1% of cells were positive for GFAP (Fig. 5); but by the third passage, staining for GFAP was negative in all cells. All cells in all cultures were positively immunostained by the anti-vimentin antibody. Newborn rat brain astrocyte and human umbilical vein endothelial cell cultures could be distinguished immunocytochemically from normal brain cultures by the presence of GFAP and factor VIII, respectively, in a large percentage of cells.

The staining patterns for fibronectin and procollagen type III in normal human brain cultures appeared to be morphologically very similar. Perinuclear, granular cytoplasmic localization and considerable extracellular fiber formation were seen (Fig. 2B). Immunocytochemical stains for laminin and collagen type IV also revealed a perinuclear "halo" of granular staining (Fig. 6); there was apparently less staining of the extracellular matrix. Immunocytochemical analysis of the extracellular residue after removal of the cell layer from all normal human brain cultures with 20 mM ammonium hydroxide showed that the matrix consisted largely of fibronectin (Fig. 7) and procollagen type III, with smaller amounts of laminin and collagen type IV.

In primary neonatal rat brain cultures, staining for GFAP was positive, revealing long intracytoplasmic fibrils, in 95% of cells. Approximately 5% of cells stained positively for fibronectin and appeared to be morphologically different from the astrocytes. By the second passage, 50% of the cells were found to have GFAP and 50% had fibronectin in a mutually exclusive fashion. Double immunocytochemical staining for collagen type IV and GFAP revealed no overlap in these two distinct cell populations (Fig. 8). By the fourth passage, none of the cells stained positively for GFAP. Concurrent with the increase in the number of cells staining positively for fibronectin after early passages, there was a proportional increase in the number of cells containing collagen type IV and procollagen type III (Table 2).

Normal human brain and leptomeningeal cultures were ultrastructurally similar. Interdigitation of plasma membranes and invagination of the cytoplasm into the nucleus were prominent features, and desmosomes and tonofilaments were abundant. All cultures displayed prominent extracellular basement membrane-like material in the form of fine filaments and granules (Fig. 9).

SDS-PAGE of the cell layer of normal human brain cultures 514W, 183G, and 514G revealed bands that were identified as the alpha$_1$ and alpha$_2$ chains of collagen type I (Fig. 10). Bands representing procollagen types I were also identified in these cultures after application of radiolabeled peak fractions from DEAE cellulose chromatography to separate vertical slab gel lanes.

DEAE-cellulose chromatography of the medium of culture 514G showed a moderate peak for procollagen type III and a much higher peak for procollagen type I (Fig. 11).

cis-Hydroxyproline (200 ug/ml) inhibited cell proliferation in both leptomeningeal and normal human brain cultures. The inhibitory effect was clearly most marked when cells were inoculated with the proline analogue on day 0 (Fig. 12).

3.4 Discussion

The properties of normal human brain cells in vitro have been well described (196,215,293,294). Our cultures of normal human brain are similar in several respects to those reported by others (196,197,282): a limited life span in vitro (15 to 20 doublings); characteristic flat cells with cytoplasmic extensions after the initial few passages; monolayer growth with striking contact inhibition; normal karyotype; terminal cell density of 5×10^4 to 7×10^4 cells/ml; and a doubling time of 24 to 30 hours. Further characterization of our normal human brain cultures by electron microscopy and indirect immunofluorescence demonstrated specialized intercellular junctions in the form of desmosomes and gap junctions and a characteristic glycoprotein profile.

To our knowledge, a glycoprotein profile such as the one we have found in our normal human brain cultures has not been described previously. The presence of fibronectin and procollagen type III in a given cell type suggest a mesodermal rather than an ectodermal origin (98). Certain ectodermal cells have, however, been reported to produce some of these matrix glycoproteins (145,272). Trelstad et al. (272) reported the synthesis of collagen chains from isolated chick spinal cords. However, because these cultures were not characterized to establish the identity of all cell types present, contaminating leptomeningeal cells and periadventitial fibroblasts may well have contributed to the synthesis of alpha$_1$ chains.

Laminin is a large, noncollagenous attachment glycoprotein closely linked to collagen type IV in the lamina densa of basement membranes (123). In the nervous system, laminin has been localized to neural crest derivatives such as the leptomeninges (225), Schwann cells (170), and neuroblastoma cells (4). Liesi et al. reported the immunocytochemical localization of laminin to early rat astrocytes in primary cultures (143). Double immunofluorescence staining clearly demonstrated GFAP and laminin in early stages in the same cell type. Although the immunofluorescence of GFAP intensified with the number of days in vitro, there was a time-dependent disappearance of laminin from these cells in culture. Our studies of neonatal rat brain cultures support these findings. Because we were using techniques to enrich for astrocytes, the first immunocytochemical analysis of these cultures was on day 15 of the primary culture. At that time, 90% of cells contained GFAP, but we were unable to demonstrate laminin in any cell type. The ability of early rat astrocytes to synthesize laminin in addition to GFAP suggests that astrocytes may play a role in the cell-cell interactions in the CNS of developing rats (143). How the transient appearance of laminin in early rat astrocytes relates to the developing human CNS remains to be determined.

The distribution of fibronectin in the central and peripheral nervous systems has been described in several reports (191,231,282). In an early study of normal human brain cultures, Vaheri et al. (282) found uniform staining of cells for fibronectin. The presence of fibronectin in both "glia-like" cells and fibroblasts was thought to relate to functional similarities between these two cell types, such as cellular mobility, cytoplasmic extensions, and their roles as supporting cells. In later studies in vivo, Schachner et al. (231) and Paetau et al. (191) localized fibronectin to sites of contact between mesenchyme and neurectoderm, including blood vessels, leptomeninges, choroid plexus, endothelial cells, and perineurium. Glial cells and neurons were not identified positively for fibronectin. Further work by Paetau et al. (192) and Liesi et al. (143) on normal human brain and neonatal rat brain cultures demonstrated that stains for GFAP and fibronectin identified completely different populations of cells. Although the GFAP-negative and fibronectin-positive cells were thought most likely to be of nonglial origin, the possibility that glial cells in vitro might de-differentiate and acquire the capacity to synthesize fibronectin could not be fully excluded (192).

A unique feature of the cytotectonics of the adult CNS is the notable absence of various collagen subtypes (243). In the present study, cultures derived from normal human brain tissue were positively identified by monoclonal antibodies to collagen type IV and procollagen type III; moreover, generous quantities of collagen chains were detected in the cell layer by SDS-PAGE and in the medium by DEAE-cellulose chromatography. Even though it might appear feasible that astrocytic foot processes, which line up against vascular basement membranes, synthesize collagen type IV, it seems unlikely that astrocytes (even in vitro) would produce the interstitial collagens, i.e., types I and III, as collagen fibers are not normally found in the parenchymal elements of the CNS. This notion is further supported by our double-immunofluorescence studies in primary neonatal rat brain cultures, in which immunostains for GFAP and either collagen type IV or procollagen type III demonstrated mutually exclusive populations of cells. In addition, the decline in the number of GFAP-positive cells concomitant with the increase in fibronectin, collagen type IV-, and procollagen type III-positive cells with progressive subcultivations suggests the outgrowth of the cultures with a nonastrocytic cell.

Vimentin, a 57 K dalton protein, is a member of the intermediate filament family of relatively insoluble cytoskeletal proteins and has been immunolocalized to a variety of cell types (78). In the CNS, vimentin immunoreactivity has been localized to embryonal rat astrocytes (17), adult rat Bergmann radial glia (48,241), fibrous astrocytes in gliotic rat optic nerves (48), human meningiomas (237), and adult rat meninges and cerebral blood vessels in vivo (50,241,303). Immunoreactivity to vimentin has also been demonstrated in primary astrocyte cultures of newborn rat brains (38), normal human brain cultures (189), malignant glioma cultures (193), and cultures of human meningiomas (148). Although vimentin was initially thought to be specific for cells of mesenchymal derivation (77), its presence in virtually all cells grown in culture, including epithelium (78) and glia (38), has raised important questions regarding the utility of vimentin as a specific characterization marker, especially under tissue culture conditions.

GFAP, the 8 to 10 nm intermediate filament that forms an integral part of the astrocytic cytoskeleton, was first isolated from homogenates of severely gliotic human brain (62) and has proven to be the most specific marker for recognizing cells of glial origin under normal and pathological conditions

(53,54,281). Because GFAP can be identified immunocytochemically in extracts of brain homogenates (16,49,86) and in astrocytes and ependymal cells in situ, the absence of GFAP in our normal human brain cultures is conspicuous. The inability to identify cells containing GFAP in cultures of normal brain, except in small percentages in the primary culture and early passages, is a common experience shared by many (79,189,196).

Because there is an inverse correlation between GFAP expression and the degree of anaplasia in astrocytic neoplasms (54), it has been presumed that, in general, culture conditions are so artificial that cells from normal human brains are forced to de-differentiate in vitro and, as a result, lose the ability to synthesize GFAP (189). However, attempts to reinduce GFAP expression by suitable manipulations of the culture conditions have failed (189). Others have postulated that normal human brain cultures are still astrocytic but are from a poorly defined subset that does not form processes or have a consistent relationship with blood vessels (144).

Recently, Sobel et al. (254) reported reduced GFAP immunoreactivity in perivascular astroglial endfeet and complete absence of GFAP in Alzheimer type II astrocytes in brain tissue, examined immunohistochemically, from patients with hepatic encephalopathy. Quantitative measurements demonstrating decreased GFAP levels in brain tissue from patients with hepatic encephalopathy compared with controls (129), and ultrastructural studies of primary astrocyte cultures treated with ammonia demonstrating loss of intermediate glial filaments (93), further support the immunohistochemical evidence of decreased GFAP in the brain in response to a pathological, non-tumorous insult to the CNS. While the generation of Alzheimer type II like astrocytes in our normal human brain cultures could conceivably account for the absence of cellular GFAP, ammonia was not added to our standard cultura medium, and the ultrastructural details of all of our normal human brain cultures did not include the cytoplasmic vacuolization, mitochondrial proliferation, or accumulation of dense bodies that have been described in human and experimental hepatic encephalopathy (93,156,187).

Gilden et al. (84) reported that 30% to 50% of cells in cultures derived from explants of adult human brain stained positively for GFAP. The intensity of staining was apparently increased in cultures from patients with certain neurological diseases (multiple sclerosis, Jakob-Creutzfeldt syndrome,

and tuberous sclerosis) and, at times, in later passage cultures. There was no mention of how long and what percentage of cells remained positive for GFAP over serial passages. More recently, Osborn et al. (189) showed that in normal human brain cultures the percentage of cells with GFA-specific filaments decreases with increasing passage number. Their initial cultures had 3% to 14% of GFAP-positive cells, but the GFA marker was lost completely by the third to eighth passage. It is largely because of the lack of GFAP-positive cells in human brain cultures that animal models, such as the neonatal rat and rabbit, which yield high percentages of GFAP-positive cells (74,92,151), are used to study glial cell function and response to a variety of agents. The reason why we identified so few GFAP-positive cells in our primary cultures of human brain may be that we used enzymatic dissociation to obtain a cell suspension, whereas most others (6,84,189,199) have used a primary explant technique. It is possible that our enzyme cocktail may be more damaging to glial cells than to other cell types.

Regardless of the technique used to initiate cell cultures from human brain, our data imply that glial cells are poorly suited to the present culture conditons: they divide slowly in vitro (if at all) and are soon overtaken by a different, more tenacious cell type. The notion that astrocytes may be overgrown by a contaminating cell type in human brain cultures is not new. Lumsden (150) recognized the early outgrowth of nonglial cells in cultures derived from adult human brain tissue and termed these cells "mesoblastic," presumably because of their resemblance to primitive, poorly defined mesodermal cells. It was thought that these cells were derived from the reticular elements of small arteries and veins, and not from capillary endothelial cells or fibroblasts. Ponten and Westermark (199) support this proposal because there is no known counterpart to this obviously mesodermal-like cell in vivo. Osborn et al. (189) and Rorke et al. (215) described the outgrowth of these cells as fibroblasts, fibroblast-like, or as being composed of certain vascular smooth muscle cells.

Our study has provided clues to the exact histogenetic origin of these undesired nonglial cells so commonly observed in cultures of adult human brain: the vigorous growth during the exponential phase and in early passages, followed by marked contact inhibition and early senesence in later passages; normal karyotype; the absence of GFAP; the expression of procollagen type III, fibronectin, and collagen type IV; the presence of

interstitial collagens in the medium and cell layer homogenates; and characteristic ultrastructural findings of desmosomes, plasma membrane interdigitation, and tonofilaments, which are not normally features of primary cultures of astrocytes (276). All of these features were identified in cultures derived exclusively from normal leptomeningeal cells in our previous study (225, Chapter 2).

Pial cells over the entire surface of the brain are separated from astrocytic foot processes by the glia limitans externa (104), and arachnoid cells follow penetrating cortical arteries in a perivascular, sleeve-like distribution from the subarachnoid space into the brain parenchyma (33). Therefore, even if the leptomeninges are carefully peeled away from brain specimens along with grossly visible blood vessels, a few leptomeningeal cells will still be present at the time of tissue dissociation. Because these cells are well suited to vigorous monolyer growth, they may eventually become the predominant cell type in normal human brain cultures.

Although other cell types (macrophages, fibroblasts, perivascular adventitial cells) are undoubtedly present in the primary culture and early passages of our normal human brain cultures, the results of this study have helped us to redefine our cultures not as "glia-like" or mesoblastic, but rather as predominantly leptomeningeal or arachnoid in origin. Thus, if leptomeningeal cells can be eliminated from culture by taking advantage of their metabolic requirements to inhibit or prevent their growth--for example with proline analogues, low O_2 concentrations, and ascorbate-free medium--attempts to grow specific glial cell types (astrocytes, oligodendroglia, and ependymal cells) from human brain tissue obtained by biopsy may be more successful and would provide more relevant <u>in vitro</u> models for the study of neurological diseases.

Despite the important negative findings of this study, the adult normal human brain-derived leptomeningeal cells should serve as well-characterized normal cell targets for the purified and glioma-derived growth factor studies to be described in Chapters VIII and IX.

(Parts of this chapter were previously published in <u>Laboratory Investigation</u> 55: 71-85, 1986. Permission to reproduce this material in this thesis was granted by the International Academy of Pathology - see Appendix)

3.5 Figure Legends and Tables

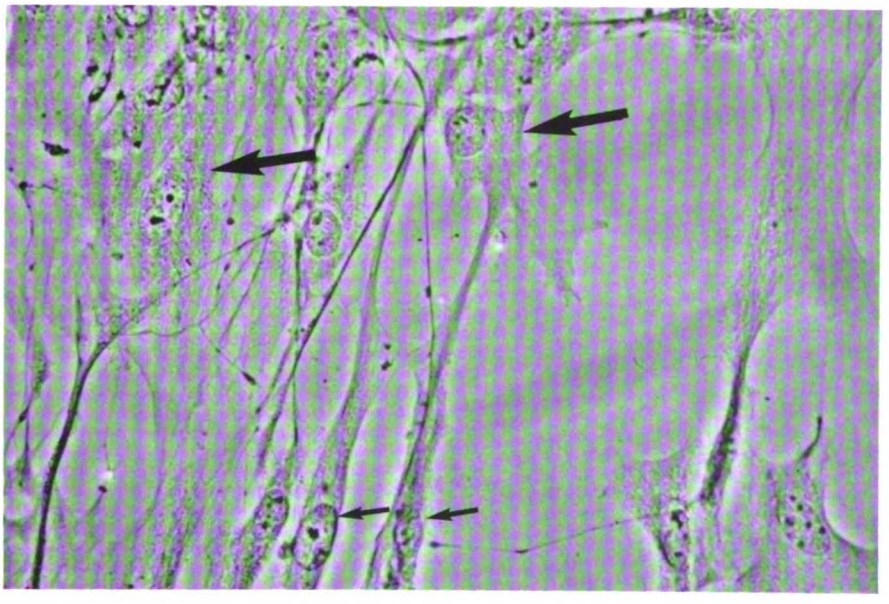


Fig. 1. Typical appearance of an early-passage normal human brain culture (514G P3) at subconfluence. Some cells (small arrows) are broadly bipolar with rounded nuclei and prominent nucleoli; others (large arrows) are polygonal with generous cytoplasm. Phase microscopy, 350X.

Fig. 2. A, Normal human brain culture [illegible] Polygonal [illegible] contact-inhibited cells are prominent. Phase [illegible] B, Normal human brain culture stained immunocytochemically [illegible] Perinuclear, granular cytoplasmic localization [illegible] present.

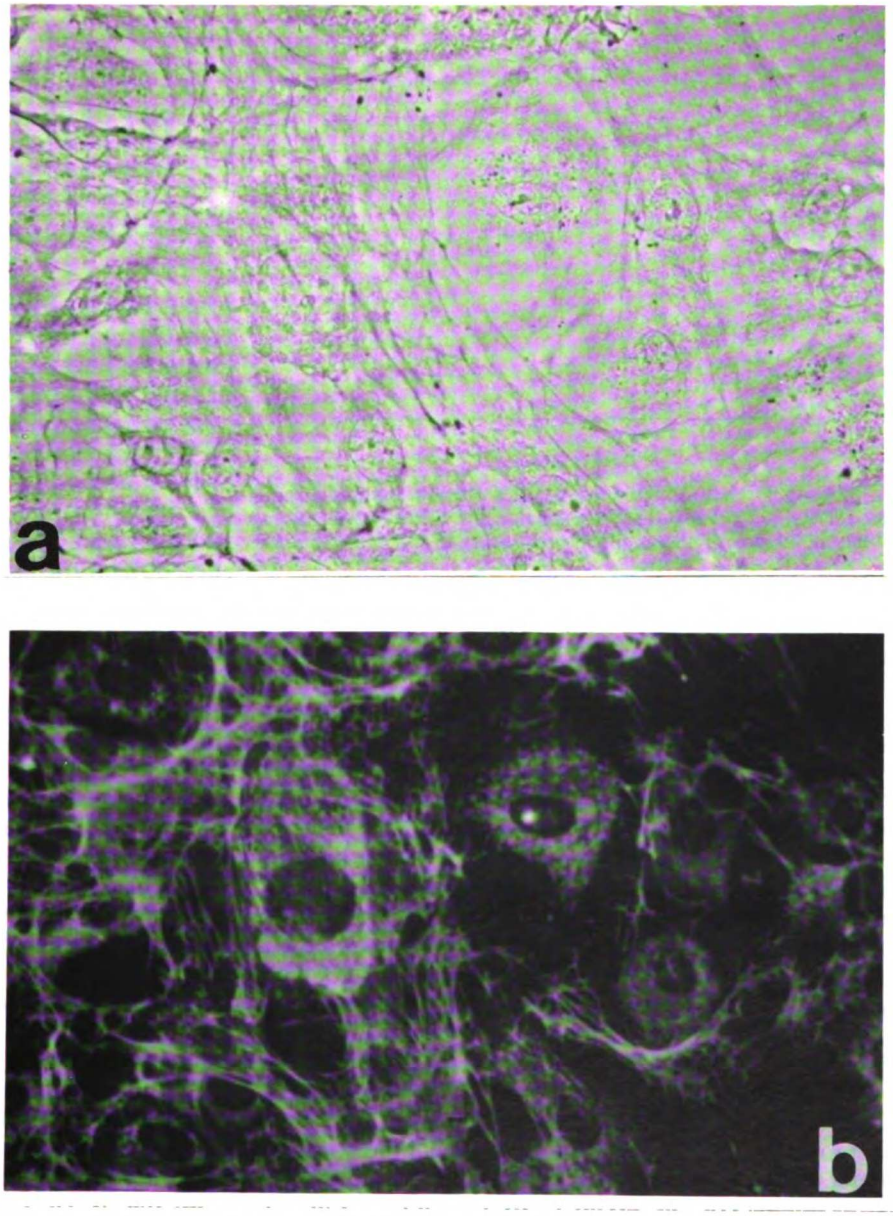


Fig. 2. A, Normal human brain culture at confluence. Polygonal, contact-inhibited cells are prominent. Phase microscopy, 350X. B, Normal human brain culture stained immunocytochemically for fibronectin. Perinuclear, granular cytoplasmic localization and extracellular fibers are present.

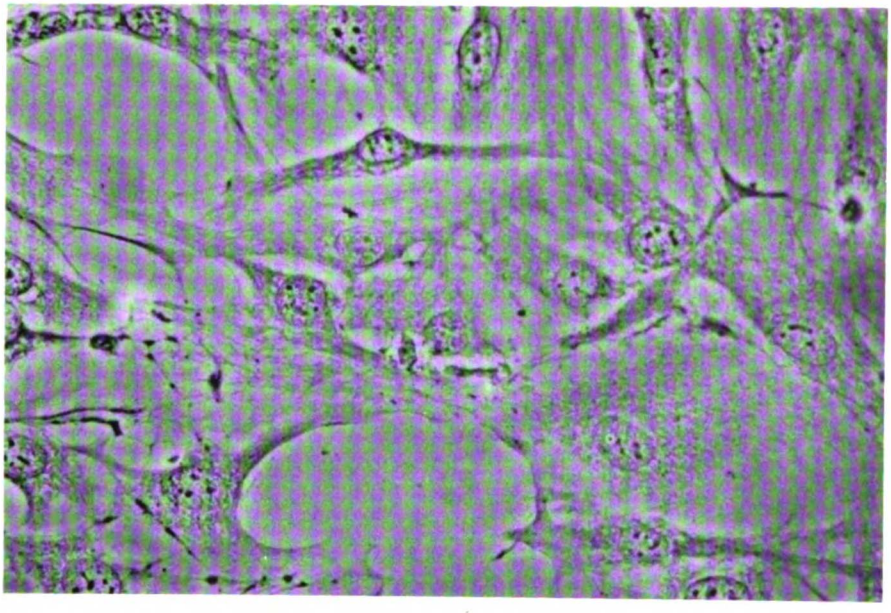


Fig. 3. Leptomeningeal culture, passage 4. The similarities to the normal human brain culture (Figs. 1 and 2) are striking. Phase microscopy, 350 X.

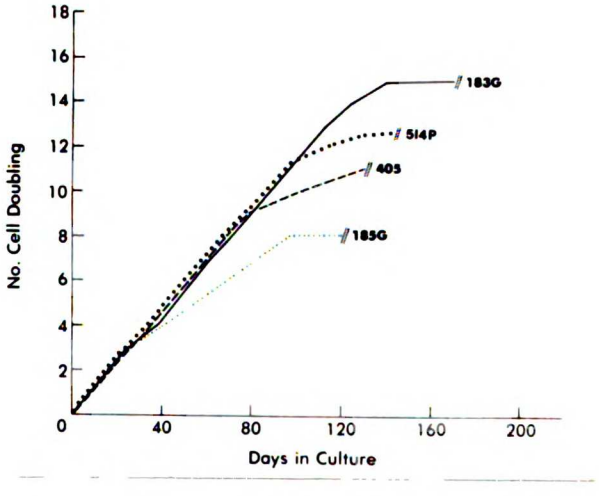


Fig. 4. Graphic time analysis of the life span of human brain cultures 183G, 405, and 185G and leptomeningeal culture 514P. After an initially rapid growth phase, all cultures senesced and degenerated.

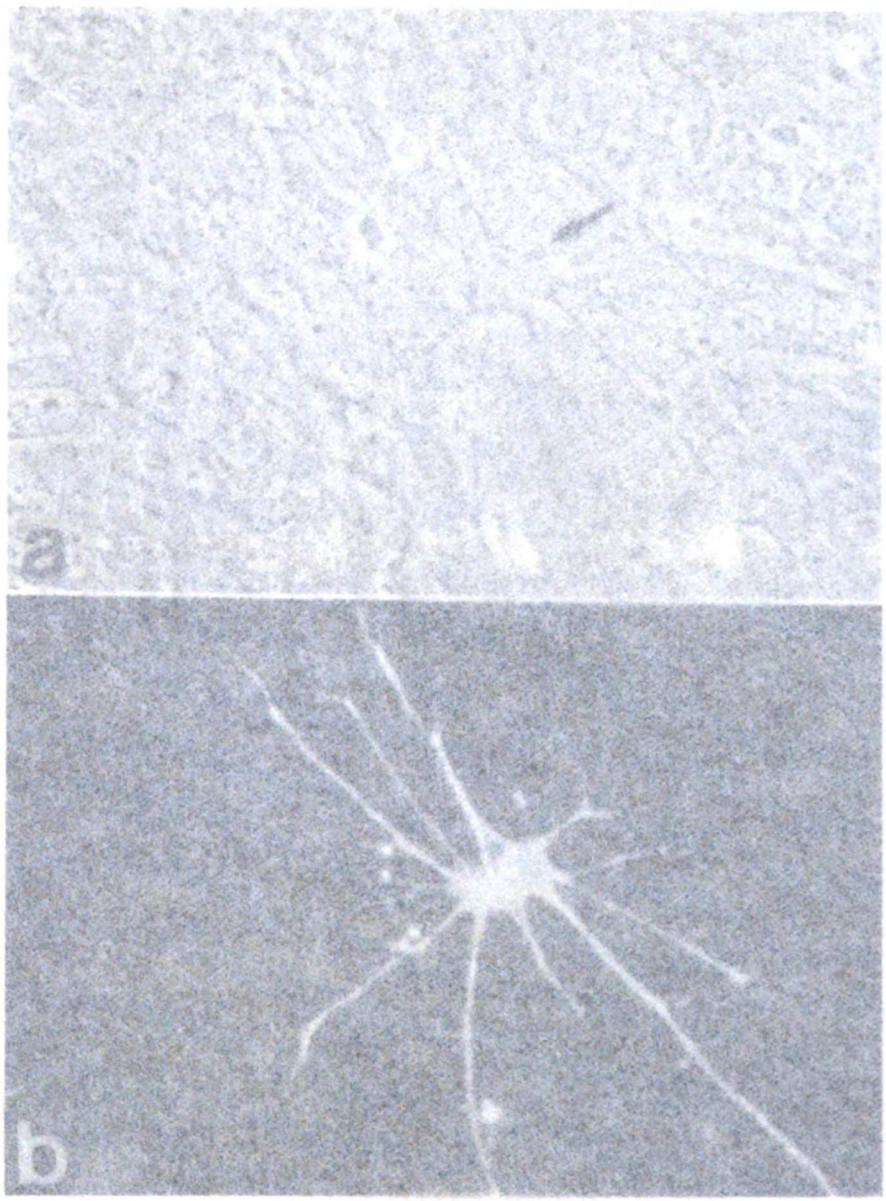


Fig. 5 Immunofluorescence [illegible] normal human brain cultures. A [illegible] typical astrocytic morphology (A, arrow) [illegible] positively [illegible] The flat, [illegible] negative for GFAP

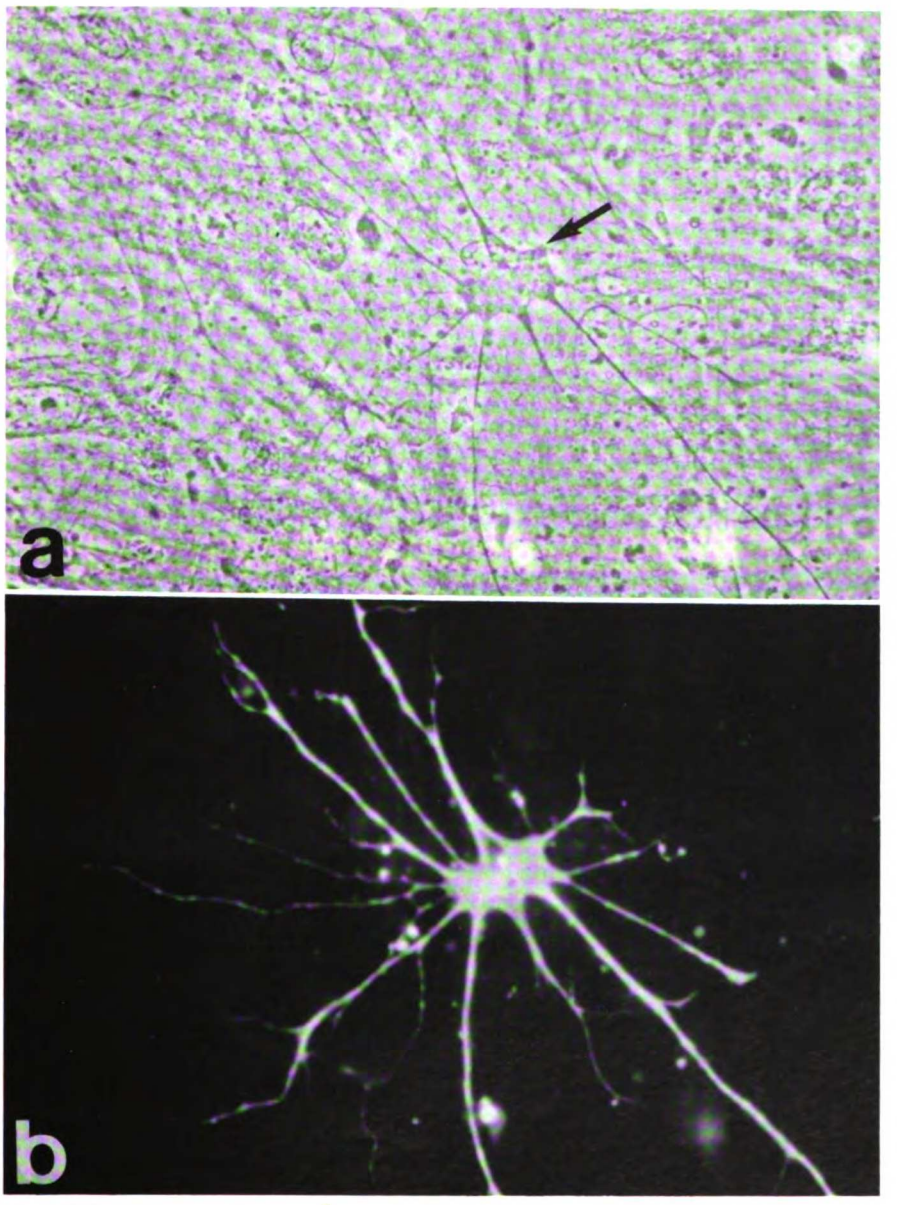


Fig. 5. Immunofluorescence staining for GFAP in a normal human brain culture. A solitary, typical astrocyte with stellate morphology (A, arrow) stains positively (B). The flat, broadly bipolar cells are negative for GFAP.

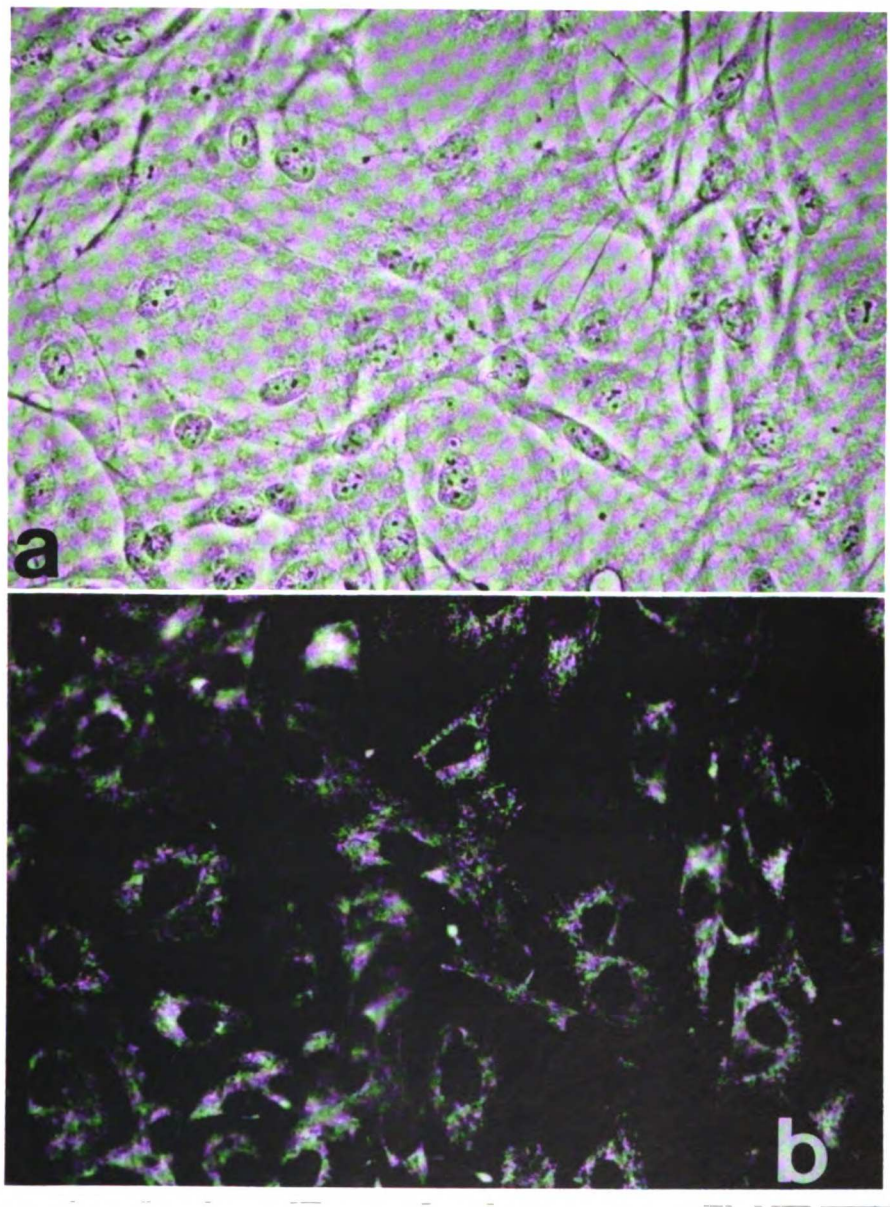


Fig. 6. Immunocytochemical localization of laminin in a normal human brain culture. Note the perinuclear, granular cytoplasmic staining in all cells. Phase and immunofluorescence microscopy, 350X.

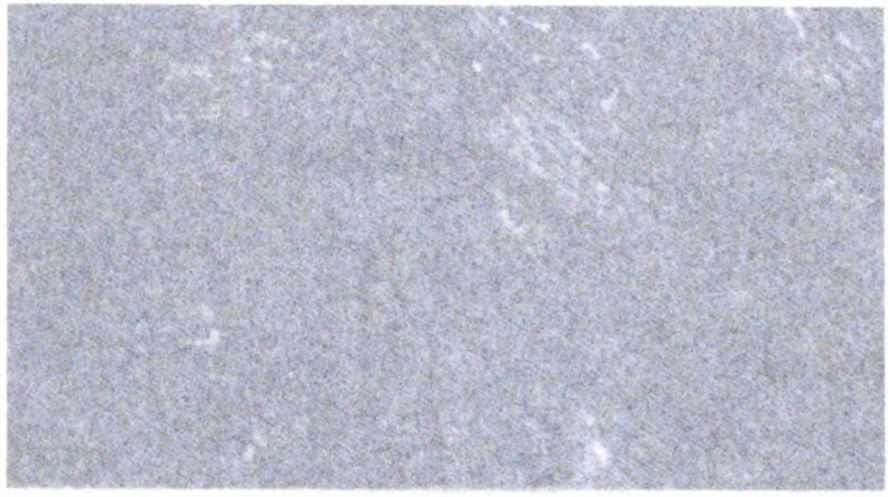


Fig. 7 [illegible] brain culture after treatment [illegible] Fluorescent stain for fibronectin [illegible] focal plaques. Immunofluorescence [illegible]

Fig. 8 Double immunofluorescent staining [illegible] type IV and CEAP in neonatal rat brain [illegible] A [illegible] 35 X. Positive staining for collagen type IV (b) [illegible] proliferation of cells separate from those staining [illegible] (small arrows)

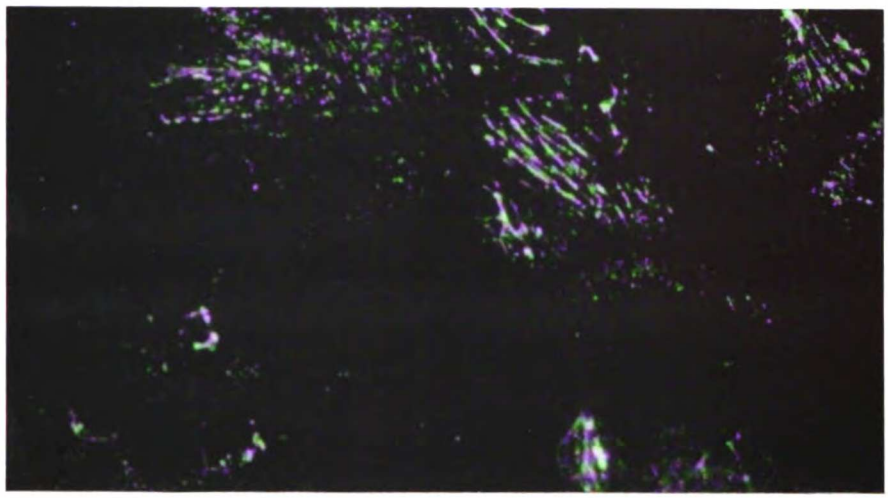


Fig. 7. Extracellular matrix of a normal human brain culture after treatment of cell layer with 20 mM ammonium hydroxide. Fluorescent stain for fibronectin reveals long fibers and granular focal plaques. Immunofluorescence microscopy, 350X.

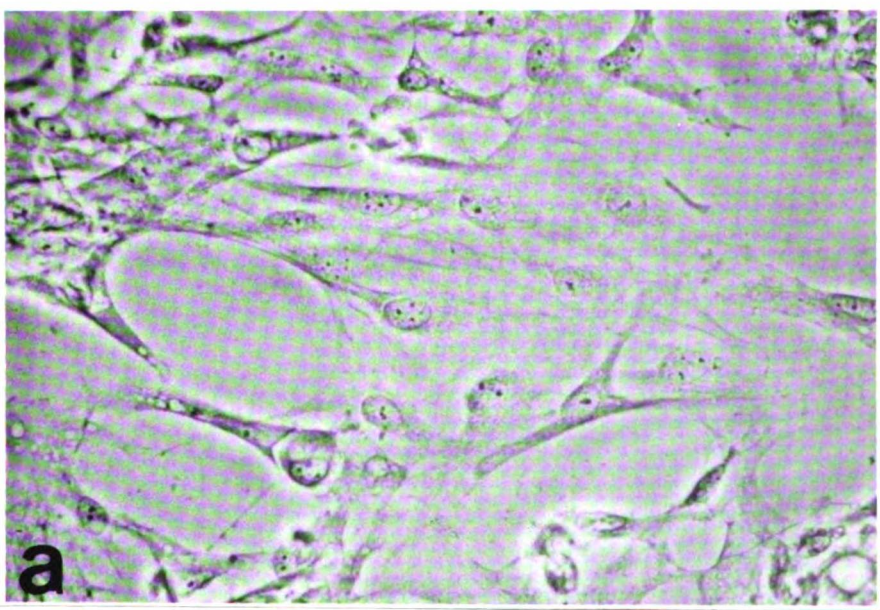


Fig. 8. Double immunocytochemical stain for collagen type IV and GFAP in neonatal rat brain cells. A, Phase microscopy, 350X. Positive staining for collagen type IV (B, large arrows) identifies a population of cells separate from those staining positively for GFAP (C, small arrows).

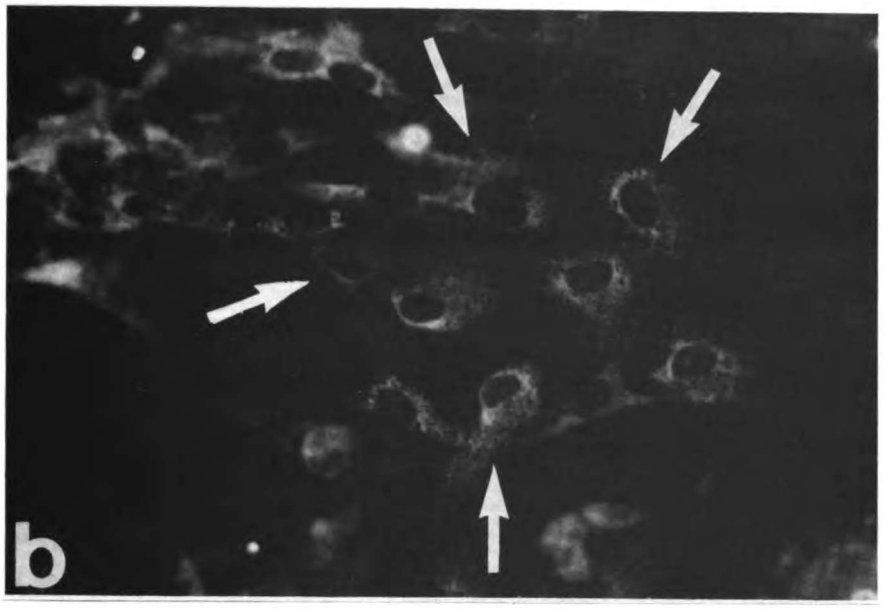


Figure 8b

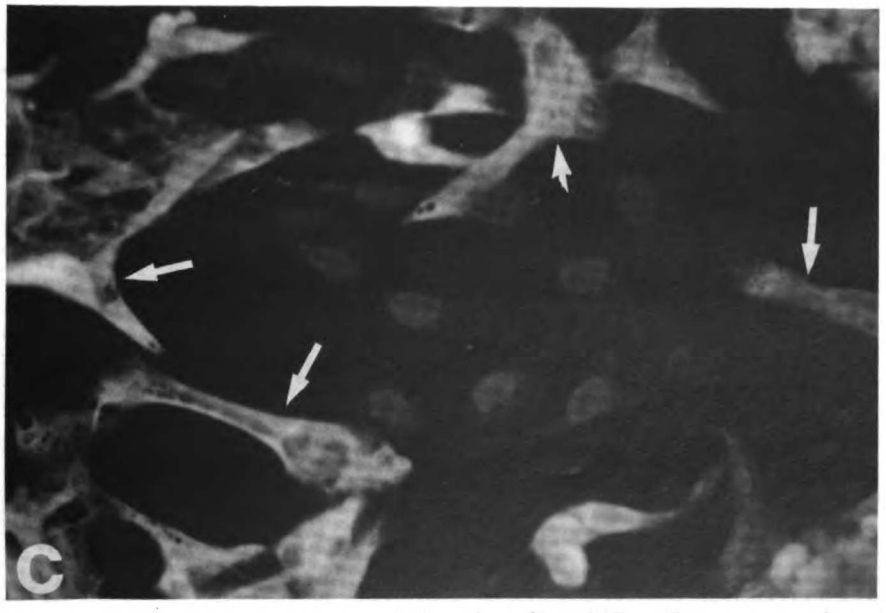


Figure 8c

Fig. 9. Ultrastructural appearance of normal human brain cultures. A, Desmosomes (small arrows) and invagination of cytoplasm into the nucleus (large arrows). Electron microscopy, 15,000X. Inset shows desmosome at higher magnification (30,000X). B, Plasma membrane interdigitation (small arrows), desmosome (large arrow), and microfilaments filling the cytoplasm (small arrowheads). In addition, granular basement membrane-like material fills the extracellular spaces (large arrowheads). Electron microscopy, 40,000X.

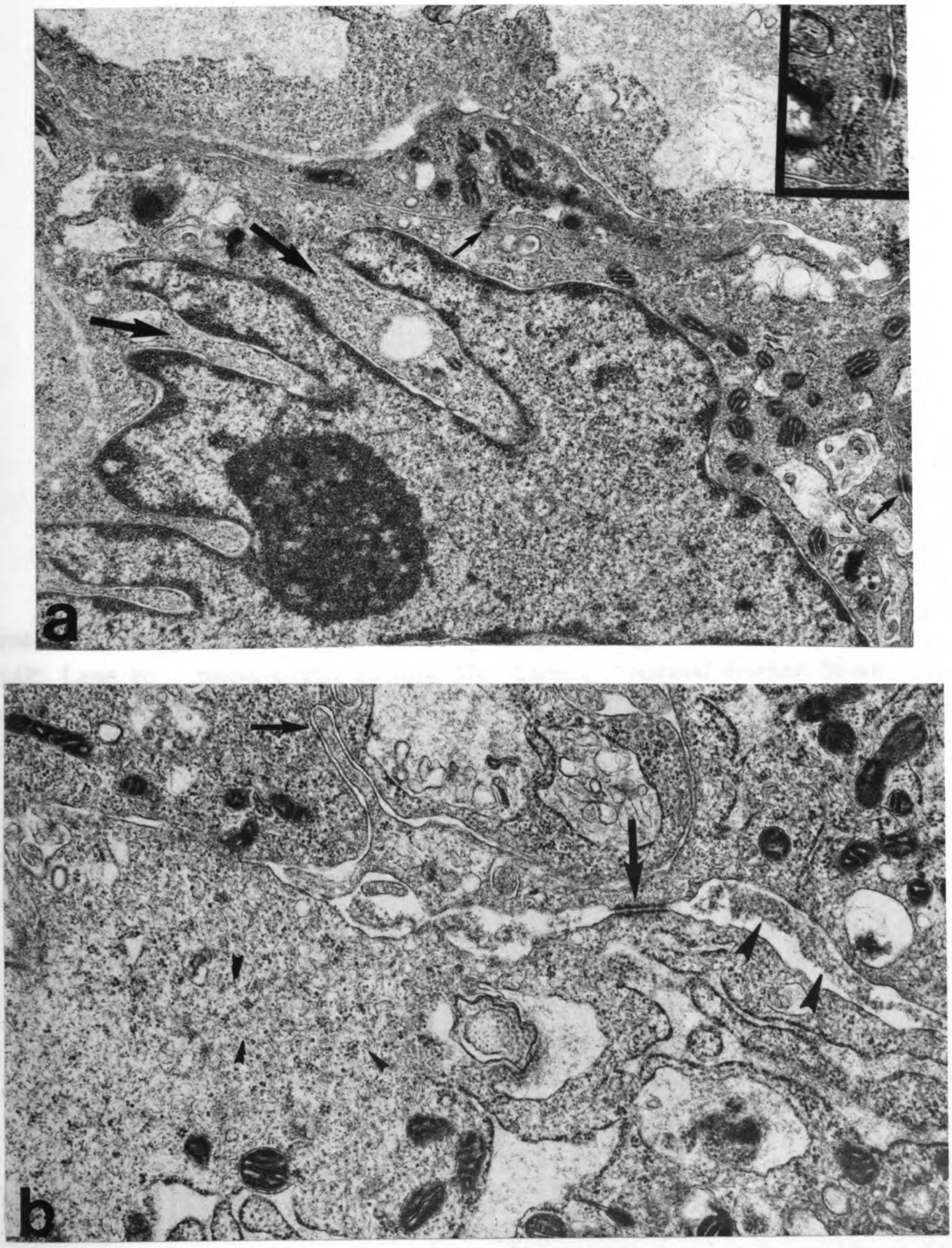
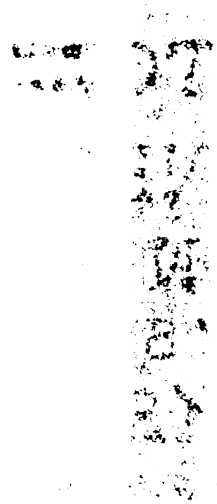


Figure 9 a & b



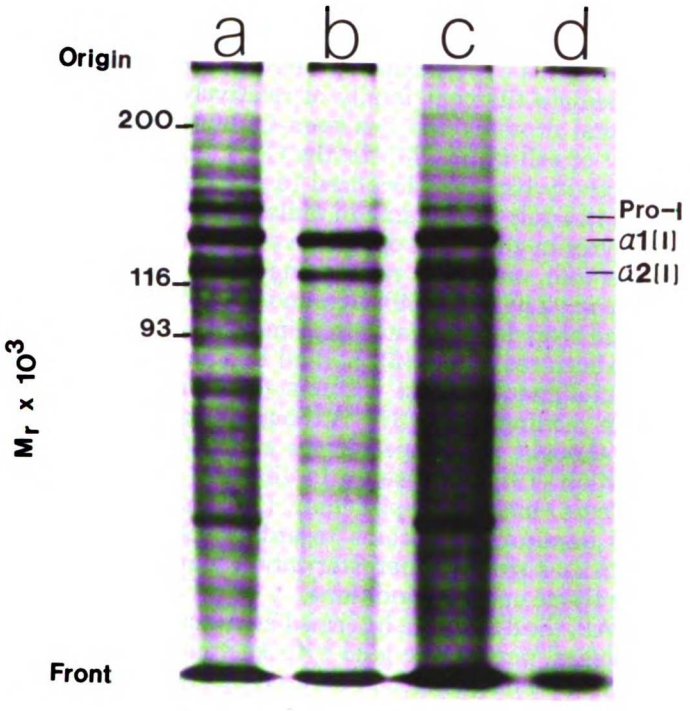


Fig. 10. SDS-PAGE analysis of ^{3}[H] proline-labeled and reduced proteins within the cell layer of cultures. Lane a: leptomeningeal culture 514P; Lane b: meningioma culture 526; Lane c: normal human brain culture 183G; Lane d: normal brain culture treated with collagenase, 10 units/ml, at 37 C for 4 hours (clostridium histolyticum, protease free; Calbiochem, San Diego). Bands representing collagen type I (alpha$_1$ (I) and alpha$_2$ (I)) are present in all three cultures. The alpha$_1$ band in each is darker than tha alpha$_2$ band. Procollagen type I band is identified above the alpha$_1$ band. All bands were absent in the collagenase-treated normal brain culture.

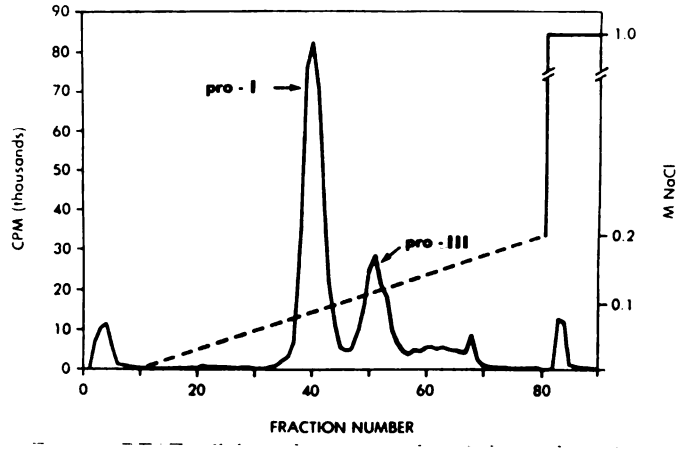


Fig. 11. DEAE-cellulose chromatography of the medium from a normal human brain culture. Peaks representing procollagen types I and III appear at fractions 40 and 50.

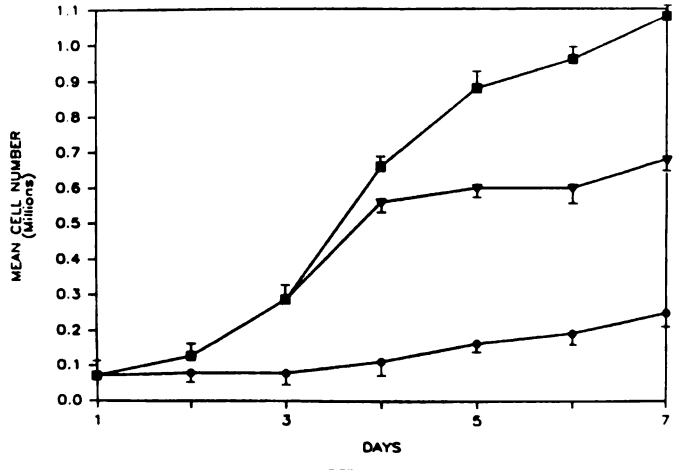


Fig. 12. Effect of cis-hydroxyproline (200 ug/ml) on cell proliferation in normal human brain culture 514G. Squares = untreated (control) flasks; circles = flasks treated on day 1; triangles = flasks treated on day 3.

TABLE 2. INTENSITY AND UNIFORMITY OF STAINING FOR GLYCOPROTEINS IN CELLS FROM NORMAL HUMAN BRAIN AND NEONATAL RAT ASTROCYTE CULTURES

Cell line	GFAP	Factor VIII	Fibronectin	Laminin	Collagen type IV	Procollagen III	Vimentin
Normal human brain							
185G P4	−[a]	−	++ (100%)[b]	++ (100%)	++ (100%)	++ (70%)	++ (100%)
233 P5	−	−	++ (100%)	++ (100%)	+−++ (100%)	++ (20%)	++ (100%)
319G P3	−	−	+++ (100%)	+++ (100%)	++ (100%)	+−++ (70%)	++ (100%)
463G P7	−	−	++ (100%)	++ (100%)	++ (100%)	+ (25%)	++ (100%)
514W (primary)	++ (0.1%)	−	++ (100%)	+++ (100%)	+−++ (100%)	++ (100%)	++ (100%)
514G (primary)	++ (0.1%)	−	++ (100%)	++ (100%)	++ (100%)	++ (100%)	++ (100%)
P3	−	−	+++ (100%)	+++ (100%)	++ (100%)	++ (100%)	++ (100%)
183G P4	−	−	+++ (100%)	++ (100%)	++ (100%)	+ (20%)	++ (100%)
183W P10	−	−	+++ (100%)	++ (100%)	++ (100%)	++ (70%)	++ (100%)
Neonatal rat astrocytes							
SDNR-II (primary)	+++ (95%)	−	++ (5%)	−	−	−	NA[c]
P1	+++ (75%)	−	++ (25%)	++ (25%)	+ (25%)	−	NA
P2	++ (50%)	−	++ (50%)	++ (50%)	++ (50%)	−	NA
P3	++ (10%)	−	+++ (100%)	++ (90%)	++ (90%)	−	NA
P4	−	−	+++ (100%)	++ (100%)	++ (100%)	−	NA

[a] Intensity of staining: −, negative; +, low; ++, moderate; +++, high.
[b] Numbers in parentheses indicate percentage of cells staining positively.
[c] NA, not assessed.

TABLE 1. DOUBLING TIME AND TERMINAL CELL DENSITY IN SELECTED CULTURES

Cell culture	TD	TCD
	hour	*cells/cm²*
514 P (Leptomeningeal)	23	4×10^4
183 G (Normal human brain)	24	4×10^4
HS27 (Fibroblast)	28	2×10^5
126 (Glioblastoma)	20	5 to 6×10^5

TD, doubling time; TCD, terminal cell density.

CHAPTER 4

CHARACTERIZATION OF FETAL HUMAN BRAIN CULTURES: DEVELOPMENT OF A POTENTIAL MODEL FOR STUDYING HUMAN GLIAL CELLS

4.1 Introduction

The major finding of Chapter 3 was that normal adult human glial cells cannot be propagated under routine culture conditions. In the present chapter, we describe our technique for selectively purifiying fetal human glial cells in culture. In addition, we describe the characterization of fetal human leptomeningeal cells in culture. Both fetal human glial and leptomeningeal cells will be used as target cells for the purified and glioma-derived growth factors in experiments to be described in Chapters VIII and IX.

The embryological complexities of the developing mammalian CNS exceed those observed in other organ systems largely because during embryogenesis the primitive neuroepithelium is thought to give rise to a number of different cell types--neurons, astrocytes, oligodendrocytes, ependymal cells--that interact in complex yet specific ways (41,247,248). In recent years, knowledge of the histogenesis of many intrinsic cell types in the developing mammalian CNS has increased through the use of electron microscopy and immunohistochemical analyses (7,16,41,138,306). Another approach has been to use tissue culture systems to characterize fetal CNS tissue (1,6,97,142,201,288). The potential advantage of such studies is that they permit morphological, biochemical, and physiological analyses in an environment that can be controlled and manipulated (203). Although there has been much work on the development and characterization of fetal brain cultures from mice and rats (74,95,97,151,166), human fetal brain cultures have not been as fully characterized (22,120,153,190).

One of the primary interests of this laboratory has been to develop highly enriched fractions of human glial cells in culture to facilitate studies of the mechanism of malignant transformation of glial cells. In a previous report, we demonstrated that cultures of adult human brain were not suited

to glial cell purification procedures; instead, the predominant cell type in these cultures was of leptomeningeal origin (227, Chapter 3). In the present study, the different cell types in cultures derived from fetal mammalian brain and fetal human leptomeninges were identified by their morphological and ultrastructural features, their ability to synthesize collagen, and their reactivity to a panel of monoclonal antibodies and antisera. Our findings demonstrate that primary fetal human brain cultures can be significantly enriched in glial cells.

4.2 Materials and Methods

4.2.1 Origin of Cell Cultures

Human fetal brains were obtained after elective second-trimester abortions; in each case, informed consent was obtained from the parents. The gestational age of each fetus was estimated by crown-rump length measurements, and varied from 16 to 24 weeks. Permission to work with the human fetal material was granted by the Human Research Committee, University of California, San Francisco. A representative portion from each of seven fetal brain specimens was fixed in Carnoy's or 10% buffered formalin solution and saved for immunohistochemical analysis as described below. The rest of the specimen was processed for tissue culture studies. Only readily identifiable and histologically proven human fetal telencephalons were used in this study.

4.2.2 Culture Method

The telencephalon was microdissected from the brain stem, cleaned of meninges, and minced. Dissociated cell cultures were prepared using a procedure based on the techniques of Manthorpe et al. (151) and Fontana et al. (74) and as described previously in detail (Section 2.2.1B). After the specimens were enzymatically and mechanically dispersed, the cell suspension was then filtered through sterile Nytex filters with a pore size of 20, 50, or 100 microns. The filtered cell suspension was seeded into 75-cm^2 flasks for continuous culture in a humidified atmosphere of 95% air and 5% CO2 at 37 C. The medium was changed once each week until the cells reached confluence. At confluence the cultures were split 1:2.

Cultures of human fetal leptomeninges were initiated separately by mincing the meninges saved from each fetal brain specimen and culturing the fragments as explants under the same culture conditions described above.

4.2.3 Electron Microscopy

The ultrastructural features of the cells in fetal human brain and leptomeningeal cultures were determined by electron microscopy using the procedure described in Section 2.2.3.

4.2.4 Antigen Expression

Immunolocalization of a variety of intracytoplasmic and extracellular matrix proteins was performed on the paraffin-embedded fetal brain and leptomeningeal specimens and on the cells in culture at various passages. The immunoperoxidase technique of Sternberger et al. (257) was used on tissue specimens, and indirect immunofluorescence microscopy was used on tissue cultures (225).

The monoclonal antibodies and antisera used in this study have been described in detail in Sections 2.2.2 and 3.2.2.

Paraffin-embedded sections 6 um thick were deparaffinized and pretreated with 0.4% pepsin (P7012, Sigma Chemical Co., St. Louis, MO) in 0.01 N HC1 for 60 min at 37 C (164,226). Fetal brain sections were then stained by the PAP technique using Dako PAP kit K548 for primary antibodies raised in rabbits and Dako monoclonal kit K660 for mouse monoclonal primary antibodies. Primary antibodies (anti-laminin, -type IV collagen, -procollagen III diluted 1:100; anti-GFAP, fibronectin, and -factor VIII diluted 1:200) were allowed to incubate overnight at 4 C. The slides were carefully rinsed in PBS after each step. The sections were counterstained with hematoxylin and mounted with Aquamount. Staining intensity, morphological localization, and background staining were evaluated for each slide. Control staining was performed by replacing the primary antiserum with either the immunoglobulin fraction from non-immunized rabbits (Dako) or ascites control fluid (Cappel, Cochraneville, PA) at dilutions identical to those of the primary antibody.

The same panel of antibodies was used for indirect immunofluorescence microscopy studies of serial monolayer cultures, beginning with the primary culture, as previously described (225,227, and

Section 2.2.4). Double immunofluorescence staining was performed as described in Section 3.2.3C. In control experiments, the fluorescein- and rhodamine-conjugated antisera did not react with each other.

Fluorescence microscopy was performed with an Olympus BHS system microscope equipped with a BH-RFL-W reflected-light fluorescence attachment (Olympus) and an HBO 100 w/2 high-pressure mercury lamp. The intensity of staining and the percentage of cells that stained positively were recorded in each case. Negative controls were examined for each stain in all studies.

4.2.5 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Human fetal brain and leptomeningeal cultures at various passages were prepared for SDS-PAGE to determine whether they produced any of the collagen subtypes (225,227). Our technique of SDS-PAGE has been described in detail (Section 2.2.6).

4.2.6 DEAE-Cellulose Chromatography

To separate the procollagens secreted into the medium from the fetal brain and leptomeningeal cell cultures, the labeled medium reserved from the SDS-PAGE experiment was processed as described previously (Section 2.2.7).

4.2.7 Addition of cis-Hydroxproline to Fetal Brain and Leptomeningeal Cultures

The proline analogue cis-4-hydroxy-L-proline (Sigma) inhibits collagen synthesis and decreases cellular proliferation in a number of cell systems (116,227, Section 3.2.3.H). To determine the effects of cis-hydroxyproline on human fetal brain and leptomeningeal cultures, 5×10^4 cells of each type were placed in different groups of 25-cm^2 flasks containing an enriched medium that consisted of minimum essential medium, glutamine, gentamicin, and 10% fibronectin-free fetal calf serum and were incubated under the same culture conditions described previously. After the cells had been seeded, cis-hydroxyproline (25, 50, 100, or 200 ug/ml) was added to cells in each group of flasks. New culture medium with fresh cis-hydroxyproline was added every other day after incubation. Growth curves were generated for control and

treated flasks of human fetal brain and leptomeningeal cultures by counting cells in a hemocytometer. Cell viability was determined by trypan blue dye exclusion. Primary cultures of human fetal brain that were initially treated with the proline analogue immediately after tissue dissociation were placed into Lab Tek chambers and characterized by the immunocytochemical techniques described above.

4.3 Results

4.3.1 Growth and Morphology of Human Fetal Brain Cultures

Viable cell growth was achieved from all seven human fetal brain specimens. Typically, a confluent monolayer was formed in the primary culture 2 weeks after the cells had been placed into the flasks; thereafter, the cells grew vigorously and could be subcultured every week. Cultures generally degenerated and were lost by the ninth passage. Several different cell types could be identified readily by phase microscopy in all primary fetal brain cultures regardless of the gestational age of the fetal sample from which the culture was derived (fig. 1). One cell type had a contracted cell soma about the nucleus from which multiple hair-like processes projected. These cells were fairly common and were usually found in clusters rather than evenly dispersed throughout the monolayer. Another cell type had a relatively large central nucleus and broadly bipolar or polygonal cytoplasm; these cells were most abundant in monolayer cultures. A third cell type was bipolar with long, thin cytoplasmic terminals extending over some distance. Although the cell population in the primary culture was heterogeneous, only broadly bipolar or polygonal cells could be found inmonolayer cultures after passage 3.

4.3.2 Electron Microscopy

The ultrastructural features of the different cell types identified in primary monolayer culture by phase microscopy were characterized by electron microscopy. One cell type had an electron-lucent nucleus and cytoplasm with distended endoplasmic reticulum filled with amorphous granular material. The cytoplasm was filled with irregular branching 8-10 nm intermediate filaments of the glial type (fig. 2A); the nucleus was large and

spherical with few invaginations and a prominent nucleolus. This cell type probably corresponds to some of the broadly bipolar cells seen by light microscopy (fig. 1) and was the most common cell type, often accounting for over half the cells seen. Another cell type had an electron-dense nucleus and a relatively electron-dense cytoplasm filled with free ribosomes and mitochondria; in cultures derived from specimens with a gestational age of 18 weeks or greater, long, electron-dense cytoplasmic extensions from these cells appeared to envelop other cell types (fig. 2B). These sinewy extensions were often laminated and had a myelin-like appearance. These cells were thought to be oligodendrocytes and accounted for about 5-10% of the cells seen in primary cultures and correspond to the cells in Figure 1 with a contracted cell soma and multiple hair-like projections. Electron microscopy showed that a variable percentage of cells had an interdigitated nucleus that was moderately electron dense and a cytoplasm filled with distended cisternae. Cell:cell epithelial type junctions were common. A rare cell type had cytoplasm that was packed with electron-dense and electron-lucent vesicles and with myelin debris; these cells were presumed to be macrophages. Neurons with a small cell soma, basal bodies, and abundant cytoplasmic microtubules accounted for about 5% of the total cell population (fig. 2B).

At passage 4, there was only one predominant cell type, which was characterized by a multiply interdigitated nucleus, distended cisternae, plasma membrane interdigitation, and epithelial-like cell:cell junctions (fig. 3A). The electron microscopic features of these cells were identical to those of cells in the leptomeningeal cultures (fig. 3B)

4.3.3 Antigen Expression

By immunohistochemistry, antisera to GFAP identified scattered astrocytes in the marginal and mantle zones in all specimens (fig. 4a). Radial glial cells were also identified by immunostains for GFAP. Immunostains for vimentin identified radial glial and vascular mesenchymal cells. The developing neuropil was negative when examined by immunostains for laminin, fibronectin, type IV collagen, and procollagen III. However, vascular mesenchymal cells and perivascular basement membrane were readily outlined by immunostains for these glycoproteins of the extracellular matrix (fig. 4b). The meninges stained positively for all extracellular matrix glycoproteins and for vimentin, but were negative for GFAP.

The results of the immunocytochemical analysis of all human fetal brain cultures are shown in Table 1. GFAP-positive cells were found in all primary cultures and represented 20-70% of the total cell population, depending on the filter size used at the time of initial dissociation. The smaller pore sizes (<50 um) did not yield a high percentage of GFAP-positive cells. With progressive subcultivation, the number of GFAP-positive cells diminished (fig. 5) until at or around passage 4, when none could be identified. Concomitant with the decrease in the number of GFAP-positive cells over serial passages was an increase in the number of cells that stained positively (both intracytoplasmically and extracellularly) for laminin, type IV collagen, fibronectin, and procollagen III (fig. 6). By double immunofluorescence, cells that showed positive filamentous staining for GFAP were not stained in a granular cytoplasmic or extracellular fibrillary fashion for laminin, fibronectin, or the collagen types (fig. 7). Nearly all cells at all passages were positive for vimentin.

4.3.4 Collagen Synthesis Studies

In primary cultures of fetal human brain, SDS-PAGE demonstrated faint bands representing the alpha chains of type I collagen; however, at later passages in the same culture, the intensity of the radiolabeled collagen bands increased (fig. 8), and the banding pattern was similar to that of the fetal human leptomeningeal cultures. DEAE-cellulose chromatography showed that both the early-passage fetal human brain and leptomeningeal cultures contained interstitial collagen types I and III (fig. 9).

4.3.5 Effect of Proline Analogue

Primary cultures of fetal leptomeningeal cells were inhibited to a significantly greater degree by cis-hydroxyproline than were primary cultures derived from fetal brain specimens. The antiproliferative effect was dose-dependent (fig. 10). The percentage of GFAP-positive cells was significantly increased in primary cultures of human fetal brain incubated with cis-hydroxyproline (fig. 11, table 1).

4.4 Discussion

Although the histogenesis of all cell types within the developing mammalian brain is imperfectly understood, most studies have shown that the primitive neural tube has a ventricular zone consisting of relatively undifferentiated pseudostratified columnar epithelial cells (41,137,247,248). With increasing gestational age and migration away from the ventricular zone, the primitive neuroepithelial precursor cells differentiate and acquire phenotypes that permit accurate characterization. Radial glia, which are thought to be processes from immature astrocytes, can be identified by immunostains for GFAP in the fetal human spinal cord by 8 weeks and in the fetal human cerebrum by 10 weeks (7,39); myelin-forming oligodendrocytes can be seen by 16 weeks of gestation (40,188), and neurons forming synapses are apparent by electron microscopy very early in embryogenesis. In addition, by about 12 weeks of gestation, the leptomeningeal epiparenchymal vascular beds have given rise to all of the endoparenchymal vessels supplying the deep and superficial regions of the telencephalon (130). Therefore, it is to be expected that a heterogeneous cell population will be found in primary cultures derived from fetal human brain specimens with a gestational age of 16-24 weeks.

In contrast to the extensively studied, well-characterized fetal and neonatal rat brain models, which have been manipulated to achieve significant enrichments of specific CNS cell types (74,97,151,166), there have been considerably fewer such studies of fetal human brain cultures. Well-characterized human neuronal and glial cell populations would be of considerable value for studying human neurological diseases. In most of the early reports, an explant technique was used to culture fetal human brain from fetal material with a gestational age of 12-24 weeks (1,6,105,190,286). In more recent years, fetal human brain cultures have been initiated with equal success from mechanically or enzymatically dissociated specimens (22,26,242). Organotypic cultures (153), which attempt to maintain the three-dimensional cytoarchitecture of the original fetal human brain specimen, have also been described. In our study, we used enzymatic and mechanical tissue dispersion and filtration through meshes of graded pore size to achieve a maximal yield of viable astrocytic (GFAP-positive) cells. In general, most studies, including the present study, have shown that the primary culture is heterogeneous, regardless of the manner in which it was initiated.

Characterization of primary fetal human brain cultures initiated from explants has revealed a cell population believed to consist of astrocytes, neurons, oligodendroglia, macrophages, and mesenchymal cells (1,6,190). The predominant cell type in these cultures appears to be of glial origin, although precise cell population counts have rarely been given. Cultures derived from dissociated fetal human brain specimens have until recently been very difficult to characterize. In his study of cultures derived from specimens with a gestational age of 16-20 weeks, Shein (242) identified only two cell types: an astrocyte-like cell (type A cell, phosphotungstic acid-hematoxylin positive), which survived serial subcultivations, and a neuronal-like cell (type S cell, silver-stain positive), which did not proliferate or survive. Bird and James (22) found that only a minority of cells in dissociated fetal human brain cultures (gestational age 16-24 weeks) could be classified with confidence. Markesbery and Lapham (153) identified immature neurons and astrocytes in fetal human brain specimens (gestational age 12-24 weeks) placed into an organotypic culture system.

The different cell types in fetal human brain cultures may be identified in part by their ultrastructural features. Neurons may be distinguished by their small cell soma and miocrotubule-filled cytoplasm (153), astrocytes by intracytoplasmic 8-10 nm glial filaments, and oligodendrocytes by a dense cytoplasmic matrix and the presence of heterochromatin and myelin. In our ultrastructural analysis of primary fetal human brain cultures, glial-type cells with readily identifiable glial filaments were the predominant cell type (20-70% of cells). Oligodendrocytes containing myelin accounted for approximately 5-10% of cells. Neurons containing microtubules were found only occasionally and in smaller numbers than oligodendrocytes. Neurons have been exceedingly difficult to grow in culture and appear to adapt poorly to tissue dissociation and to the conditions of monolayer growth (286). Other cell types included macrophages with cytoplasms filled with lysosomes and myelin-debris and cells that resembled those found in leptomeningeal cultures. Identification of cells by electron microscopy alone, however, has limitations, and many cells cannot be characterized sufficiently by this method. Immunocytochemical techniques and antibodies to specific cells of the CNS have facilitated the identification of many cell types in fetal human brain cultures.

Kennedy et al. (120) identified many of the major types of cells in fetal human brain cultures (gestational age 15-21 weeks) using cell-type-specific antibodies. In their study, the marker used to identify astrocytes was GFAP; other markers included galactocerebroside for oligodendrocytes; tetanus toxin for neurons; and Thy-1 and fibronectin for fibroblasts or leptomeningeal cells. Double immunolabeling studies showed that antibodies against GFAP, galactocerebroside, and tetanus toxin identified different populations of cells. In addition, Raff et al. (202) and Kennedy et al. (119) have used immunocytochemical techniques to show that two population of GFAP-positive astrocytes (GFAP-positive/A2B5-negative and GFAP-positive/A2B5-negative) can be delineated in rat and human brain cultures. In these studies, none of the cells identified as oligodendrocytes, neurons, or astrocytes stained positively for fibronectin. Although there was no mention of the number of cells that were fibronectin positive and potentially of fibroblastic or leptomeningeal origin in these studies, other investigators have identified mesenchymal-like cells in relatively high percentages in fetal human brain cultures (26,190). Since neuronal and glial cells do not normally produce collagen (32,153), we assessed the ability of fetal human brain cells in culture to synthesize various collagen subtypes. We also tested our cultures for the expression of other glycoproteins of the extracellular matrix, such as laminin and fibronectin.

To guide our interpretation of the positive immmunostaining seen in tissue cultures of fetal human brain, we first analyzed the paraffin-embedded specimens from which the cultures were derived for the distribution of the various markers. Our results parallel those of Choi and Lapham (41) in that immunohistochemical stains for GFAP were predominantly found in radial glia and, to a lesser extent, in stellate marginal and cortical astrocytes. Vimentin, which has been localized in situ to immature glia and neurons in fetal rat brain (16,50), was immunolocalized to the cerebral vasculature, the leptomeninges, and radial glia in all of our specimens. Laminin, fibronectin, type IV collagen, and procollagen III were immunolocalized to the leptomeninges, the basement membrane of the cerebral vasculature, and the glial limitans externa; intrinsic neuronal and glial cell populations were not stained by these antibodies.

Immunostains for GFAP and types IV collagen or procollagen III recognized nonoverlapping populations of cells. In primary cultures, under

our optimum conditions for cultivating astrocytes, GFAP-positive cells were usually the largest single population of cells in monolayer cultures; however, with progressive subcultivation, the number of GFAP-positive, collagen-negative cells decreased and the number of GFAP-negative, collagen-positive cell increased. Cultures derived directly from the leptomeninges had a staining profile identical to that of later-passage fetal human brain cultures. Furthermore, examination of later-passage fetal human brain cultures by electron microscopy demonstrated a single cell type with a large cytoplasm, distended cisternae, invaginated nucleus, and cell:cell junctions; this cell type was identical to the predominant cell type in cultures derived exclusively from the fetal human leptomeninges. These results strongly suggest that with progressive subcultivation, the main cell type in fetal human brain cultures is a mesenchymal cell derived from the leptomeninges.

This interpretation is further supported by our collagen biosynthesis studies, which showed that fetal human brain cultures at later passages had interstitial collagen profiles similar to those of fetal human leptomeningeal cultures. Despite reports suggesting that muscle formation and collagen synthesis can occur from primitive neuroectodermal precursors (102,136,272,296), we feel our in vivo and in vitro antigen expression and biosynthesis data are more elegantly explained by invoking collagen synthesis by mesenchymal cells derived from the leptomeninges, rather than by the pluripotentiality of primitive neuroectodermal cells.

A major finding in this study was that the growth of collagen-synthesizing fetal human leptomeningeal cells in culture is inhibited by cis-hydroxyproline. In primary fetal human brain cultures treated with this agent, we could achieve a 90% enrichment for glial cells, presumably by preventing the proliferation of contaminating collagen-producing cell types. Kennedy et al. (119) recently described the purification of human fetal astrocytes using the methods of Noble et al. (184), who treated fetal rat brain cultures with cytosine arabinoside and obtained a highly enriched astrocyte population.

We were also interested in this study in examining the fetal human brain and leptomeningeal cultures for their immunoreactivity to antisera to laminin and fibronectin. Laminin and fibronectin immunoreactivity was confined to flat, polygonal cells in fetal human brain and leptomeningeal cultures. Fibronectin-positive and laminin-positive populations of cells

increased in proportion to the increase in collagen-positive cell populations. This suggests that fibronectin and laminin may be synthesized by leptomeningeal-derived mesenchymal cells in culture. Our double immunofluorescence studies provide more direct evidence concerning the identify of fibronectin- and laminin-positive cell populations: GFAP and laminin or fibronectin recognized mutually exclusive cell populations. These results in human fetal brain cultures differ from those in neonatal rat cultures, in which it was shown that astrocytes can express both GFAP and fibronectin or laminin (142,143).

In summary, this study has shown that primary cultures derived from mechanically and enzymatically dissociated specimens of fetal human brain contain a heterogeneous cell population. With progressive subcultivation, a mesenchymal cell derived from the leptomeninges became predominant. Although electron microscopy and immunostains for GFAP showed that the largest cell population often consisted of astrocytes, the percentage of astrocytes could be increased by pretreating the primary cell dissociate with cis-hydroxyproline. The resulting relatively pure population of GFAP-positive cells may serve as a useful model with which to study human glial cells.

4.5 Figure Legends and Tables

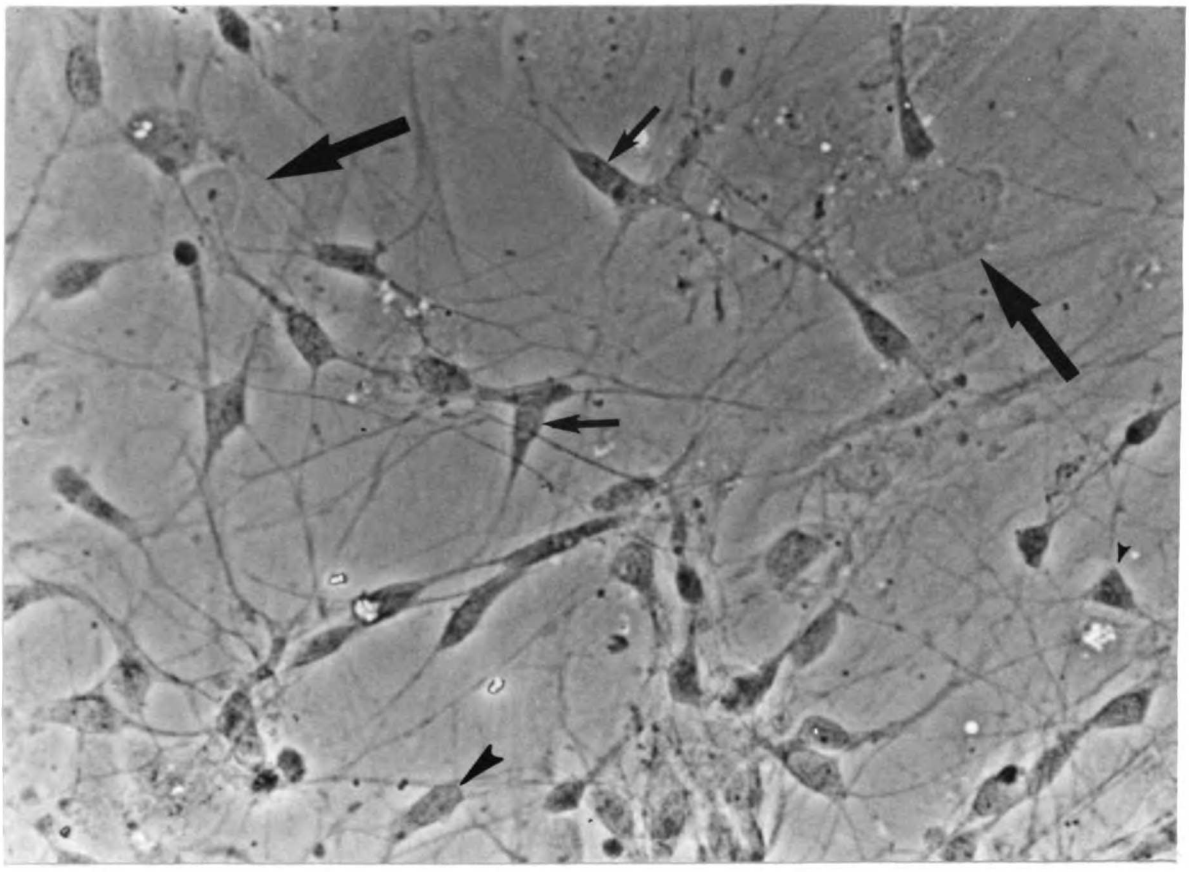


Fig. 1. Morphological features of the different cell types in a primary culture (SF 673) derived from a fetal human brain specimen with a gestational age of 18 weeks. Approximately 5-10% of the cells had a cytoplasm contracted about the nucleus from which multiple thin processes emanated (small arrows); these cells were usually located in clusters. The majority of cells were broadly bipolar or polygonal and had well-defined nuclei and an extensive cytoplasm forming sheets (large arrows). About 20% of cells were bipolar with a large, central nucleus and identifiable cytoplasm (large arrowheads). About 5% of cells were also bipolar, but had a cytoplasm contracted about the nucleus and cell processes extending outward from oppostive ends of the nucleus (small arrowheads). Phase microscopy, x 200.

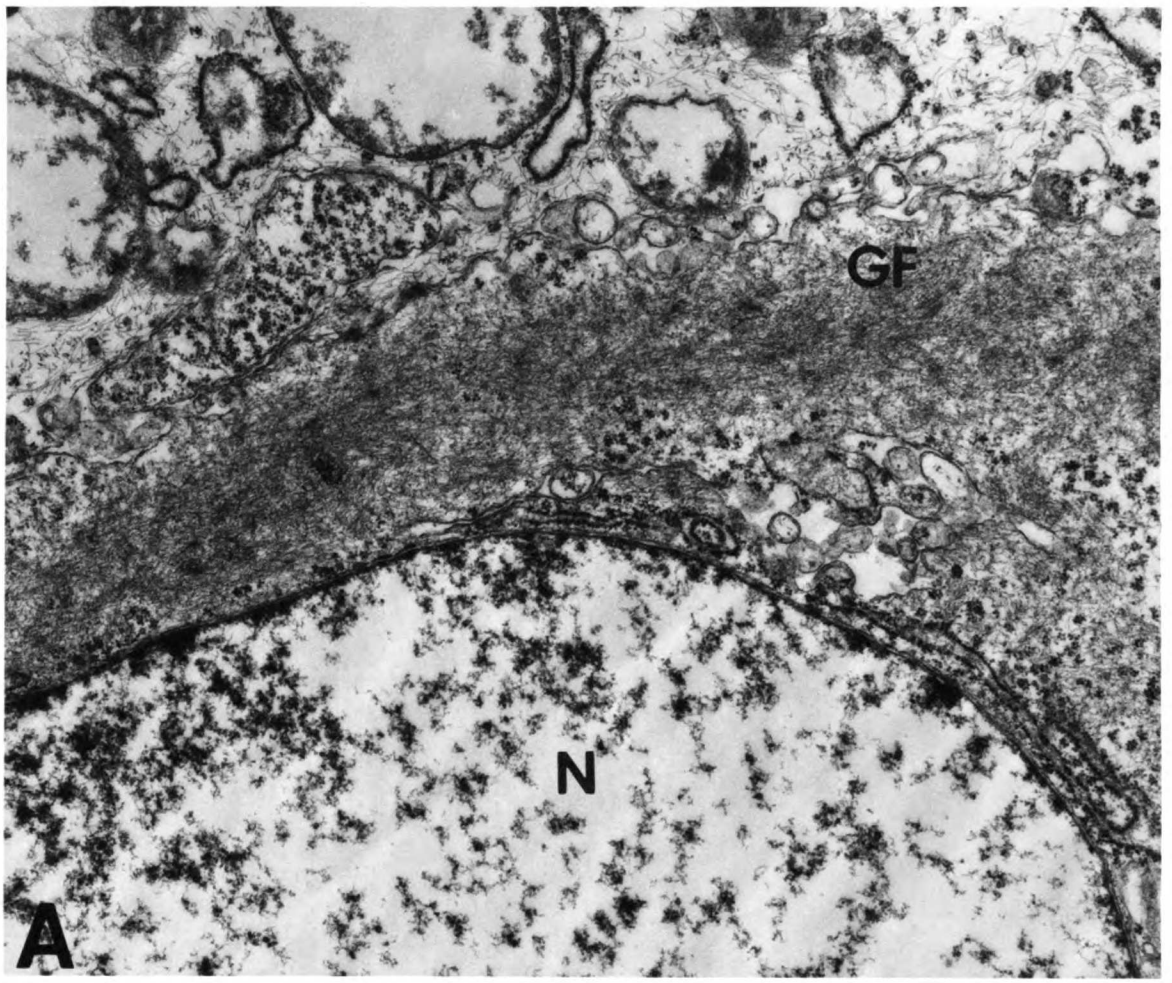


Fig. 2A Ultrastructural features of primary fetal human brain cultures SF 621 (gestational age 19 weeks). The majority of cells in this culture were recognized as astrocytes by the presence of well-developed and characteristic 8-10 nm intracytoplasmic glial filaments (GF). These filaments are short, branched, and course around the nucleus (N). Electron microscopy, x 20,000.

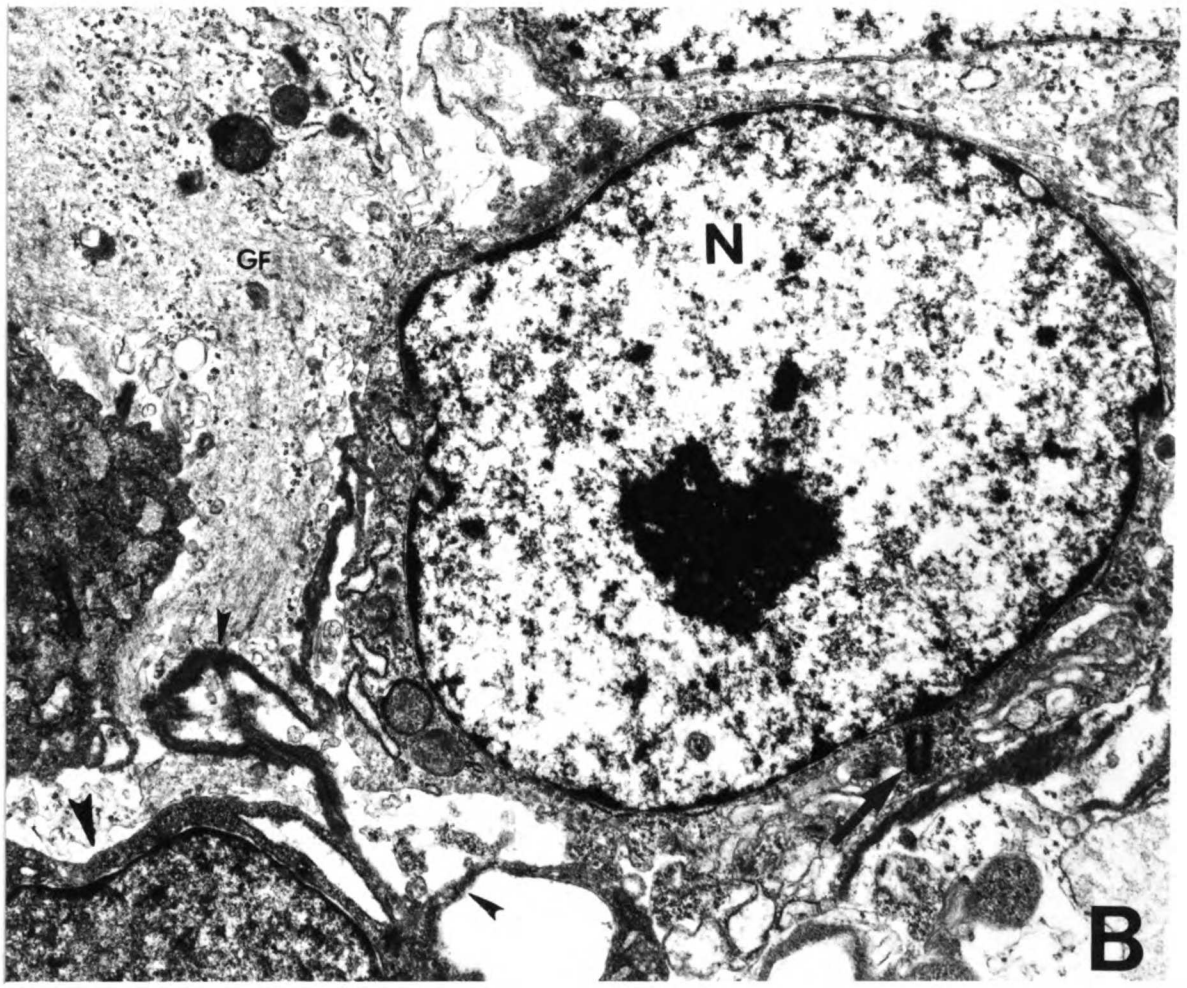


Fig 2B Ultrastructural features of primary fetal human brain culture SF 621 (gestational age 19 weeks). About 5% of cells in this primary culture had an electron-dense cytomatrix and electron-dense heterochromatin (large arrowhead). Thin, electron-dense cytoplasmic extensions emanated from these cells (small arrowheads) and enveloped other cell bodies and processes. The cell processes were often laminated and had the density of myelin. These cells were thought to resemble oligodendrocytes most closely. A neuron is recognized by its prominent nucleus (N), sparse cytoplasm containing a basal body (large arrow), and microtubules. Glial filaments (GF) are present in the cytoplasm of an interposed astrocyte. Electron microscopy, x 10,000.

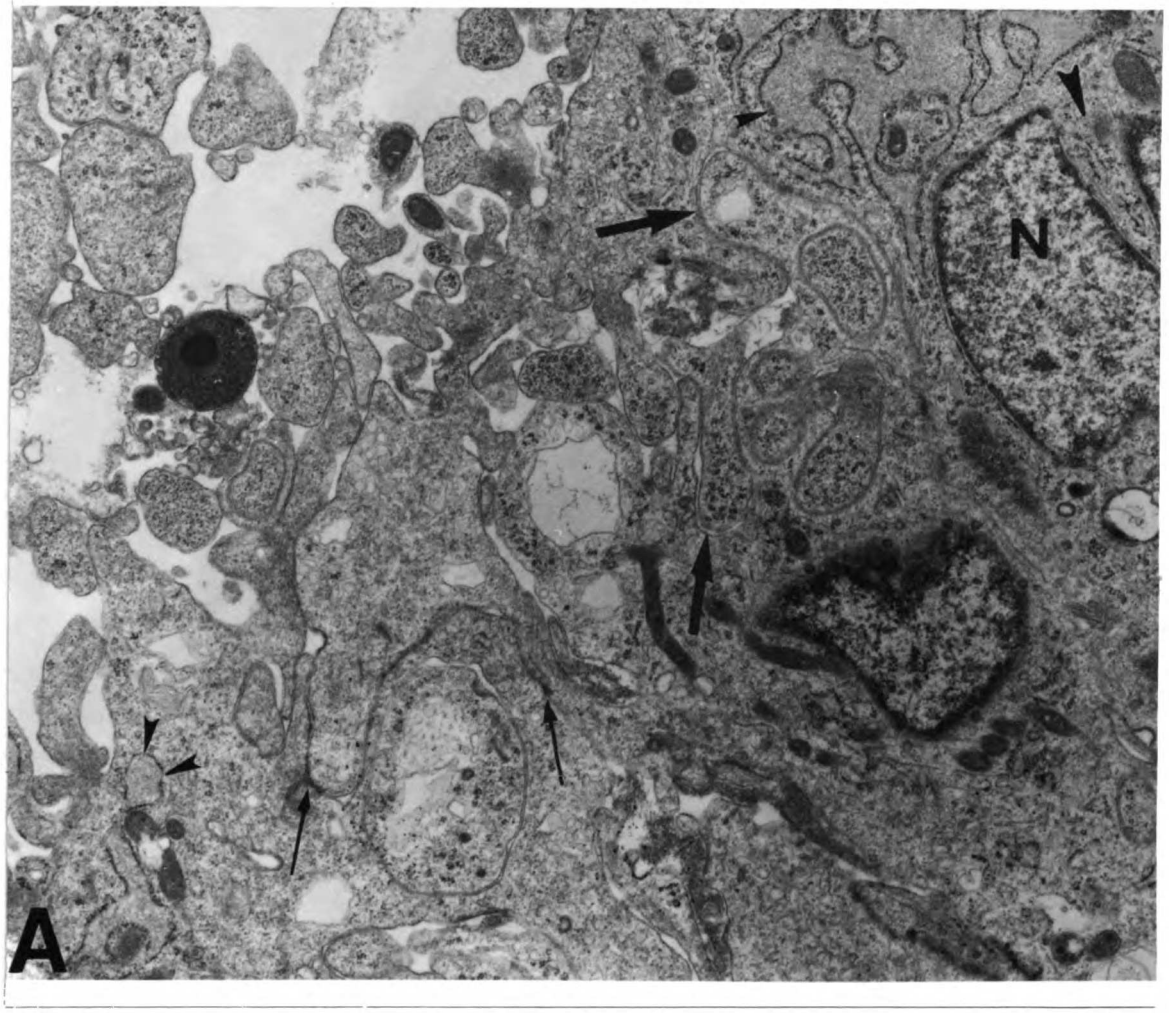


Fig. 3A Ultrastructural features of fetal human brain culture SF 622, passage 4. Only one cell population is present. These cells have distended cisternae (small arrowheads), a cytoplasm that invaginates into the nucleus (large arrowheads), epithelial-like cell:cell junctions (small arrows), and pronounced interdigitation of plasma membranes (large arrows).

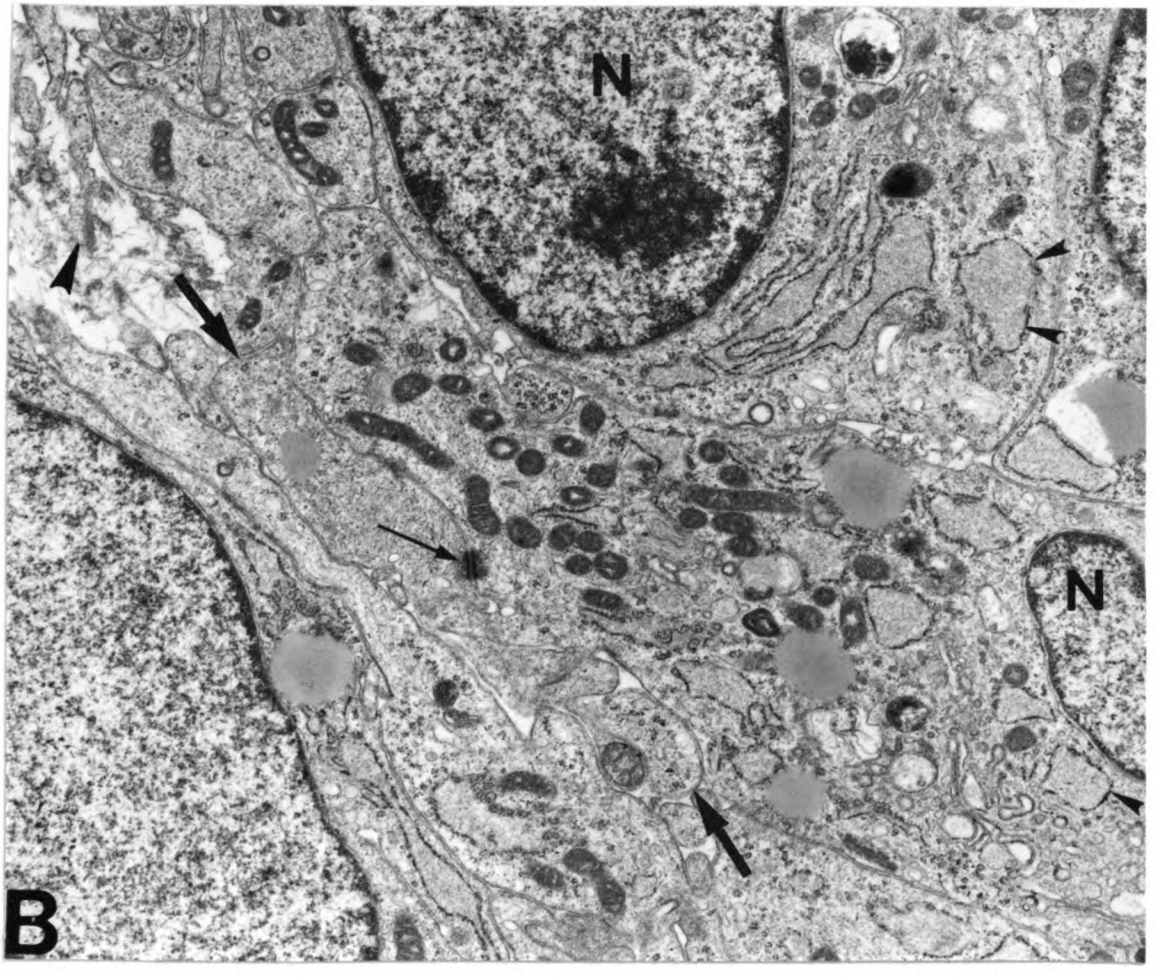


Fig 3B Ultrastructral features of fetal human leptomeningeal culture SF-622, passage 1. The monolayer is formed by cells that have distended cisternae (small arrowheads) and form desmosomes (small arrow). There is marked interdigitation of the plasma membrane (large arrows). Fine, filamentous basement membrane-like material is found in the extracellular spaces (large arrowhead). N = nucleus. A, B Electron microscopy, x 10,000.

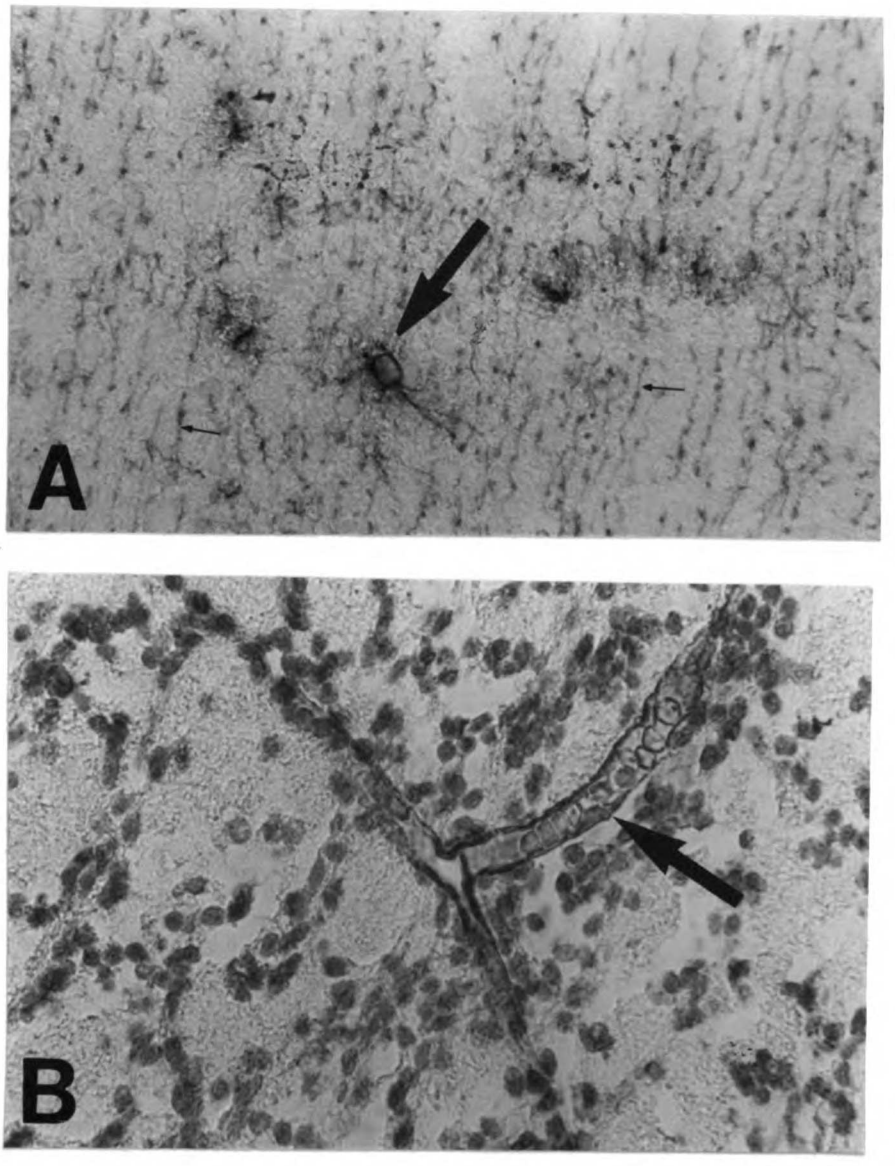
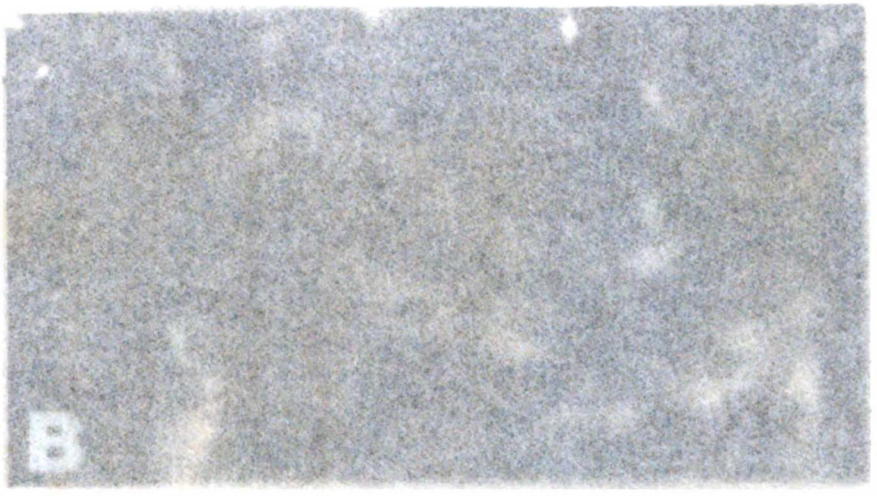
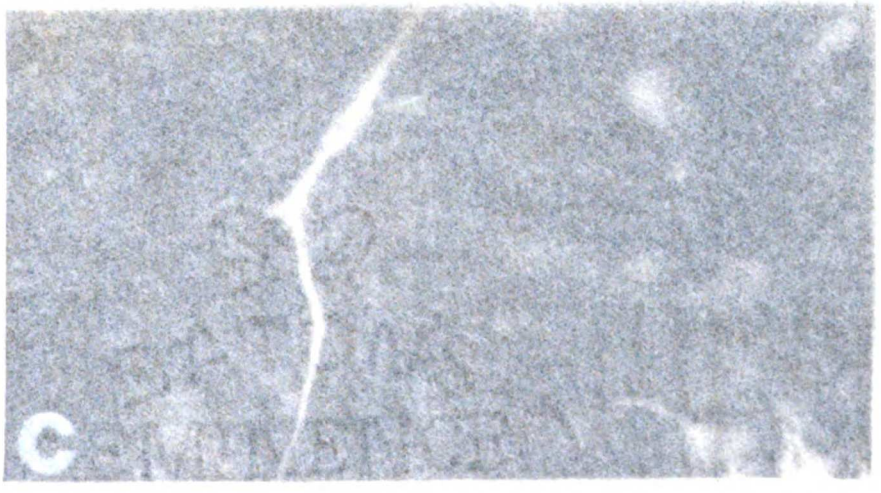


Fig.4. Fetal human brain specimen analyzed by immunohistochemistry. a A solitary stellate cell in themarginal zone (large arrow) of a specimen with a gestational age of 18 weeks is recognized as an atrocyte by immunostains for GFAP. The radial glia (small arrows) are positively identified by the anti-GFAP antiserum. Light microscopy, x 100. b Fetal human brain specimen, gestational age 19 weeks. Intracerebral vessels in longitudinal section (large arrow) and cross section (small arrow) are positively immunostained for type IV collagen. The neuropil is unstained. Light microscopy, x 150.

Fetal human brain culture SF-611, passage 2. Broadly
[illegible] row and large, polygonal cells [illegible]
all cells in this culture are positive
[illegible] ural antibody to type IV collagen. The [illegible]
[illegible] clear, and largely confined to the cytoplasm (arrow
[illegible] same field as in panels a and b is immunostaining
[illegible] um (arrow). This cell was not positively immunostained
[illegible] tional antibody to type IV collagen. a-c Phase and d
[illegible] orescence microscopy, x 500

Fig. 5. Fetal human brain culture SF-611, passage 2. a Broadly bipolar cells (small arrows) and large, polygonal cells (large arrows) are shown. b Virtually all cells in this culture are positively immunostained with monoclonal antibody to type IV collagen. The staining is granular, perinuclear, and largely confined to the cytoplasm (arrows). c Only one cell in the same field as in panels a and b is immunostained with anti-GFAP antiserum (arrow). This cell was not positively immunostained with the monoclonal antibody to type IV collagen. a-c Phase and double indirect immunofluorescence microscopy, x 500.


B


C

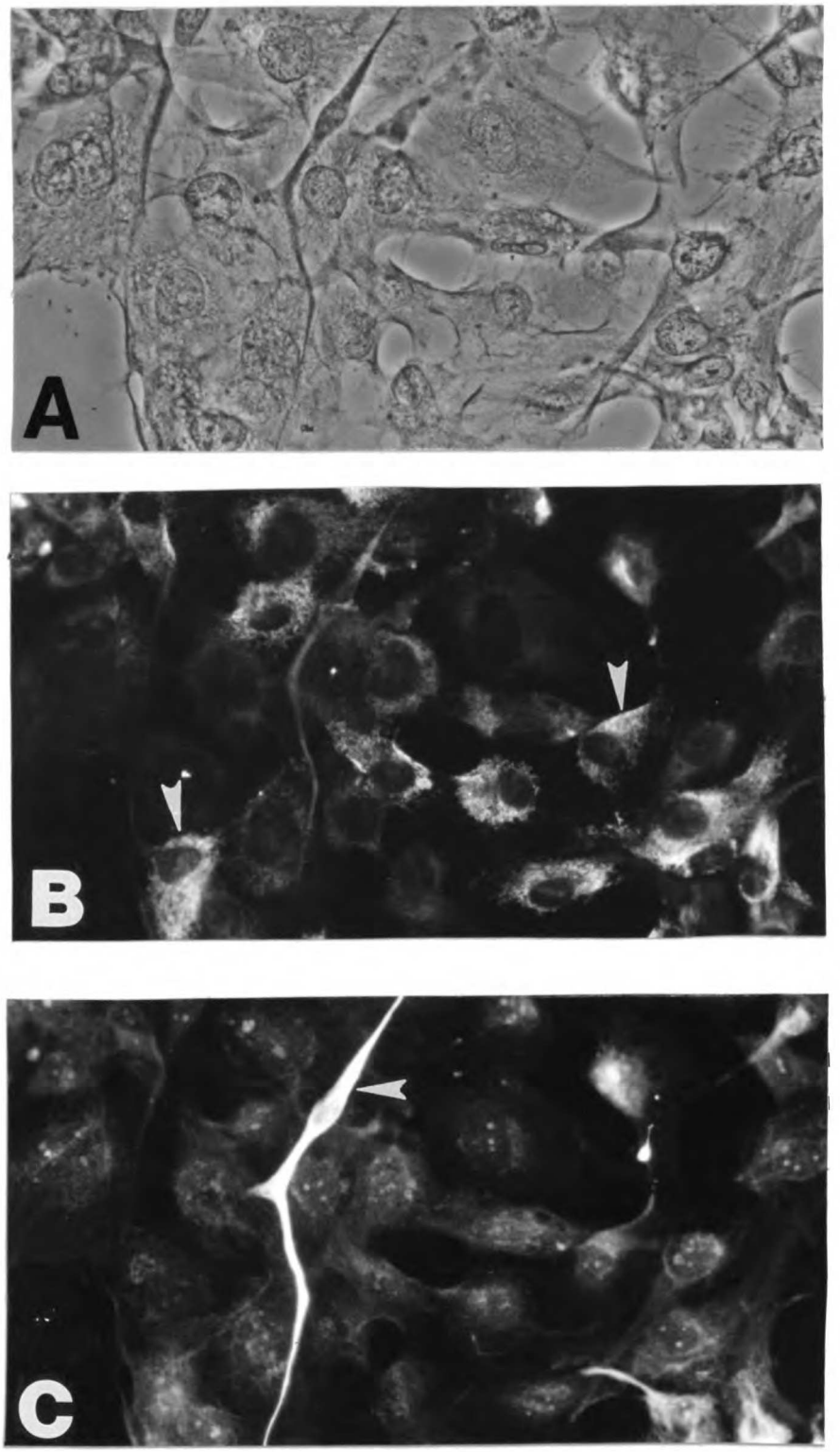


Figure 5 A-C

Fetal human brain
cells are shown. b
served in nearly all
monoclonal antibody. In
positively identified.
microscopy, ×5
and br
staining is
the
(large arrows) is
fluorescence

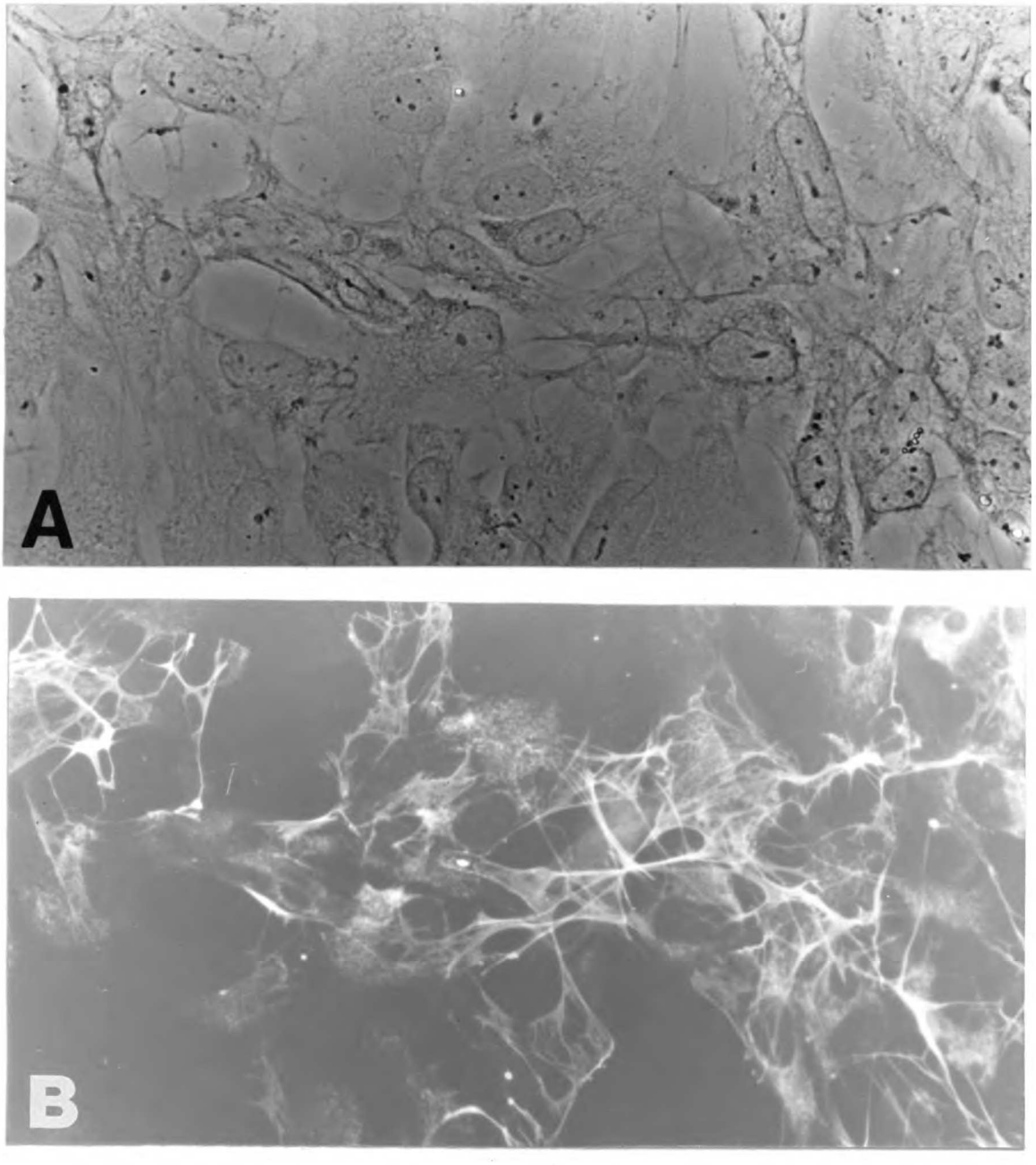


Fig. 6. **Fetal human brain culture SF-621, passage 2. a Polygonal** and broadly bipolar cells **are shown. b Intense granular, cytoplasmic immunostaining** is observed in nearly all cells (small arrows) with the procollagen III monoclonal antibody. In addition, the extracellular matrix (large arrows) is positively identified. a, b Phase and indirect immunofluorescence microscopy, x 500.

7. Double immunofluorescence analysis of SF-673. passage 2. a Pha-
roscopy shows a homogeneous population of polygonal cells.
ensions for fibronectin identify the pericellular matrix (large arrow)
as the cytoplasm of many of the cells (small arrows). c A single cell
microscopic field is positively identified by the anti-GFAP antiserum
s cell is not positively identified for fibronectin. a-c Phase and indire
munofluorescence microscopy, x 500.

Fig. 7. Double immunofluorescence analysis of SF-673, passage 2. a Phase microscopy shows a homogeneous population of polygonal cells. b Immunostains for fibronectin identify the pericellular matrix (large arrow) as well as the cytoplasm of many of the cells (small arrow). c A single cell in this microscopic field is positively identified by the anti-GFAP antiserum. This cell is not positively identified for fibronectin. a-c Phase and indirect immunofluorescence microscopy, x 500.

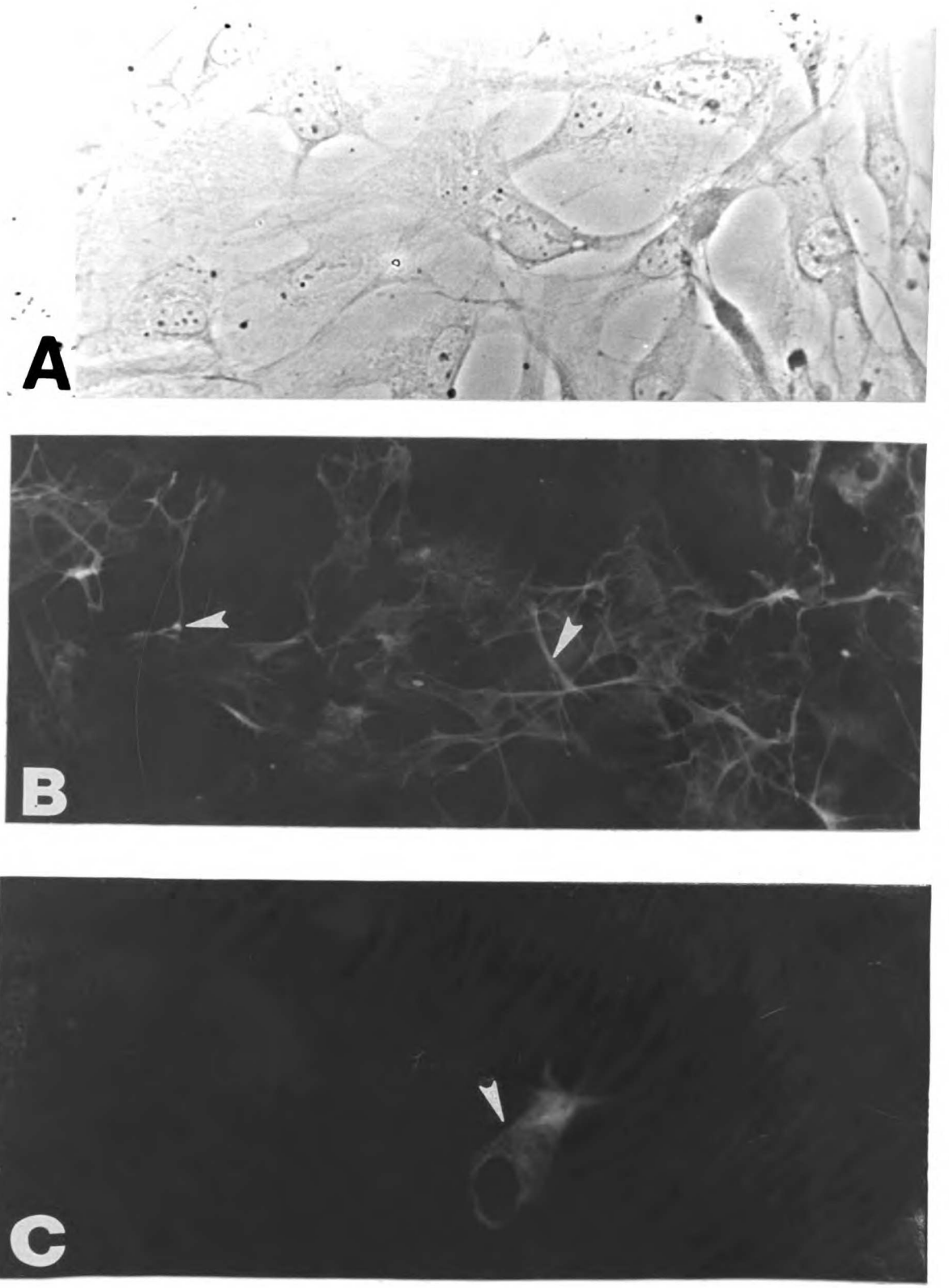


Fig 7 A-C

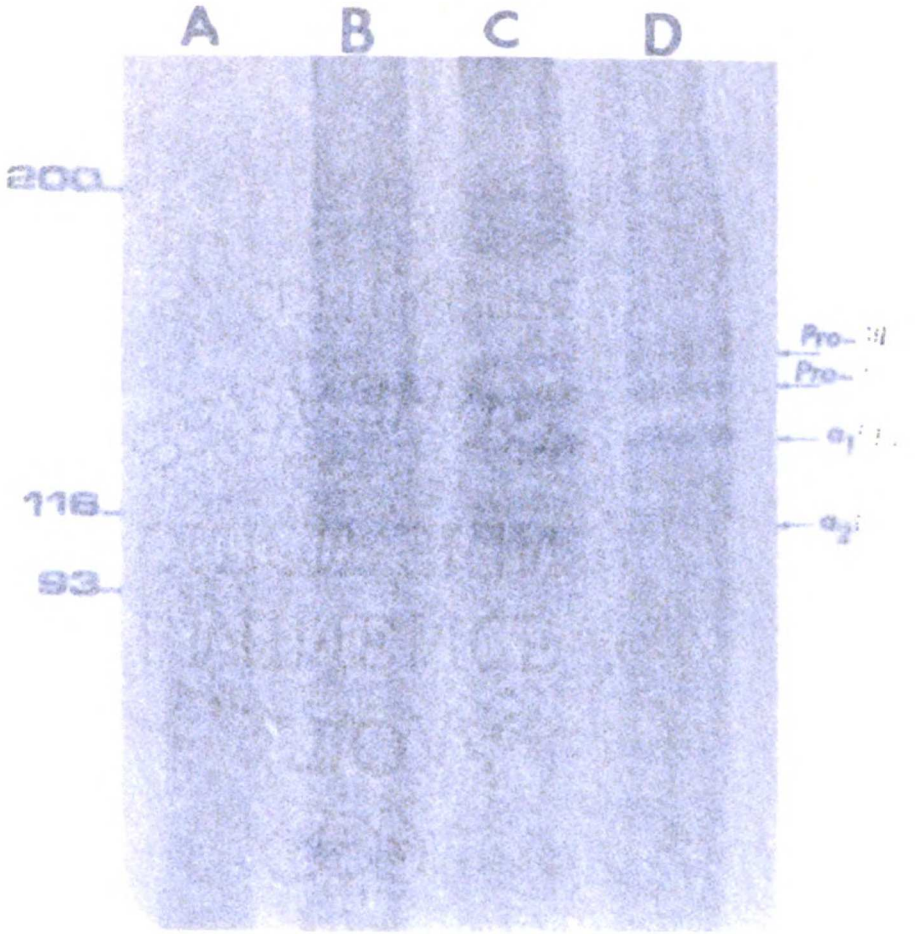


Fig 8. SDS PAGE analysis of
Lane a: Fetal human b
10 U/ml at 37 C for 4
Calbiochem, San Diego, California
621, passage 2. Lane c: Human fetal
Lane d: Human fetal brain cultures
collagen (alpha-1 (I) and alpha-2
labeled collagen bands are faint
passages. Procollagen types I
Treatment with collagenase
labeled collagen bands. M
as shown.

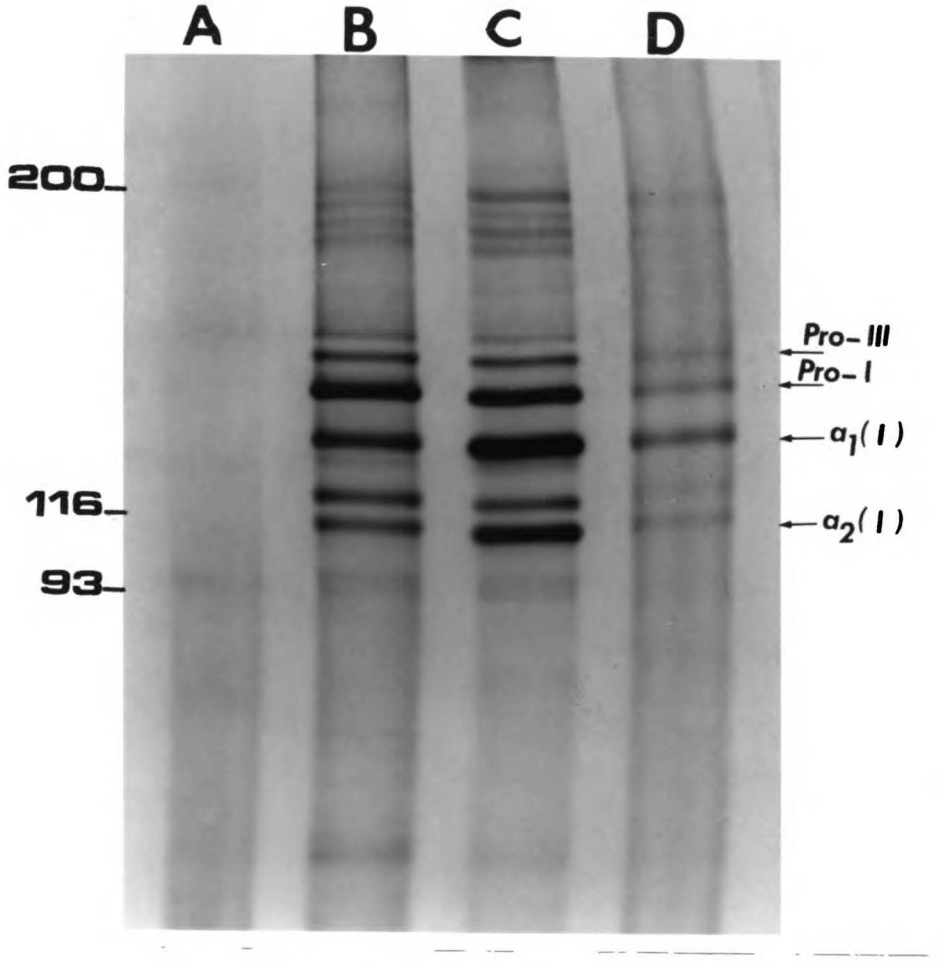


Fig. 8. SDS-PAGE analysis of ^{3}[H]-proline-labeled and reduced proteins bands. Lane a: Fetal human brain culture SF-621, passage 2, treated with collagenase, 10 U/ml at 37 C for 4 hours (clostridium histolyticum, protease free; Calbiochem, San Diego, California). Lane b: Human fetal brain culture SF-621, passage 2. Lane c: Human fetal leptomeningeal culture SF-621, passage 1. Lane d: Human fetal brain culture, primary. Bands representing type I collagen (alpha-1 (I) and alpha-2 (I)) are present in lanes b, c, and d. Proline-labeled collagen bands are fainter in the primary culture than in subsequent passages. Procollagen types I and III are identifed above the alpha-1 bands. Treatment with collagenase (lane a) results in disappearance of the proline-labeled collagen bands. Molecular weight standards are as shown.

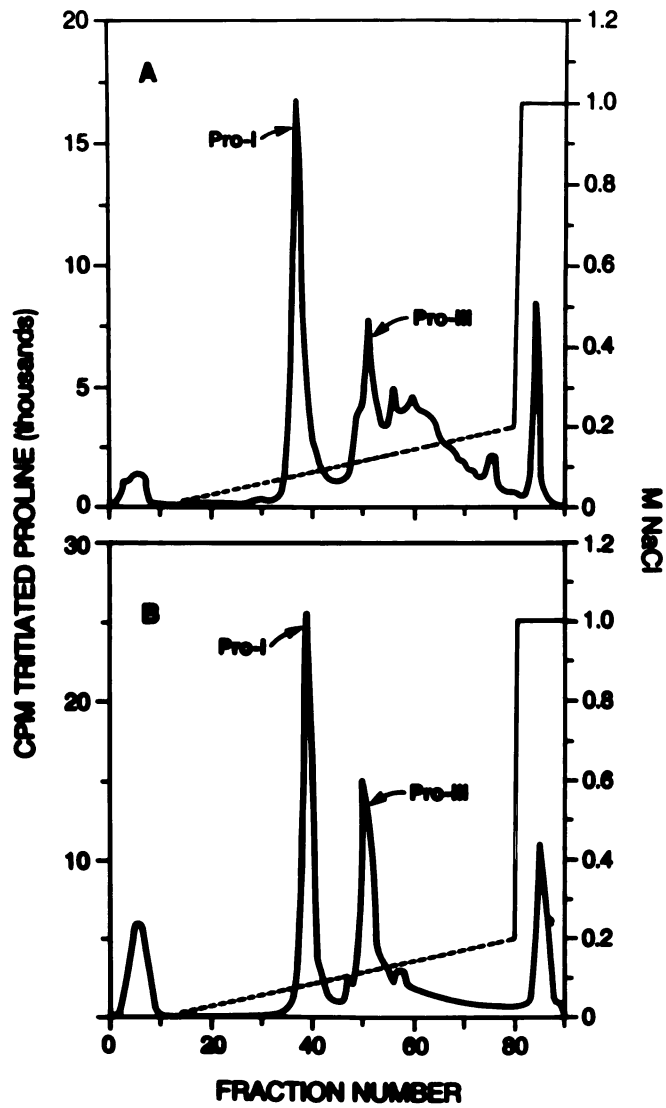


Fig. 9. DEAE-cellulose chromatography of the medium from fetal human brain culture SF-673, passage 1 (a), and fetal human leptomeningeal culture SF-673, passage 1 (b). Both cultures synthesized soluble procollagens I and III. Separation of the procollagens was accomplished with an increasing salt gradient (dotted line).

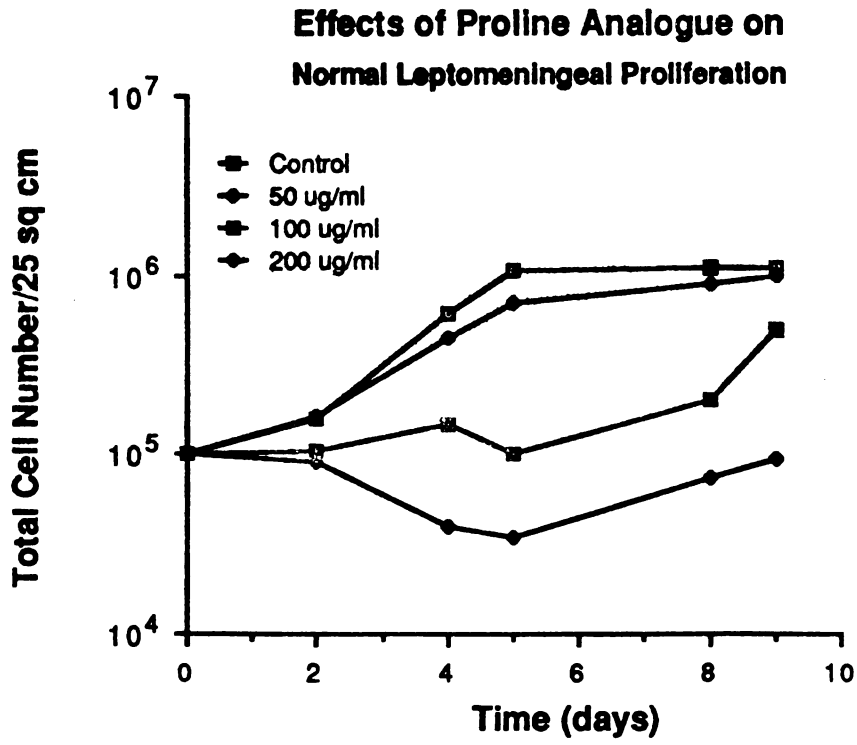


Fig. 10. Effects of cis-hydroxyproline on fetal human leptomeningeal cell proliferation. Compared with untreated cultures (open squares), there is a dose-dependent decrease in cell proliferation with increasing concentrations of cis-hydroxyproline. Open circles, 50 ug/ml; filled squares, 100 ug/ml; filled circles, 200 ug/ml. Symbols represent the mean total number of leptomeningeal cells counted in a 25 sq cm flask. Error bars represent the standard deviation of four separate cell counts.

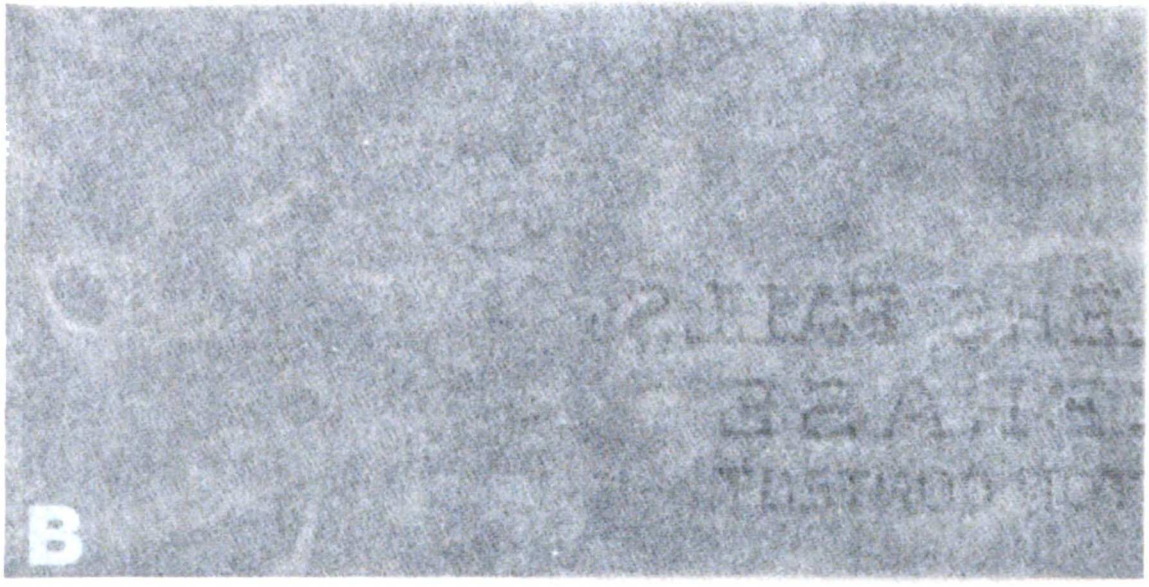


Fig. 11. Primary human fetal brain cells grown with cis-4-hydroxyprolines, 200 ug/ml. a. A heterogeneous culture of fetal brain cells. b. Immunostaining for GFAP demonstrates that the culture is highly enriched with GFAP-positive astrocytes (more than 90% of cells).

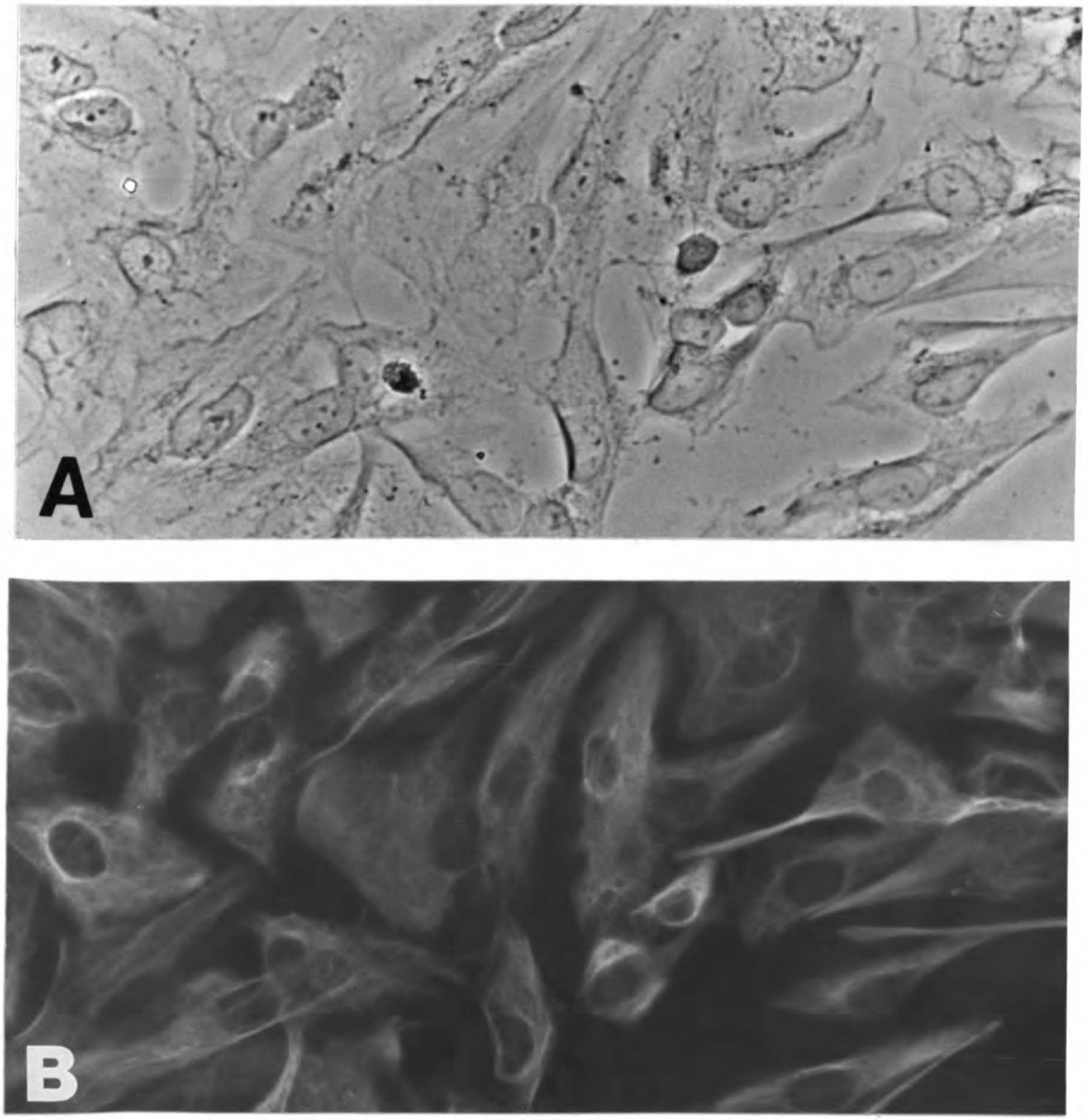


Fig. 11. Primary human fetal brain culture SF-673 treated with cis-hydroxyprolines, 200 ug/ml. a A homogeneous colony of fetal brain cells. b Immunostaining for GFAP demonstrates that the culture is highly enriched with GFAP-positive astrocytes (more than 90% of cells).

Control
50 ug/ml
100 ug/ml
200 ug/ml

TABLE 1
IMMUNOCYTOCHEMICAL CHARACTERIZATION OF HUMAN FETAL BRAIN AND LEPTOMENINGEAL CULTURES

Culture	Gestational Age (wks)	GFAP	Factor VIII	Fibronectin	Laminin	Procollagen III	Collagen Type IV	Vimentin
Fetal Brain								
SF-609	19							
Primary		++ (40)	-	++ (40)	++ (40)	++ (40)	++ (40)	NA
P1		++ (20)	-	++ (70)	++ (70)	++ (70)	++ (70)	NA
P2		-	-	++ (95)	++ (95)	++ (95)	++ (95)	NA
P3		-	-	++ (100)	++(100)	++ (100)	++ (100)	NA
SF-611	21							
Primary		++ (60)	-	++ (30)	++ (30)	++ (30)	++ (30)	NA
P1		++ (10)	-	++ (90)	++ (90)	++ (90)	++ (90)	++ (95)
P2		++ (1)	-	++ (95)	++ (95)	++ (95)	++(90)	++ (100)
P3		+ (0.5)	-	++ (100)	++ (100)	++ (100)	++ (100)	++ (100)
SF-612	16							
Primary		++ (30)	-	++(60)	++ (60)	++ (60)	++ (60)	++ (95)
P1		+ (1)	-	++ (99)	++ (99)	++ (99)	++ (99)	++ (99)
SF-621	19							
Primary		+++ (60)	-	++ (30)	++ (30)	++ (30)	++ (30)	++ (90)
P1		++ (30)	-	++ (60)	++ (60)	++ (60)	++ (60)	++ (95)
P2		+ (5)	-	++ (95)	++ (95)	++ (95)	++ (95)	++ (100)
SF-622	24							
Primary		++ (20)	-	++ (80)	++ (80)	++ (80)	++ (80)	++ (90)
P1		++ (10)	-	++ (90)	++ (90)	++ (90)	++ (90)	++ (95)
P2		++ (5)	-	++ (95)	++ (95)	++ (95)	++ (95)	++ (100)
SF-673	18							
Primary		++ (50)	-	++ (40)	++ (40)	++ (40)	++ (40)	++ (95)
P1		++ (20)	-	++ (70)	++ (70)	++ (70)	++ (70)	++ (95)
P2		+ (1)	-	++ (95)	++ (95)	++ (95)	++ (95)	++ (95)
Primary cis-Pro		++ (90)	-	+ (5)	+ (5)	+ (5)	+ (5)	++ (95)
SF-684	20							
Primary		++ (40)	-	++ (50)	++ (50)	++ (50)	++ (50)	++ (90)
Primary cis-Pro		++ (90)	-	+ (5)	+ (5)	+ (5)	+ (5)	+ (95)
Fetal Leptomeninges								
SF-621 P1	19	+ (0.1)	-	+++ (99)	+++ (99)	++ (99)	++ (99)	+++ (100)
SF-622 P2	24	-	-	+++ (100)	+++ (100)	+++ (100)	+++ (100)	+++ (100)
SF-673 Primary	18	-	-	+++ (100)	+++ (100)	+++ (100)	+++ (100)	+++ (100)

Intensity of staining: - = negative; + = low; ++ = moderate; +++ = high.
Numbers in parentheses indicate the approximate percentage of cells that stained positively.

CHAPTER 5

ESTABLISMENT AND CHARACTERIZATION OF A CELL LINE FROM A HUMAN GLIOSARCOMA

5.1 Introduction:

The previous three chapters have dealt with establishing reproducible model systems for propagating well-characterized normal human leptomeningeal and glial cells in culture. In order to test the hypothesis that malignant glioma cells secrete soluble factors responsible for the malignant transformation of previously normal cells, well-characterized malignant glioma cell lines are needed to serve as the sources for the transforming growth factor studies (Chapters VIII and IX). The present chapter and the next chapter (Chapter VI) describe the establishment and characterization of several malignant glioma cell lines. In the experiments described below we describe the complete characterization of a cell line derived from a human gliosarcoma.

Non-teratomatous tumors consisting of two dissimilar neoplastic tissues have been termed "mixed" tumors. Some well-described examples of these rare neoplasms include the mixed Mullerian tumor of the uterus, the mixed tumor of the salivary gland, and the nephroblastoma (Wilms' tumor). The most common mixed tumor of the CNS is the gliosarcoma, which occurs in approximately 2% to 8% of all glioblastoma multiformes (69,172,223). We define gliosarcoma as the admixture of two distinct neoplasms in the CNS-glioblastoma multiforme and sarcoma (usually fibrosarcoma)--each of which may be diagnosed independently of the other.

In general, the pathogenesis of mixed tumors is poorly understood. The recognition of gliosarcoma as a distinct neuropathological entity has led

to a number of theories pertaining to the origin of the mixed tumor cell populations. Most studies have supported the hypothesis that the glioblastoma induces the sarcomatous transformation of the vascular endothelium and/or the perivascular adventitial cells (67,169,177,246,251). Determining the mechanism by which glioblastoma cells contribute to the induction of neoplasia in previously non-neoplastic tissues will be of fundamental importance in increasing our understanding of the behavior of primitive glial tumors and the responses they evoke in neighboring tissues within the CNS. An in vitro model system that faithfully reproduces the elements of the gliosarcoma as they occur in vivo would be helpful in answering such mechanistic questions. In this study, we characterized a cell line derived from a human gliosarcoma from the time of inception to the time of establishment (monolayer passage greater than 30). Although in early passages, the culture displayed both gliomatous and sarcomatous elements, at establishment, the predominant tumor cell type was a collagen-producing spindle-cell sarcoma.

5.2 Materials and Methods

5.2.1 Origin of the Cell Line

Cell line SF-539 BT was derived from a recurrent, right temporoparietal glioblastoma multiforme. The patient was a 34-year-old white woman who had undergone two subtotal resections followed by external beam radiation therapy (6000 rads) and chemotherapy (BCNU, 5-fluorouracil, hydroxyurea, and 6-mercaptopurine). Histopathologic analysis of a tumor specimen from the first operation showed a highly cellular tumor with marked nuclear and cytoplasmic pleomorphism, areas of pseudopalisading and necrosis, a high mitotic index, and proliferation of vascular mesenchymal cells. Many cells were positively immunostained by antiserum to GFAP. The histopathologic features of the tumor resected at the second operation were similar to those of the first tumor, except that the proliferation of the vascular elements was much greater. Neither tumor showed sarcomatous changes. Increasing tumor size and progressive neurological dysfunction prompted a third operation to reduce the mass of the tumor 15 months after her initial presentation. The patient continued to deteriorate and died 17 months after the first operation. The autopsy, restricted to the head and intracranial contents, revealed a right temporal lobe tumor extending into the right frontal, parietal, and occipital lobes; tumor was also found in the right basal

ganglia and diffusely throughout the corpus callosum. The culture and cell line were initiated from a biopsy specimen obtained during the third operation. The pathological diagnosis at that time was glioblastoma multiforme with spindle-cell sarcoma (gliosarcoma).

5.2.2 Monolayer Cell Culture

A representative portion of the tumor specimen was fixed in formalin, embedded in paraffin, cut into thin sections, and stained with hematoxylin and eosin, phosphotungstic acid-hematoxylin, and reticulin stains to confirm the diagnosis of gliosarcoma. The remainder of the specimen was processed within 4 hr after the biopsy as previously described (218, Section 2.2.1B). The medium was changed twice weekly until the cells reached confluence, when the cultures were split 1:2. A growth curve and the cell line doubling time were generated by counting cells daily in later passages (greater than 40) in 25 sq cm flasks; trypan blue staining was used to determine cell viability. The morphological features of cells in culture were recorded with an Olympus PM-10 phase microscope and camera.

5.2.3 Growth in Soft Agar

The ability of the cell line to grow in an anchorage-independent fashion was assessed by the method of Courtenay and Mills (45). Briefly, soft agar cultures were set up in triplicate in 10-ml tubes by adding 0.2 ml of red blood cells (washed and inactivated August rat red blood cells diluted 1:8), 0.2 ml of cell suspension, and 0.6 ml of 0.5% agar in Ham's F12 medium with 15% fetal calf serum. After the cells had incubated for 5 to 7 days in a 5% oxygen atmosphere, 1 ml of complete medium was added to each tube. Two weeks later, the cells were fixed for 24 hr with INT stain (Eastman Kodak, Rochester NY) and colonies of more than 50 cells were counted under a stereomicroscope. The plating efficiency was expressed as the number of colonies scored as a percentage of the total number of cells seeded. Growth in agar was assessed in the primary culture and at passages 4, 7, 19, and 45.

5.2.4 Tumorigenicity in Athymic Mice

The tumorigenicity of SF-539 BT was determined by subcutaneous injection of 5×10^6 to 2×10^7 cells in 0.3 ml of PBS into 5 30-day-old Swiss/c-nu-nu female nude mice (Animal Care Facility, University of California, San

Francisco). The tumors were harvested when they reached 0.5 to 1.0 sq cm, usually between 2 and 12 weeks after inoculation. Each tumor was dissected free of connective tissue, fixed and processed for examination by light and electron microscopy.

5.2.5 Chromosome Analysis

Culture SF-539 BT and a culture (SF-539 SLT) initiated from a separate peripheral portion of the tumor, which was obtained during the patient's third operation and which showed only sarcomatous features histologically, were harvested for cytogenetic analysis using the procedure described by Trent (275). Briefly, monolayer cultures were incubated for 1.5 hr at 37 C in the presence of Colcemid, 0.05 ug/ml (Sigma). The cells were trypsinized, washed, and resuspended in 37 C KCl for 18 min. Slides were prepared by standard air-drying techniques and the cells were G banded (305). Chromosome abnormalities are described in accordance with the International System for Human Cytogenetic Nomenclature (111).

5.2.6 Electron Microscopy

The ultrastructural features of the cell line at passages 19 and 40 were assessed by electron microscopy as described in Section 2.2.3. Tumor specimens from athymic mice were fixed and embedded in a similar fashion.

5.2.7 Antigen Expression

Immunolocalization of a variety of intracytoplasmic and extracellular matrix proteins was performed on the paraffin-embedded gliosarcoma specimen from the third surgery and on the cells in culture at various passages. The immunoperoxidase technique of Sternberger (257) was used on tissue specimens and indirect immunofluorescence was used on tissue cultures (225). The characteristics of all monoclonal antibodies and antisera used in this study have been described in detail (Sections 2.2.2 and 3.2.2).

Paraffin-embedded sections 6 um thick were deparaffinized and pretreated with 0.4% pepsin (Sigma, P7012) in 0.01 N HCl for 60 min at 37 C (164). Tumor sections were then stained with the PAP technique as described in Section 4.2.4.

The same panel of antibodies was used to perform indirect immunofluorescence on serial monolayer cultures, beginning with the

primary culture, and on cells capable of growing in soft agar, as previously described (225,227, Section 2.2.4).

5.2.8 Chemosensitivity of the Cell Line

The sensitivity of the cell line to BCNU was determined in monolayer and in soft agar systems at passages 11, 14, 19, and 45. The monolayer assay was performed as described previously (218). Treatment with BCNU in vitro at 5 ug/ml in the presence or absence of fetal calf serum was considered equivalent to the maximum clinically achievable dose of BCNU in vivo (Levin V: personal communication). The choice of a cell kill of 40% as the discriminating point between sensitivity and resistance of cells in vitro was based on correlation with the in vivo tumor response (217).

For the Courtenay agar assay, 1.0×10^5 to 5.0×10^5 tumor cells were trypsinized, counted, and placed into 15-ml centrifuge tubes containing 1 ml Ham's F-12 media. BCNU was added at concentrations of 11, 23, and 47 uM for 2 hr in 20% oxygen; the flasks were shaken every 15 min. The tubes were then filled with PBS and centrifuged for 5 min at 1000 rpm. The supernatant was decanted and 1 ml of fresh medium was added. The control cells and those that received the highest dose of BCNU were then counted. The cells in each tube were diluted to the desired test concentration. Then, 0.2 ml of cells were seeded into 15-ml tubes containing 0.2 ml of red blood cells and 0.6 ml of agar. The cells were incubated for 14 days at 37 C in 5% oxygen. Colony formation, plating efficiency, and surviving fractions were determined as in the monolayer assay.

5.2.9 Collagen Synthesis Studies

Tumor cells at passages 8 and 35 were prepared for SDS-polyacrylamide gel electrophoresis to determine whether they produced any of the collagen subtypes. Briefly, the cells were incubated in 10 ml of proline- and glutamine-free RPMI medium supplemented with ascorbate (25 ug/ml; Calbiochem) and beta-aminopropionitrile (50 ug/ml; Sigma) for 4 hr. Radiolabeled proline (L-2,3,4,5-^{3}[H] proline, 102 Ci/mmol; Amersham, Arlington Heights, IL), 40 uCi/ml, was added to the medium after the volume had been reduced to 6 ml by aspiration, and the cells were allowed to incubate for 24 hr. The medium was withdrawn and saved for analysis by DEAE-cellulose chromatography. The cell layer was rinsed in 0.5% Nonidet P 40 for 100 minutes, scraped off the

flask, and homogenized. A 4.5% stacking gel and 5% to 7.5% separating gels were used to separate the radiolabeled proteins into bands (133). The gel processing and the separation of the procollagens in the medium by DEAE-cellulose chromatography have been reported in detail elsewhere (225,227).

5.3 Results

5.3.1 Growth Characteristics and Cell Morphology

SF-539 BT grew vigorously in early-passage monolayer culture. Initially, two types of cells were apparent: a broadly bipolar cell with a large central nucleus and intracytoplasmic vesicles (Fig. 1) and a stellate cell with multiple long cytoplasmic extensions (Fig. 2A and 2B). After serial subcultivations, however, stellate cells were no longer present. The cell doubling time at passage 45 was 32 hours, and the saturation density was 1.3 x 10^6 cells/25 sq cm flask. The plating efficiency in monolayer culture at passage 45 was 22%. At establishment, the cells did not exhibit contact inhibition, rapidly overgrew the culture, and detached from the flask at confluence. Although cells from the primary culture did not grow in soft agar, cells from passages 4, 7, 19, and 45 did form colonies in agar; the plating efficiency was 0.2%. Currently, the highest passage of the cell line is 85.

5.3.2 Tumorigenicity in Athymic Mice

Mice inoculated with 0.3 ml of a tumor cell suspension containing more than 1.0 x 10^7 cells grew palpable, enlarging, subcutaneous tumors. Grossly, the tumors were well vascularized, contained a central core of necrosis, and had a viable external capsule of variable thickness. Hematoxylin-eosin staining of the formalin-fixed xenotransplanted tumors revealed a highly cellular tumor forming linear arrays (Fig. 3). The cells showed cytoplasmic and nuclear pleomorphism and had large central nuclei with prominent nucleoli. The tumor vasculature was thin-walled. Pathologically, the tumor most closely resembled a spindle-cell sarcoma.

5.3.3 Karyotype and Chromosome Analysis

The results of the chromosome banding analysis of SF-539 BT and SF-539 SLT are presented in Table 1 and Fig. 4. G-banding revealed numerous clonal structural and numeric alterations common to both cultures, including

del(2)(q12), der(3)(p12), t(2;4)(q12:p16), t(6;?)(q27;?), and del(12)(p11), as well as clonal chromosomal alterations unique to each line. These results strongly support the common origin of both cultures from the same original tumor.

5.3.4 Electron Microscopy

The ultrastructural features of SF-539 BT were similar at passages 19 and 40. Large round cells with multiple thin villus-like cytoplasmic extensions were evident. A solitary nucleus, usually central and containing a prominent nucleolus, was seen in all cells. The cytoplasm was filled with fine microfilaments, distended rough endoplasmic reticulum filled with amorphous material, and prominent Golgi apparatus. A few small lipid inclusions were also present. Multivesicular bodies were conspicuous (Fig. 5) and may be the ultrastructural correlates of the cytoplasmic vesicles seen by light microscopy. Fine, filamentous basement-membrane-like material was also present in the extracellular spaces. There were no junctional complexes or prominent intermediate type filaments.

The tumors grown in athymic mice showed extracellular collagen fiber formation (Fig. 6) and other ultrastructural features similar to those described above. Lipid inclusions were more prominent in the mouse-grown tumor cells than in those grown in monolayer culture. Multivesicular bodies were not present in the tumor cells grown in mice. Instead, many membrane-bound electron-lucent bodies were found throughout the cytoplasm.

5.3.5 Antigen Expression

The panel of monoclonal antibodies and antisera showed that the immunohistochemical profile of the paraffin-embedded gliosarcoma specimen (Fig. 7) was similar to that found in other gliosarcomas (25). The gliomatous areas were stained positively by immunostains for GFAP and negatively by stains for fibronectin, laminin, collagen type IV, procollagen type III, and reticulin. Conversely, areas in which the spindle-cell sarcoma predominated were richly positive for laminin, fibronectin, collagen type IV, and procollagen type III. Although most of the staining was extracellular, some intracytoplasmic staining for fibronectin and laminin was seen in the sarcomatous component. Giant cells located at the border of the areas of glioblastoma and sarcoma were negative for GFAP and positive for laminin,

collagen type IV, and fibronectin. Anti-factor-VIII antiserum recognized luminal endothelial cells in all areas of the tumor.

The results of the immunocytochemical analysis of SF-539 BT are shown in Table 2. Glial cells could not be identified by the presence of GFAP after passage 3. Concomitant with the decrease in the number of GFAP-positive cells over serial passages was an increase in number of cells that stained positively (both intracytoplasmically and extracellularly) for laminin, collagen type IV, fibronectin, and procollagen type III (Fig. 8). At establishment of the cell line, the immunolocalization of the panel of proteins remained basically unchanged. No cells in culture stained positively for factor-VIII-related antigen. The characterization profile of cells taken as clones from the agar system was identical to that of cells in monolayer culture.

5.3.6 Chemosensitivity of the Cell Line

The cell kill at the maximal clinically achievable dose of BCNU (23 uM) was 2% in monolayer culture at passage 42; in the agar system (passage 42), the cell kill at the same dose of BCNU was 26%.

5.3.7 Collagen Synthesis Studies

SDS-PAGE analysis of the proteins labeled with ^{3}[H]-proline revealed bands representing the alpha 1 and alpha 2 bands of collagen type I (Fig. 9). Bands of higher molecular weight representing procollagen types I and III were also recognized after application of radiolabeled peak fractions from DEAE-cellulose chromatography to separate vertical slab gel lanes.

DEAE-cellulose chromatography of the medium of culture SF-539 BT showed a moderate peak for procollagen type III and a much higher peak for procollagen type I (Fig. 10). This procollagen profile was basically unaltered when tested at intervals of 30 passages.

5.4 Discussion

In establishing this cell line from a human gliosarcoma, we were interested primarily in characterizing the change in tumor cell populations over time and with progressive subcultivations. Using specific characterization techniques that enabled us to identify, with a relatively high degree of certainty, the separate tumor cell populations, we examined the

composition of the monolayer and soft agar cultures over serial passages to determine which type of cell would eventually predominate. Our findings provide evidence that the sarcomatous element of the gliosarcoma is the predominant cell type at establishment of the cell line and that the malignant gliomatous element is lost after progressive subcultivations.

Most gliosarcomas are thought to arise as dependent tumors in which one neoplastic component precedes and induces the other (67,68,101,134,169,177,220,251). Other possible derivations include simultaneous transformation of glial and mesenchymal elements (65,182) and tumor formation as a result of external influences such as radiation therapy (134,185). Most authors believe that the glioblastoma induces the proliferation and malignant transformation of the intradural vascular mesenchymal cells (220,251). However, there are no firm experimental data that would prove this hypothesis.

Vascular mesenchymal proliferation (defined as proliferation of perivascular and endothelial cells) is a frequent concomitant not only of malignant astrocytomas (67,68,113,169,251,287), but also of oligodendrogliomas (66,194) and metastatic carcinoma of the brain (1). The recognition of vascular endothelial hyperplasia in malignant gliomas is important because a direct correlation between the degree of histological malignancy and the degree of vascular mesenchymal proliferation has been reported (68,91,147).

The cell of origin responsible for vascular mesenchymal proliferation in glioblastoma multiforme has been the subject of some debate. There are morphologic, immunohistochemical, and ultrastructural data to support the notion that the endothelial cell is the likely precursor cell (223,232,251,289). However, in our study and in the immunohistochemical analysis of malignant gliomas reported by McComb et al. (169), factor-VIII-related positivity was demonstrated only in endothelial cells bounding the vascular lumen; the mesenchymal cells forming the remainder of the hypercellular blood vessel wall were unstained. Therefore, while one interpretation of these data is that vascular mesenchymal proliferation consists solely of hyperplastic endothelial cells, some of which have lost their ability to express factor-VIII-related antigen, it is distinctly possible that vascular mesenchymal proliferation is a direct reflection of a mitogenic influence on a different adventitial cell population (e.g., pericyte, fibroblast, leptomeningeal)

altogether. It is clear that additional endothelial cell markers such as Ulex europaeus I lectin may prove useful in more fully characterizing the proliferating vascular cell pool in glioblastoma and gliosarcoma (222,251).

The cell line that we have established from a human gliosarcoma is a tumor insofar as it possesses an abnormal karyotype with marker chromosomes, grows in soft agar, and is tumorigenic in athymic mice. If our immunocytochemical methods have, in fact, accurately identified separate tumor cell populations, SF-539 BT at establishment resembles the sarcomatous portions of the original mixed tumor. SF-539 BT expressed collagen type IV, fibronectin, laminin, and procollagen type III-- the proteins that localized solely to the sarcomatous regions of the surgical specimen--but regions of glioblastoma were not identified positively by immunostains for those proteins. Although the gliosarcoma biopsy specimen had strongly staining islands of GFAP positivity, the culture derived from it rapidly lost cells capable of expressing GFAP. It seems unlikely that the malignant gliomatous element of our tumor should lose the ability to express GFAP when placed in culture and subsequently gain the ability to express extracellular matrix proteins that have not yet been conclusively localized immunohistochemically to the gliomatous areas of a gliosarcoma. Rather, we believe our immunochemical data suggest that only sarcoma cells are present at establishment of the cell line, either because their cell kinetics (higher labeling index and growth fraction) are more favorable or because they adapt more readily to the culture conditions than do cells containing GFAP. Further support for this hypothesis comes from our ultrastructural studies, which revealed large cells with dilated rough endoplasmic reticulum and extracellular collagen fiber formation, and from the production of a tumor resembling a spindle-cell sarcoma in athymic mice inoculated with SF-539 BT. In future studies, use of the organ culture or three-dimensional matrix system may eliminate the loss of markers of differentiation in glioma cells grown in monolayer cultures and preserve the proportions of glioma and sarcoma cells in the original tumor (5).

The demonstration of collagen synthesis by SF-539 BT in monolayer culture is an additional indication that the cell line is sarcomatous, since we have found no clear proof that well-characterized malignant gliomas can synthesize the interstitial collagens. Alitalo et al. (5) demonstrated the biosynthesis of collagen type IV in a human astrocytoma cell line (U-251 MG)

after labeling the cells with ^{3}[H]-proline; however, this cell line was found not to contain collagen types I, II, III, and V. Rabson et al. (200) reported the increased synthesis of procollagen I relative to procollagen type III in a glioblastoma cell line treated with hexamethylene bisacetamide; however, because this cell line (CBT) has the histological appearance of a sarcoma and has produced a fibrosarcoma in nude mice, it appears to be of mesenchymal rather than of glial origin and may have arisen from malignant mesenchymal cells present in the original glioblastoma multiforme. Similarly, Maunoury (162) reported that three of five cell lines he established from malignant human brain tumors consisted of spindle- shaped cells that stained positively with Masson's trichrome and reticulin stains. In addition, extracellular collagen fibers were demonstrated by electron microscopy in all three cell lines, one derived from a meningeal sarcoma and two from malignant gliomas. None of these cell lines expressed GFAP or S-100 protein. In our study, DEAE-cellulose chromatography and SDS-PAGE demonstrated that SF-539 BT has a reproducible procollagen and collagen profile.

Tissue culture studies of gliosarcomas have been reported previously (172,251). In each study, the sarcomatous element in vitro appeared to outgrow the gliomatous element. However, the distinctions between the cell types were based on the findings of light microscopy only. McKeever et al. (172) used immunocytochemical methods to analyze the cells derived from explants of a gliosarcoma culture at passage 8. More than half the cells were positive for fibronectin, but no cells containing GFAP were identified. The present study is the first in which the cell populations of a gliosarcoma culture have been carefully characterized over serial passages and to demonstrate the autonomous tumorigenicity of the sarcomatous element at establishment of the culture. Interestingly, and perhaps coincidentally, the pathological behavior of the tumor paralleled the history of the culture derived from it in that the glioblastoma was eventually outgrown by the spindle-cell sarcoma. Similarly, the relative resistance of SF-539 BT to BCNU in monolayer and soft agar systems in early passages appears to reflect that patient's lack of response to chemotherapeutic agents, including BCNU. If the sarcoma and glioblastoma cell populations differ in their sensitivity to radiation and chemotherapy in vivo, the more resistent cells should have a selective growth advantage and eventually become predominant. Several reports have alluded to the increased growth potential of the sarcoma relative

to the glioma (66,67,172,177,246). The sarcoma often forms the bulk of the neoplasm even if the original tumor was diagnosed as a glioblastoma multiforme. And when a gliosarcoma metastasizes, the sarcomatous component and not the gliomatous component is almost always found in the metastasis (36,236). It is uncertain whether postoperative cranial irradiation and systemic chemotherapy play a role in the pathogenesis of the sarcomatous element. The findings in the patient we describe and in several patients reported elsewhere (65,69,134,169,185,251) provide anecdotal evidence of tumor induction by external influences.

In conclusion, by establishing and characterizing SF-539 BT, we have contributed to the understanding of the growth dynamics and identification of the tumor cell types in a human gliosarcoma culture. We have generated a resistant cell line that most closely resembles the spindle-shaped sarcoma of the original tumor and is currently being used to study mechanisms of tumor resistance. In the future, the two types of tumor cells in a gliosarcoma should be cloned to yield pure glioma and sarcoma populations from the same tumor. Well-characterized tumor clones will lend themselves to more direct experiments designed to isolate the soluble factor(s) responsible for tumor induction in the mesenchymal elements of the CNS and will provide better models for studying the interactions between glioma and sarcoma cells.

(Part of the material in this chapter has been previously published in Cancer Research 46: 5893-5902, 1986. Permission to reproduce the data here has been granted by the American Association of Cancer Research - see Appendix)

5.5 Figure Legends and Tables

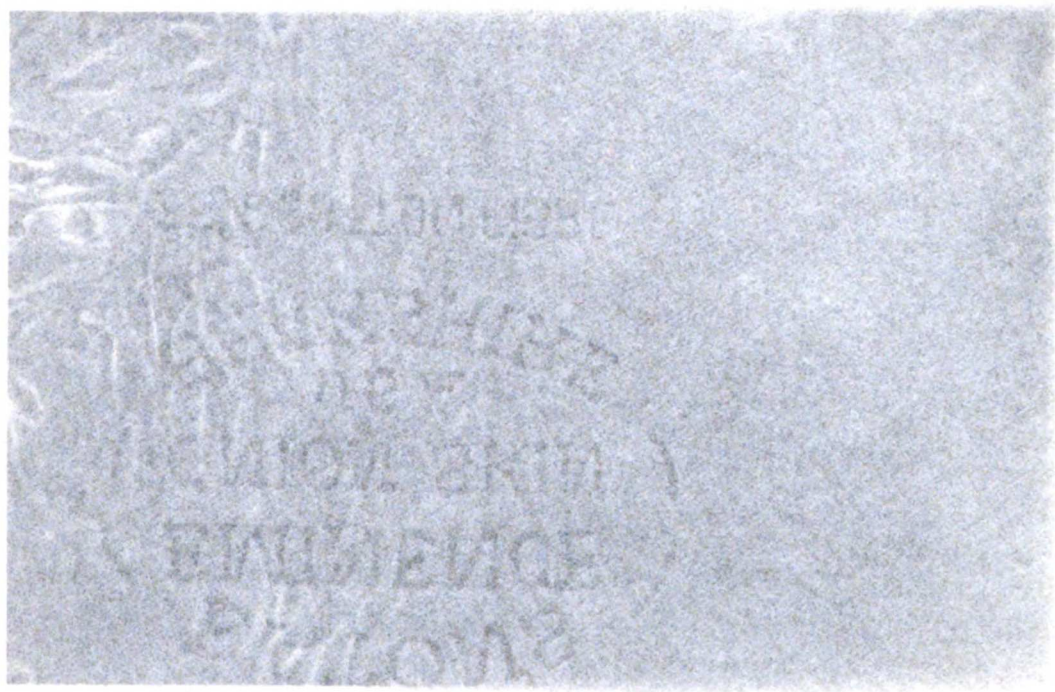


Fig. 1. Sr 509 BL, p
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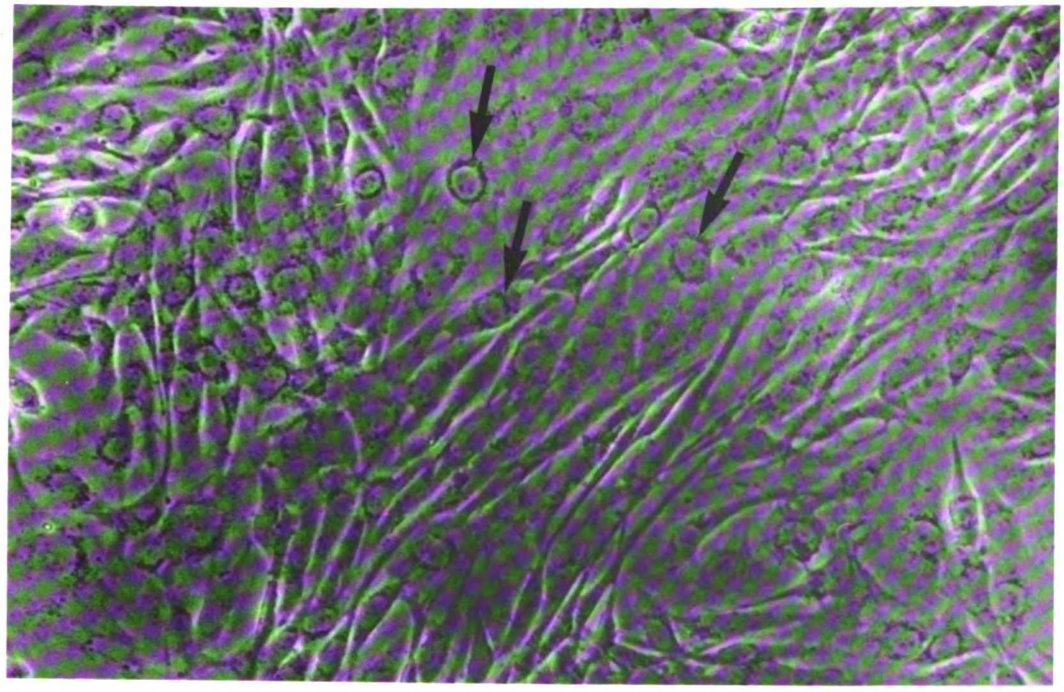


Fig. 1. SF-539 BT, passage 3. Broadly bipolar cells with large central nuclei and generous cytoplasm represent one cell type seen in early-passage cultures. Multiple intracytoplasmic vesicles (arrows) could be seen by light microscopy. Phase microscopy, X100.

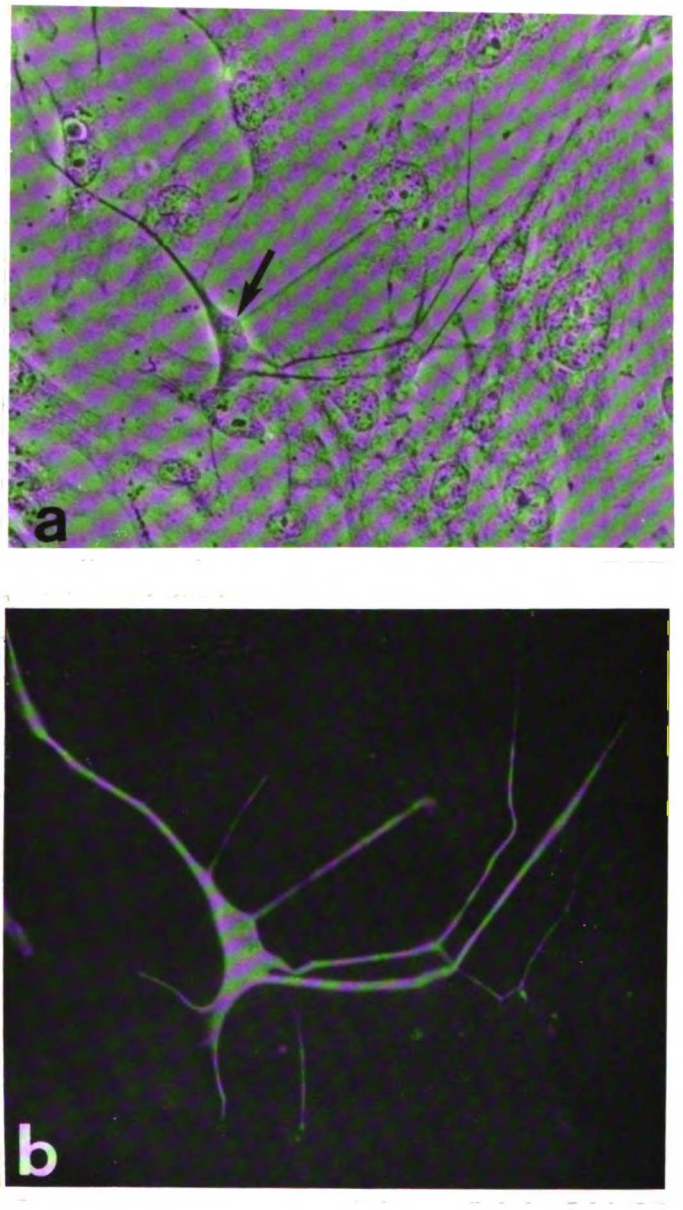


Fig. 2. SF-539 BT, passage 2. a, A single stellate cell with multiple slender processes and minimal cytoplasm (arrow) is seen against a background of broadly bipolar cells. b, Only the stellate cell is positively identified by immunostaining for GFAP. Phase microscopy and indirect immunofluorescence, X500

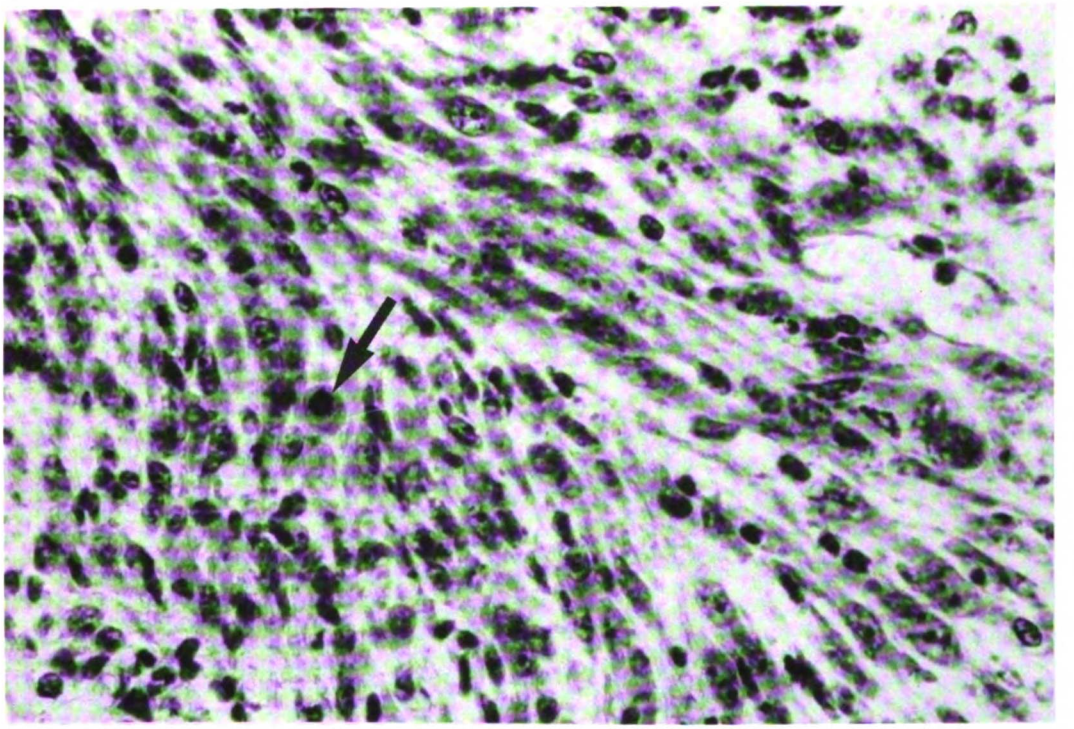


Fig. 3. Histologic section of tumor grown in athymic mice. The tumor is hypercellular; the cells show a modest amount of nuclear pleomorphism and are arranged in linear arrays. Arrow indicates a mitotic figure. The morphology of the tumor most closely resembles the spindle-cell sarcoma of the original tumor. Hematoxylin-eosin, X150.

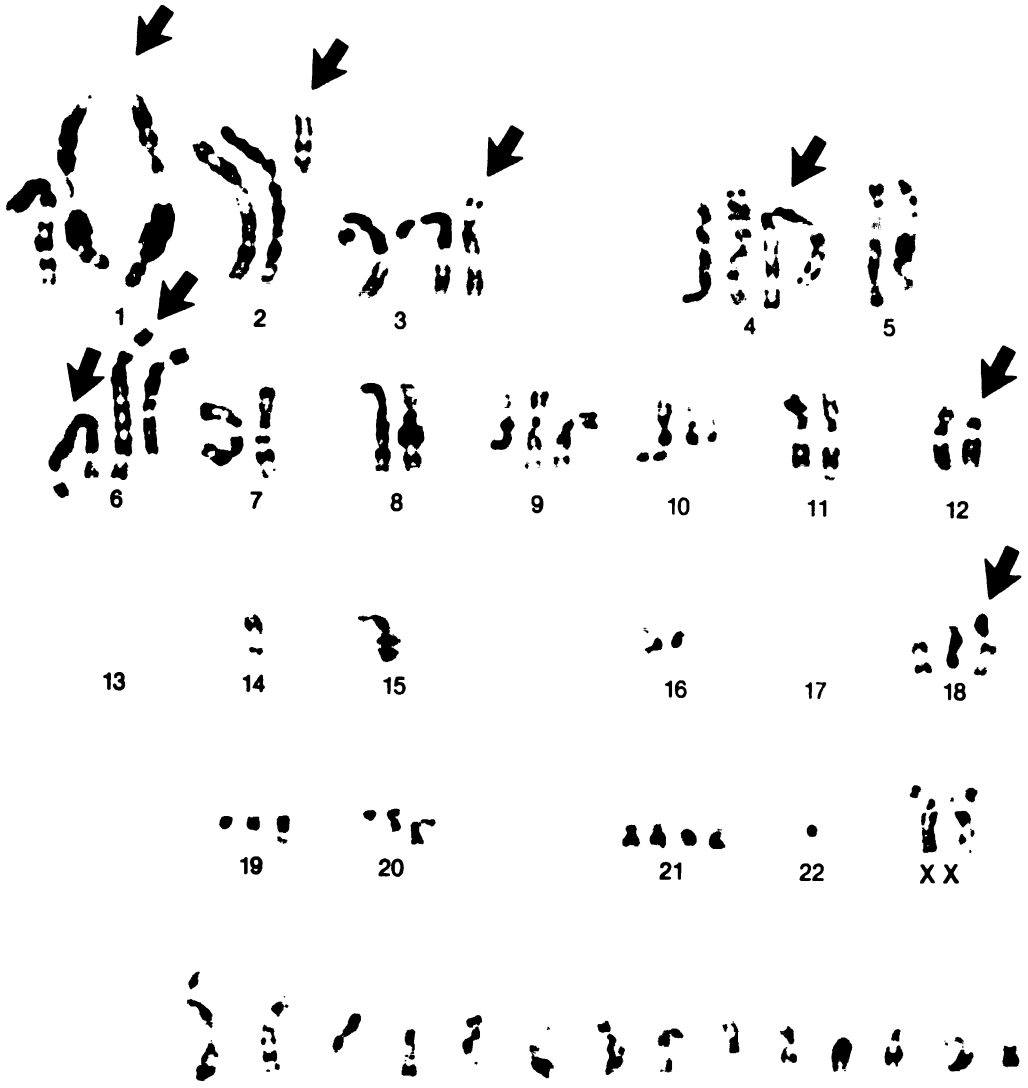


Fig. 4. G-banded karyotype from SF-539 BT. Arrows indicate structural alteration; unidentifiable markers (Umars) are located in the bottom row.

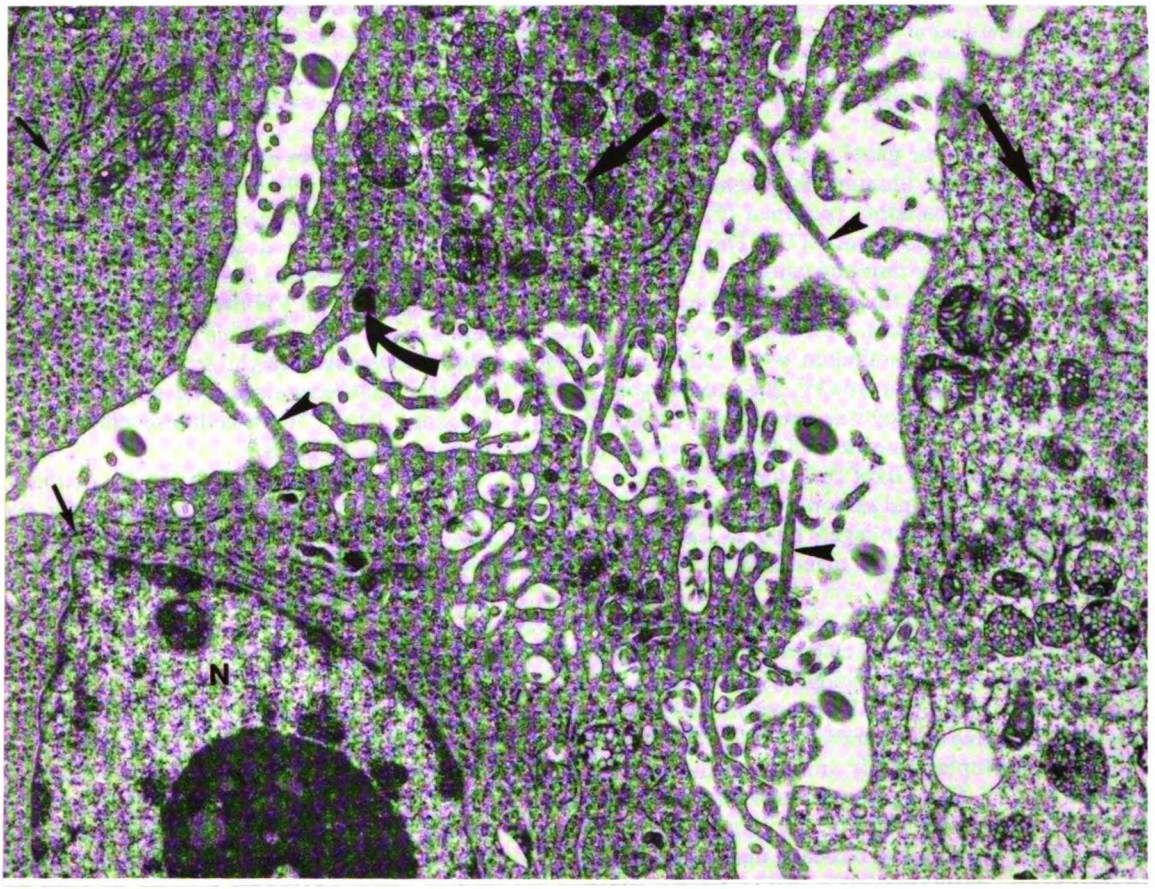


Fig. 5. Ultrastructural detail of SF-539 BT in culture, passage 40. The tumor cells are large with multiple thin cytoplasmic extensions forming filopodia (arrowheads). The nucleus (N) is centrally placed and the nucleolus is prominent. Multivesicular bodies are present throughout the cytoplasm (large arrows). The rough endoplasmic reticulum is prominent (small arrows). Lipid inclusions are also present (curved arrow). Electron microscopy, X10,000.

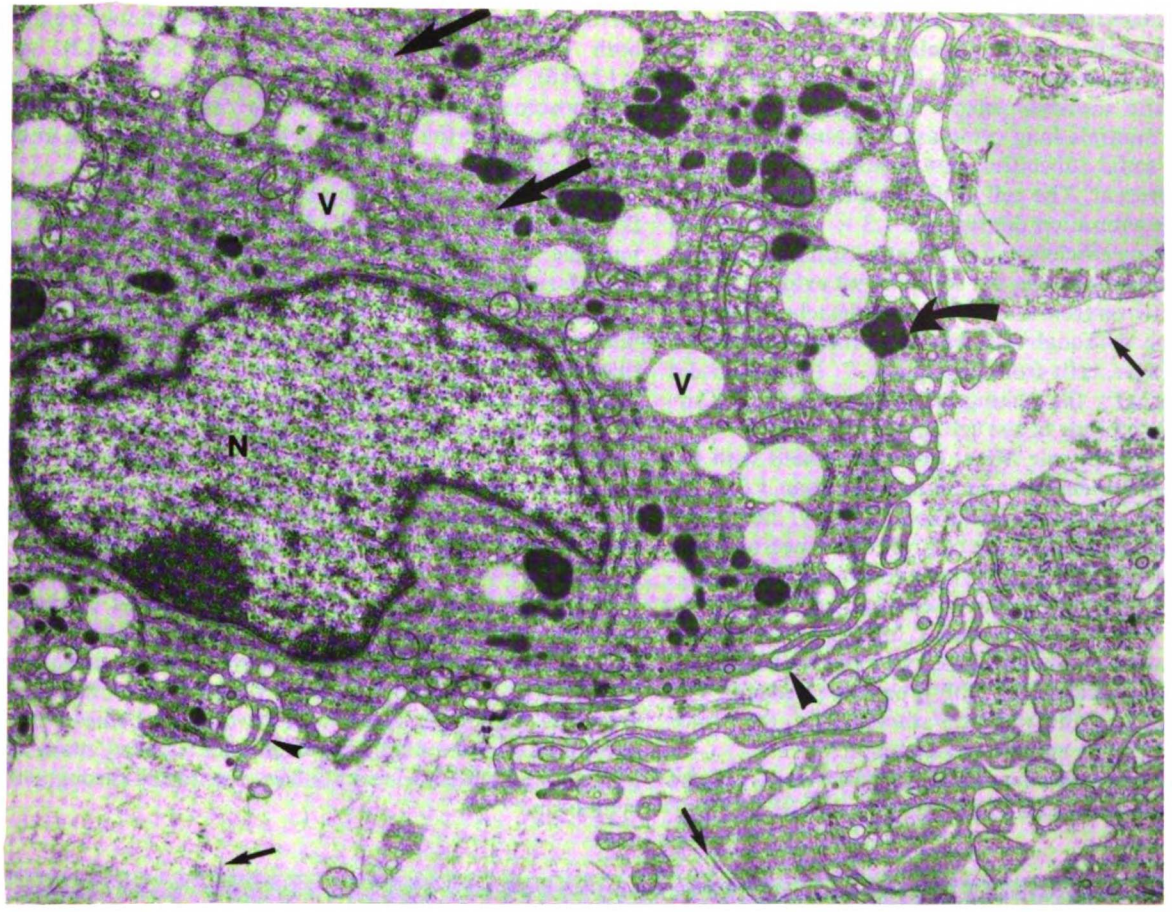


Fig. 6. Ultrastructural detail of xenotransplanted SF-539 BT. The features resemble those seen in Fig. 5. Large tumor cells with cytoplasmic extensions forming filopodia are also present. Spherical, membrane-bound, electron-lucent bodies (V) are found throughout the cytoplasm. Filopodia (arrowheads) are prominent. Tumor cells grown in athymic mice also showed more abundant filament formation (large arrows) and lipid inclusions (curved arrow) within the cytoplasm than did tumor cells grown in monolayer culture. Also, xenotransplanted tumors contained extracellular collagen fibers (small arrows). N = nucleus. Electron microscopy, X10,000.

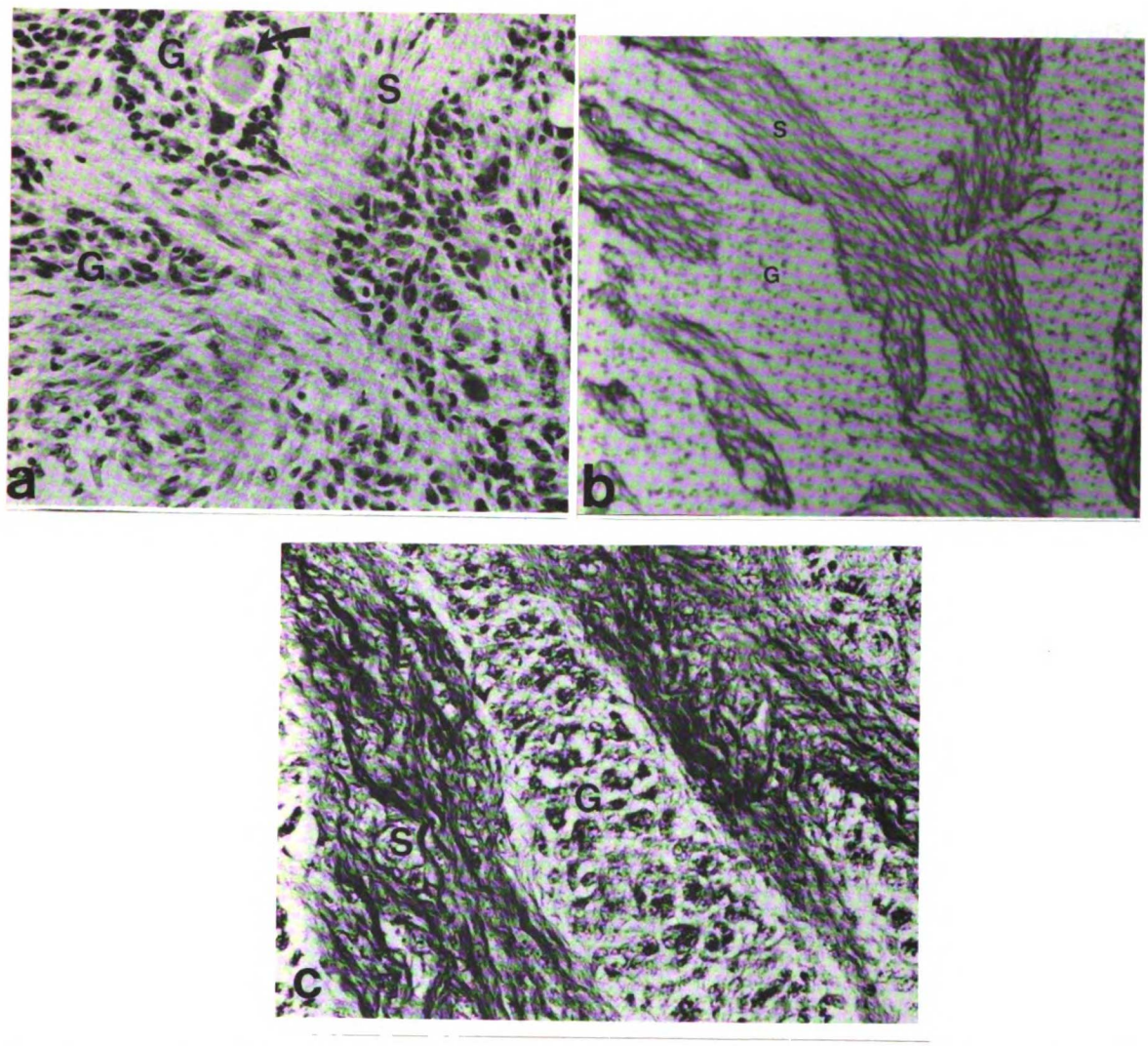
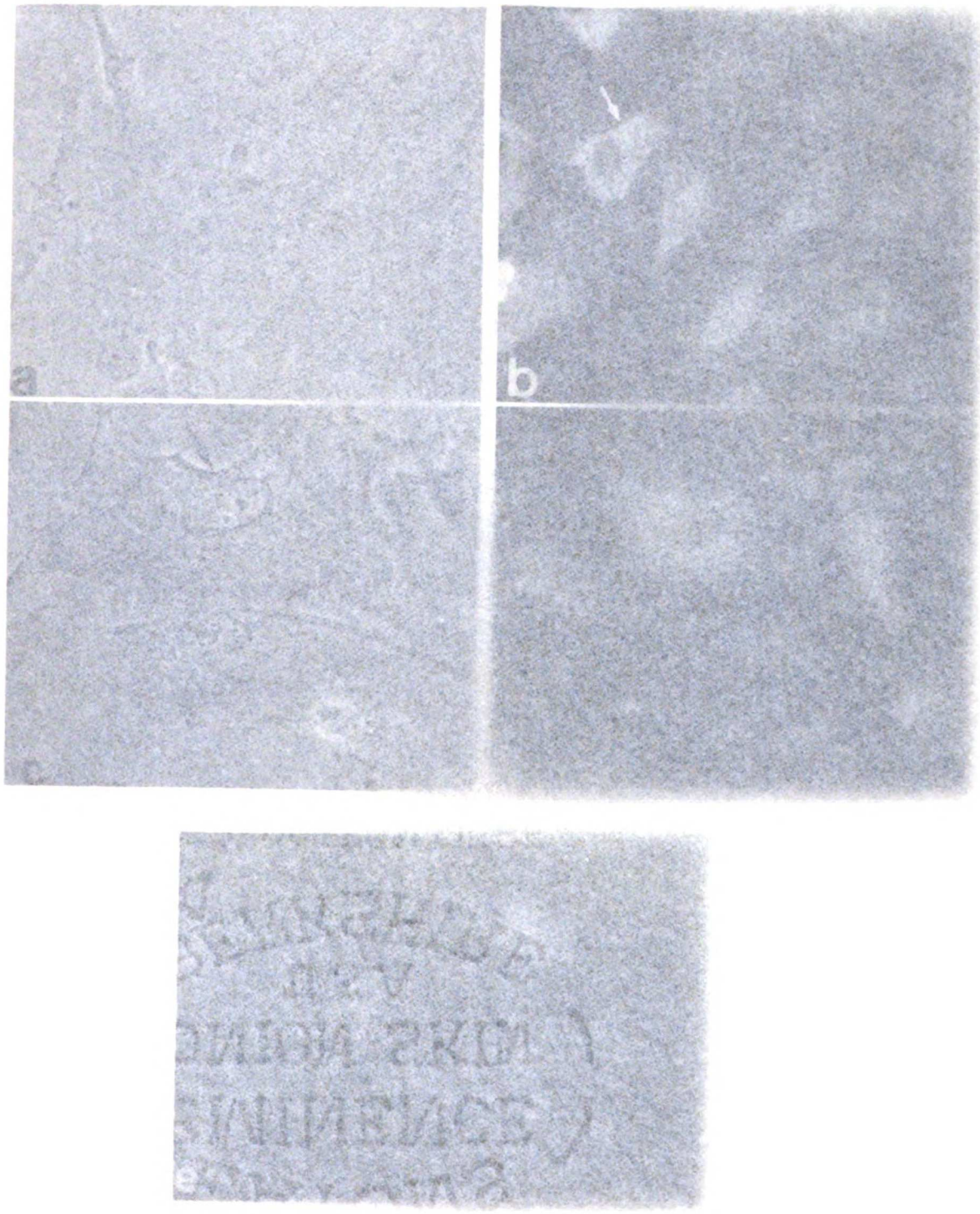


Fig. 7. Immunohistochemical characterization of the gliosarcoma. a, Islands of glioma (G) stain positively with immunostains for GFAP; regions of sarcoma (S) are not identified with the anti-GFAP antiserum. A giant multinucleated cell at the border of gliomatous and sarcomatous elements (curved arrow) is negative by GFAP immunostains. Light microscopy, X150. b, Regions of sarcoma are positively identified as dark linear bands by stains for reticulin. The gliomatous areas are negative for reticulin. Light microscopy, X75. c, Immunostain for laminin within the gliosarcoma. The sarcomatous element stains positively in an extracellular, fibrillary fashion. The gliomatous regions are unstained. Light microscopy, X500.

Fig. 8. Immunocytochemical analysis of SF-539 BT. a, b, Passage 2. All cells are positively identified by anti-laminin antibody. Staining is granular, perinuclear, and largely cytoplasmic (arrow). Phase and indirect immunofluorescence microscopy, X500. c, d, e, Passage 2. Phase microscopic examination (c) reveals a variety of different cell morphologies; large arrows indicate cells that will stain positively for GFAP, while small arrows indicate cells that will stain positively for collagen type IV. In (d), immunostaining for GFAP identifies three cells that show characteristic intracytoplasmic filament formation (small arrows); a long cytoplasmic process is also positively identified (large arrow). In (e), granular perinuclear cytoplasmic staining is seen in three cells immunostained with collagen type IV antibody (arrows). Therefore, by double immunofluorescence, GFAP and collagen type IV immunostains recognized completely separate populations of cells. In control experiments, the fluorescein-and rhodamine-conjugated antisera did not react with each other. Phase and indirect immunofluorescence microscopy, X500.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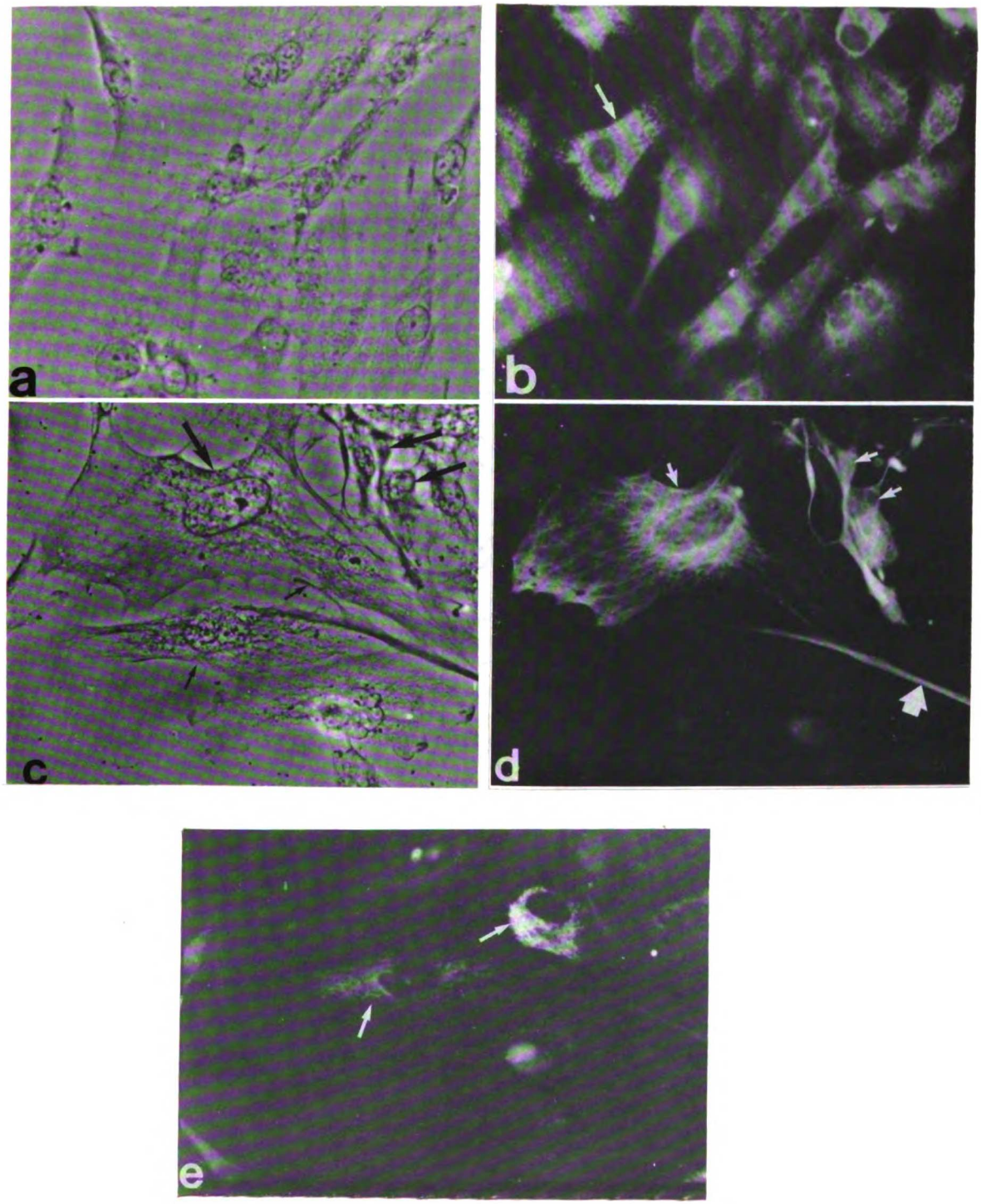

Figure 8 a - e

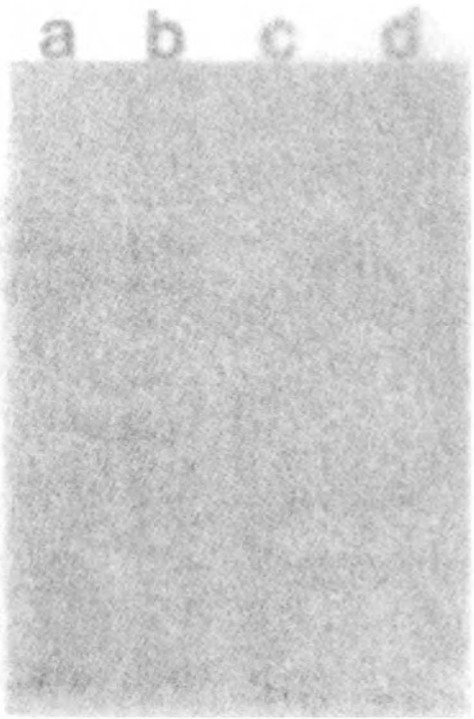

Fig. 8. SDS-PAGE and
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representing type I collag
bands i. each is darker the
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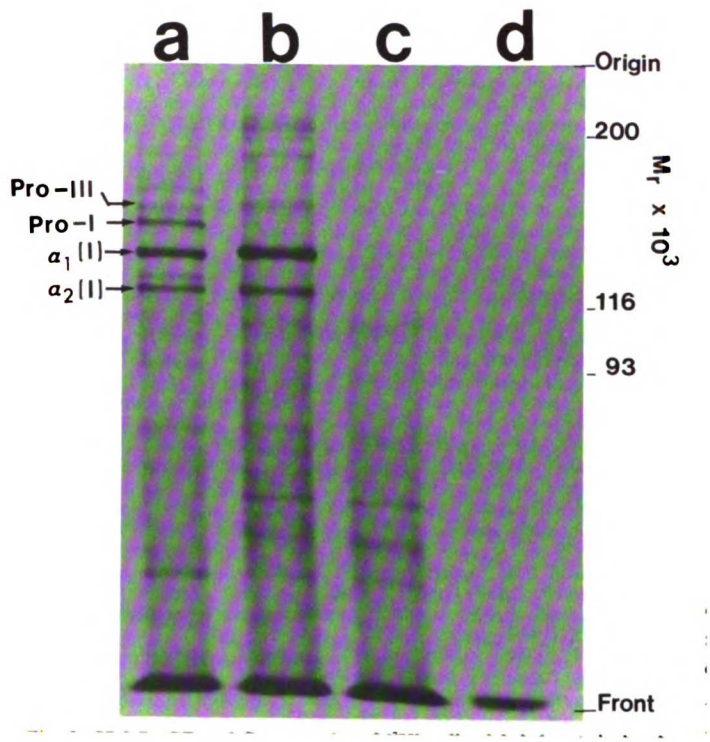


Fig. 9. SDS-PAGE and fluorography of ^{3}[H]- proline-labeled protein bands. Lane a: SF-526 (meningioma). Lane b: SF-539 BT (gliosarcoma). Lane c: U-251 MG (malignant astrocytoma). Lane d: SF-539 BT incubated with collagenase (Sigma, type V), 10 U/ml, for 4 hr at 37 C. In lanes a and b, bands representing type I collagen (alpha $_1$ (I) and alpha $_2$ (I)) are present. The alpha $_1$ band in each is darker than the alpha $_2$ band. Procollagen types I and III are identified above the alpha 1 bands. U-251 MG (lane c) does not appear to make any of the interstitial collagens. Treatment with collagenase (lane d) results in disappearance of the proline-labeled collagen bands. Molecular weight standards are shown.

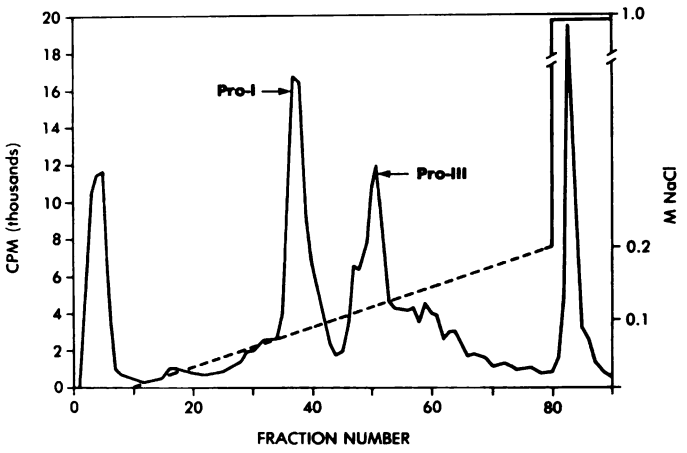


Fig. 10. DEAE-cellulose chromatography of SF- 539 BT. A moderate peak for procollagen type III is seen at fraction 50; a more prominent peak representing procollagen type I is seen at fraction 40. Separation of procollagens was achieved with an increasing salt gradient, as shown (dotted line).

Table 2 *Immunocytochemical characterization of cell line SF-539 BT over multiple passages*

Intensity of staining, scored as negative (−), low (+), moderate (++), or high (+++). Numbers in parentheses indicate the percentage of cells that stained positively.

Culture type	Passage	GFAP	VIII:RAg	Fibronectin	Laminin	Collagen type IV	Procollagen III
Monolayer	Primary	+++ (50)	−	++ (50)	+ (10)	++ (10)	++ (5)
Monolayer	1	+++ (12)	−	++ (85)	++ (85)	++ (85)	++ (40)
Monolayer	2	+++ (3)	−	+++ (95)	++ (95)	++ (95)	++ (50)
Monolayer	3	+++ (0.05)	−	++ (99)	++ (99)	++ (80)	++ (25)
Monolayer	4	−	−	++ (100)	++ (100)	++ (90)	++ (25)
Monolayer	7	−	−	++ (100)	++ (100)	++ (90)	++ (25)
Monolayer	42	−	−	++ (100)	++ (100)	++ (80)	++ (20)
Agar	4	++ (1)	−	++ (95)	++ (95)	++ (70)	++ (25)
Agar	7	−	−	++ (100)	++ (100)	++ (80)	++ (20)

Table 1 *Summary of cytogenetic analysis of SF-539 BT and SF-539 SLT*[a]

Cell line	Cells counted	Range	Mode	Common clonal structural alterations	Unique clonal structural alterations
SF-539 BT[b]	50	40–184	64	del(2)(q12) der(3)(p12) t(2;4)(q12;p16) t(6;?)(q27;?) del(12)(p11)	t(9;?)(p2?;?) t(18;?)(p11;?)
SF-539 SLT[c]	50	54–127	61	del(2)(q12) der(3)(p12) t(2;4)(q12;p16) t(6;?)(q27;?) del(12)(p11)	del(7)(q21) iso8q t(12;?)(q24;?) t(3;12)(p12;p11)

[a] del, deletion; der, derivative; iso, isochromosome; t, translocation.

[b] Established from portion of brain tumor with both gliomatous and sarcomatous features histologically.

[c] Established from portion of brain tumor with only sarcomatous features histologically.

CHAPTER 6

ESTABLISHMENT AND CHARACTERIZATION OF CELL LINES DERIVED FROM HUMAN MALIGNANT GLIOMAS

6.1 Introduction

It is clear from the data presented in Chapter 5 that the gliosarcoma cell line SF-539 BT will not serve as an appropriate glioma cell line from which to harvest serum-free conditioned medium to perform the transforming growth factor assays described in Chapter 9. Rather, we have shown quite cogently that SF-539 BT resembles the sarcomatous portion of the original mixed tumor. The present chapter describes the characterization of several malignant glioma-derived cell lines, some of which will be appropriate for use in the TGF studies described in Chapters 8 and 9.

The properties and characteristics of several series of cell lines derived from malignant gliomas have been well described (18,162,196,259,296). A valuable feature of some glioma-derived cell lines is the ability of the tumor cells in culture to retain some of the phenotypic properties of the original tumor. If cell culture systems are to continue to serve as useful models for studying tumor cell biology, further efforts to characterize cell lines derived from malignant gliomas are needed because little is known about the histogenesis of many of the different cell types in these tumors (19).

In 1977, Maunoury established and characterized five cell lines from human malignant gliomas. Masson and reticulin staining and electron microscopy unexpectedly demonstrated that four of the cell lines synthesized collagen in vitro (162). It was suggested that these collagen-producing cell lines might have been derived from hyperproliferative vascular elements rather than from glial elements in the original tumors. Therefore, in this study, we established and characterized cell lines from six human gliomas and determined their ability to synthesize extracellular matrix proteins and interstitial collagen.

6.2 Materials and Methods

6.2.1 Origins of the Cell Lines

The age and sex of the patients, the tumor location, and the histopathological features of the surgical specimens from which the cell lines were initiated are described in Table 1. The surgical specimens were processed as described below within 4 hours of surgery. In addition, three well-characterized glioma-derived cell lines (U- 251 MG, U 373 MG, and U 343 MG-A) were used in the antigen expression and collagen synthesis studies (18,199).

6.2.2 Monolayer Cell Culture

To obtain a tumor diagnosis, a representative portion of each tumor specimen was fixed in formalin, embedded in paraffin, cut into thin sections, and stained with hematoxylin and eosin. The remainder of the specimen was processed as described previously (218, Sections 2.2.1B, 3.2.1A). The medium was changed twice weekly until the cells reached confluence. At confluence, the cultures were split 1:2. After establishment (monolayer passage greater than 30), growth curves and cell doubling times were generated by counting cells daily in 25 sq cm flasks; trypan blue staining was used to determine cell viability. The morphological features of the cell lines were recorded with an Olympus PM-10 phase microscope and camera. All cell lines were routinely tested for Mycoplasma contamination as described by Russell et al. (224).

6.2.3 Growth in Soft Agar

The ability of each cell line to achieve anchorage-independent growth was assessed by the method of Courtenay and Mills (45). Our technique for performing the soft agar assay has been described in Section 5.2.3. The plating efficiency was expressed as the number of colonies scored as a percentage of the total number of cells seeded.

6.2.4 Tumorigenicity in Athymic Mice

The tumorigenicity of all cell lines was determined by subcutaneous injection of 5×10^6 to 2×10^7 cells in 0.3 ml PBS into 30-day-old Swiss/c-nu-nu female nude mice (Animal Care Facility, University of California, San Francisco). The tumors were harvested when they reached 0.5-1.0 sq cm. Each tumor was dissected free of connective tissue, fixed, and processed for examination by light and electron microscopy.

6.2.5 Chromosome Analysis

Established cell lines were harvested and banded for cytogenetic analysis using the procedure described by Trent (274). Briefly, monolayer cultures were incubated for 1.5 hours at 37 C in the presence of Colcemid, 0.05 ug/ml (Sigma, St. Louis, MO, USA). The cells were trypsinized, washed and resuspended in KCl at 37 C for 18 min. Slides were prepared by standard air-drying techniques and the cells were G- and Q-banded (305). Chromosome abnormalities are described in accordance with the International System for Human Cytogenetic Nomenclature (111).

6.2.6 Electron Microscopy

The ultrastructural features of all cell lines were assessed by electron microscopy. Glioma cells were processed for electron microscopy using the technique described previously in Section 2.2.3.

6.2.7 Antigen Expression

Several intracytoplasmic and extracellular matrix proteins were immunolocalized in the six paraffin- embedded tumor specimens and in the tumor cells in early- and late-passage cultures. The immunoperoxidase technique of Sternberger (257) was used on tissue specimens and indirect immunofluorescence was used in tissue cultures (225,227).

The monoclonal antibodies and antisera used in this study have been completely characterized and described previously (Sections 2.2.2, & 3.2.2). Rabbit anti-glutamine synthetase antisera was a gift from Dr. M. Norenberg (University of Florida, Gainesville, FL, USA). The specificity of the anti-glutamine synthetase antisera was determined by radioimmunoprecipitation (157).

Immunohistochemistry was performed on all paraffin-embedded glioma sections using the panel of monoclonal antibodies and antisera described previously. Our technique for immunohistochemistry has been outlined in detail in Sections 4.2.4 and 5.2.7.

Indirect immunofluorescence staining was performed on all glioma cell lines using the antibodies decribed above and the rabbit anti-glutamine synthetase antisera. Our method for performing indirect

immunofluorescence has been described in detail elsewhere (225,227, Section 2.2.4).

6.2.8 Chemosensitivity Assays

The sensitivity of the six glioma-derived cell lines to BCNU was determined in monolayer and in soft-agar systems. The details of the monolayer and soft agar chemosensitivity assays have been described (218, Section 5.2.8).

6.2.9 Collagen Synthesis Studies

All glioma cell lines were prepared for gel electrophoresis (Section 2.2.6) to determine whether they produced any of the collagen subtypes. The medium was withdrawn and saved for analysis by DEAE-cellulose chromatography (225,227, Section 2.2.6, & 2.2.7).

6.3 Results

6.3.1 Growth Characteristics and Cell Line Morphology

According to the classification system used at UCSF for the malignant gliomas (147), four of the tumors from which the cell lines were derived were glioblastomas multiforme, one was a highly anaplastic astrocytoma, and one was a gliosarcoma. Nuclear and cytoplasmic pleomorphism, pseudopalisading, vascular mesenchymal proliferation, hypercellularity, and a high mitotic index were features common to the glioblastomas (Fig. 1); the highly anaplastic astrocytoma did not exhibit pseudopalisading or pronounced vascular mesenchymal proliferation, and the gliosarcoma consisted of regions of glioblastoma and spindle-cell sarcoma.

The growth and kinetic characteristics of each cell line are shown in table 2. All cell lines grew vigorously in monolayer culture even at earlier passages. SF-188 has the shortest doubling time (28 hours) and the greatest plating efficiency in agar. All cell lines demonstrated marked crowding and loss of contact inhibition at confluence. Our glioma-derived cell lines are morphologically diverse, and once established, have maintained their respective morphologies over serial passages. Only one cell line (SF-295) had a glia-like morphology (Fig. 2). All cell lines grew anchorage independently in soft agar.

6.3.2 Tumorigenicity in Athymic Mice

Cell line SF-539 gave rise to palpable, enlarging spindle-cell sarcomas in nude mice (227). None of the other cell lines produced tumors in the athymic mice.

6.3.3 Karyotype and Chromosome Analysis

Detailed cytogenetic analyses have been reported previously for all cell lines except SF-295 (12,226). Karyotypic results of SF-295 were determined by Dr. Ward Peterson, Children's Hospital, Detroit, MI. G- and Q-banding analysis revealed clonal structural and numerical alterations. Two of the cell lines (SF-210 and SF-268) revealed hyperdiploid modal chromosome numbers. SF-539 was hypotriploid, and SF-126, SF-188, and SF-295 were hypertriploid. The most common clonal numerical changes were gains in chromosomes 7 and 20, and losses of the sex chromosomes, and less frequently losses of chromosomes 10, 12, and 13. SF-126, SF-188, and SF-295 contained double minutes.

6.3.4 Electron Microscopy

The ultrastructural features of all cell lines are presented in Table 3. All tumor cells had central nuclei with prominent, and often multiple, nucleoli. Microvilli were abundant in SF-539, SF-188, SF-210, and SF-268. Epithelial-like cell junctions were seen in SF-126, SF-188, and SF-268 (Fig. 3). The cytoplasm of all cells contained abundant microfilaments. Glial-like intermediate filaments were not observed in any cell line. Collagen fibrils were seen in SF-126 and SF-539.

6.3.5 Antigen Expression

The glial origin of each glioma specimens placed into culture was confirmed by immunostains for GFAP, which demonstrated positive intratumoral staining for the astrocyte-specific marker in each case (Fig. 4). In the immunohistochemical analysis, antisera to factor-VIII, fibronectin, and laminin and monoclonal antibodies to collagen type IV and procollagen type III revealed intense immunostaining in regions of vascular mesenchymal proliferation (Fig. 5) and at the border between glial and mesenchymal elements. No direct staining of tumor cells or intratumoral components was seen.

The results of the immunocytochemical analysis of all the glioma-derived cell lines are shown in Table 4. Only cell lines SF-539 and SF-295 contained GFAP-positive cells in early-passage cultures (Fig. 2); in both cell lines, however, the astrocytic marker was lost with progressive subcultivation. Immunostains for glutamine synthetase were negative in all six cell lines in both early and late passages. Immunostains for fibronectin and laminin were positive in all six glioma-derived cell lines in early-passage culture (Fig. 6), but in later passages, expression of these two glycoproteins remained low or was dimished or completly lost in SF-188, SF-210, SF-268, and SF-295. Cell lines SF-126, SF-188, and SF-539 were positively immunostained by monoclonal antibodies to collagen type IV in both early- and late-passage cultures, whereas expression of type IV collagen was lost in later passages of cell lines SF-210, SF-268, and SF-295. At establishment, only SF-539 and SF-126 expressed procollagen type III to any significant degree (Fig. 7). No cell line was positively immunostained by antisera to factor VIII. At establishment cell lines SF-295 and SF-268 failed to react with any of the antibodies selected for use in this study.

6.3.6 Chemosensitivity Assay

The results of chemosensitivity assay are shown in Table 2. As defined by the surviving fraction of cells at the highest clinically achievable dose of BCNU, four of the cell lines were resistant and two were sensitive in monolayer culture. The sensitivity or resistance of these cell lines did not change when tested in the soft agar assay.

6.3.7 Collagen Synthesis Studies

DEAE-cellulose chromatography demonstrated interstital collagen synthesis in three of the six cell lines (Fig. 7). The medium of SF-539 was enriched in procollagen I relative to procollagen III, whereas SF-126 showed preferential production of procollagen III. SF-210 contained very low levels of the radiolabeled interstitial collagen precursors at low passage only. SF-268, SF-295, SF-188, U-251 MG, and U 343 MG-A synthesized neither procollagen I nor procollagen III.

Analysis of the cell layers by SDS-PAGE complemented the results of DEAE-cellulose chromatography. SF-539 and SF-126 contained radiolabeled

protein bands identified as types I and III collagen. The other cell lines were negative when examined for these collagen subtypes (Fig. 8).

6.4 Discussion

The success rates in establishing cell lines from human malignant gliomas placed into culture have varied, but are in general relatively high (196,199,292). In vitro, malignant glioma cells differ from cultures of normal human brain in that the tumor cultures have an infinite life span, a greater saturation density, and decreased contact inhibition. The growth characteristics of our glioma-derived cell lines are similar to those described by others (18,162,196,259,292).

Although the growth and kinetic properties of cell lines derived from malignant gliomas are similar from series to series, there has been an unexpected degree of genotypic and phenotypic diversity from cell line to cell line (19,27,75,199,239,297,298,304). Put in other terms, two cell lines established from two histologically similar glioblastomas multiforme will probably differ dramatically with respect to their genotypes and phenotypes. Wikstrand et al. (298) and Shapiro et al. (239) have shown that such heterogeneity also exists within a given cell line and its clones. The heterogeneity of the six cell lines in our study included differences in karyotype, tumorigenicity, morphology, ultrastructural features, antigenic expression, and response to BCNU.

Established glioma-derived cell lines demonstrate widespread karyotypic heterogeneity, including gains of chromosomes 7 and 14, and decreases of chromosomes 4, 10, 12, 22, and the sex chromosomes (18,21,24,152,162,199,297,302). Gains of chromosome 7 and loss of the sex chromosomes observed in our series are in good agreement with this profile. Additional copies of chromosome 20 has not been reported previously as characteristic of human malignant gliomas, and may represent a random occurrence observed in our small series. Occurrence of double minutes has been demonstrated in approximately 50% of human gliomas. Double minutes represent cytologically recognizable chromosomal changes associated with amplified cellular genes. Three of our cell lines contained double minutes. SF-188 has been previously reported to be amplified for c-myc (273).

All six glioma-derived cell lines achieved anchorage-independent growth in soft agar, but only one formed a tumor in athymic mice. The difficulties of successfully heterotransplanting human glioma-derived cell lines have been described previously (18,20,115,162,163,204,239). From all accounts, established glioma-derived cell lines have a less than 1 in 4 probability of forming representative tumors in athymic mice. A much greater success rate (near 90%) has been reported for direct inoculation of malignant glioma specimens into athymic mice (115,204,240). In contrast to the results of others (20), nuclear configuration, percent colony formation in agar, and saturation density were not good predictors of the tumorigenicity of malignant glioma cell in our study. The inability of many aneuploid glioma-derived cell lines to form tumors in athymic mice, however, cannot be used as evidence against the neoplastic nature of the cell lines (21,162,199).

Light and electron microscopy have shown that the morphological features of cell lines derived from human gliomas are heterogeneous (18,24,199). In our study of six cell lines, no specific cell type appeared consistently at establishment. Moreover, there was ultrastructural variability between cell lines in the number and distribution of intracytoplasmic organelles, filaments, and cell:cell junctional complexes. Two of the cell lines formed collagen fibrils in vitro.

Perhaps nowhere is the heterogeneity of human malignant glioma-derived cell lines more apparent than in the many studies that have examined the complex antigenic expression of malignant glioma cells in culture (18,59,75,115,162,233,259,297,298). Attempts to establish unequivocally the identity of most glioma-derived cell lines with GFAP, the marker that is specific for glial cells, have met with disappointment. In a study by Bigner et al., only one of 15 glioma-derived cell lines expressed GFAP over serial passages (18). Maunoury established five cell lines from 50 intracranial tumors, none of which expressed GFAP (162). Studer et al. recently reported four cell lines that appear to express GFAP over several passages (259); although there was a spectrum of GFAP positivity in each glioma-derived cell line, the stability of GFAP expression over time was not specifically stated. In our study, all pathological tumor specimens placed into culture had readily identifiable regions of GFAP-positive cells. In early-passage cultures, however, GFAP expression was found in only a small percentage of cells in two cell lines (SF-539 BT and SF-295), and with progressive subcultivation,

GFAP-positive cells could no longer be identified. At establishment, SF-295 became an antigenically null cell line, and SF-539 BT resembled a spindle-cell sarcoma (226). The absence of GFAP-positive cells in most glioma-derived cell cultures may be explained on the basis of better in vitro adaptability of originally GFAP-negative glial tumor cells; the loss of the ability of originally GFAP-positive cells to synthesize GFAP because of dedifferentiation in the artificial, in vitro environment; or the selective culture advantage of GFAP-negative non-glial reactive or tumor cells (e.g., hyperproliferative vascular cells or sarcomatous elements) present in the original glial tumor.

The limited usefulness of GFAP immunocytochemistry in positively identifying the majority of glial-derived tumor cell lines has prompted the search for additional astrocyte-specific markers. One such marker that is largely confined to astrocytes is glutamine synthetase (157,186,195,234) Glutamine synthetase has been immunolocalized to anaplastic gliomas containing poorly fibrillated cells, to poorly differentiated neural tumors, and to the astrocytic element of different mixed gliomas (195). However, in our analysis we were unable to demonstrate glutamine synthetase immunoreactivity in any of our glioma-derived cell lines. Although no tumor-cell-specific markers have yet been found, several monoclonal antibodies that recognize glioma-associated antigens in a highly restricted pattern of reactivity have been developed (27,31,34,233,297,298). Antibodies such as these may prove useful in further characterizing glioma- derived cell lines.

Most malignant glioma specimens placed into culture will display an initial outgrowth of tumor cells, normal and reactive parenchymal cells, and normal stromal cells that presumably arise from the meninges and vascular connective tissue. That reactive or stromal cells may be established from malignant glioma specimens placed in culture was demonstrated in a study by Manoury (162), in which a cell line inoculated into athymic mice induced a tumor that had features similar to the hyperplastic vasculature of the original glioblastoma multiforme and that synthesized interstitial collagen. Based on these morphological observations, he therefore proposed that this cell line might be derived from vascular rather than glial elements. Rabson et al. (200) reported the synthesis of interstitial procollagens by a glioblastoma cell line; however, because this cell line had the histological appearance of a sarcoma and produced a fibrosarcoma in nude mice, it also might have arisen from

malignant mesenchymal cells present in the original tumor. Other xenotransplantation studies have shown that glioma- derived cell lines may give rise to tumors with sarcomatous rather than glial features (91,299).

In our study, DEAE-cellulose chromatography, SDS-PAGE, and immunocytochemistry demonstrated interstitial collagen synthesis in two cell lines. One of them, SF-539, was derived from a gliosarcoma and has been characterized in vitro as a spindle-cell sarcoma (226); the other, SF-126, was derived from a glioblastoma multiforme that exhibited marked vascular mesenchymal proliferation in vivo. In both tumors, the collagen types were immunolocalized solely to regions of vascular proliferation. If tumor cells in vitro do retain some of the phenotypic characteristics of their tissue of origin (31), then in vitro interstitial collagen synthesis may be a useful marker for mesenchymal cells. Recently, mesenchymal tumors have been identified by their secretion of types I and III collagen (3). Despite claims of the pluripotentiality of primitive neuroectodermal cells (102,136,207,272,296), collagen synthesis by mesenchymal cells from the original tumor specimen provides a simpler and more elegant explanation for our in vivo and in vitro antigen expression data.

Our immunohistochemical analysis of antigen expression showed that fibronectin, laminin, and type IV collagen were present almost exclusively in intratumoral perivascular regions rather than in tumor cells. All our cell lines reacted positively with anti-fibronectin antisera, but immunoreactivity decreased with increasing cell passage. Glioma-derived cell lines have been reported to synthesize fibronectin, especially after serial passage in athymic mice (297). Double immunocytochemical studies have shown that after passage in athymic mice, about 10% of U-251 MG malignant glioma cells contain both fibronectin and GFAP (115). Laminin and type IV collagen were similarly immunolocalized to variable degrees to several of our cell lines. Laminin has been found in GFAP- positive neonatal rat astrocytes (143), and type IV collagen has been found in U-251 MG tumor cells (3) and neuroblastoma cells (4). Since glycoproteins of the basement membrane have also been found in fibrosarcomas, meningiomas (225), and schwannomas, their occurrence in our glioma-derived cell lines neither proves nor disproves the cell line's origin from a glial precursor. Recently, McKeever et al. reported the presence of laminin, fibronectin, types I and IV collagen in two human glioma-derived cell lines (171). However, because neither of

these cultures expressed GFAP (18,24) and because immunohistochemical studies were not performed on the surgical specimens from which these cultures were initiated, the glial origin of these cells lines remains unproved. Proteins of the extracellular matrix appear to play a fundamental role in the antigenicity of glioma-derived cell lines, because monoclonal antibodies to these tumor cells recognize antigens present largely in the intratumoral extracellular spaces (27,168).

We conclude that cell lines that synthesize interstitial collagen are probably derived from the proliferative or transformed mesenchymal cells often found in glioblastomas multiforme. The loss of GFAP expression and other biochemical markers of differentiation of neuroectodermal cells and the expression of proteins such as the collagen types, which are not normally associated with differentiated glia, are evidence of the greater in vitro adaptability of mesenchymal cells in some cell lines derived from malignant gliomas. Therefore, an important conclusion from this study is that glioma cell lines that synthesize interstitial collagens should be excluded from consideration in testing the gliosarcoma hypothesis (Chapter 9).

6.5 Figure Legends and Tables

Fig. 1. Histopathological features of the surgical specimens of tumors from which cell lines SF-188 (A) and SF-126 (B) were derived. Photomicrographs show hypercellularity, and nuclear and cytoplasmic pleomorphism. (A) An example of pseudopalisading in a glioblastoma multiforme is shown in which tumor cells cluster around an area of incipient necrosis (N). H & E x250. (B) Vascular mesenchymal proliferation (V) is shown in this glioblastoma multiforme. A mitotic figure in the glial element is seen (arrow). H & E, x500.

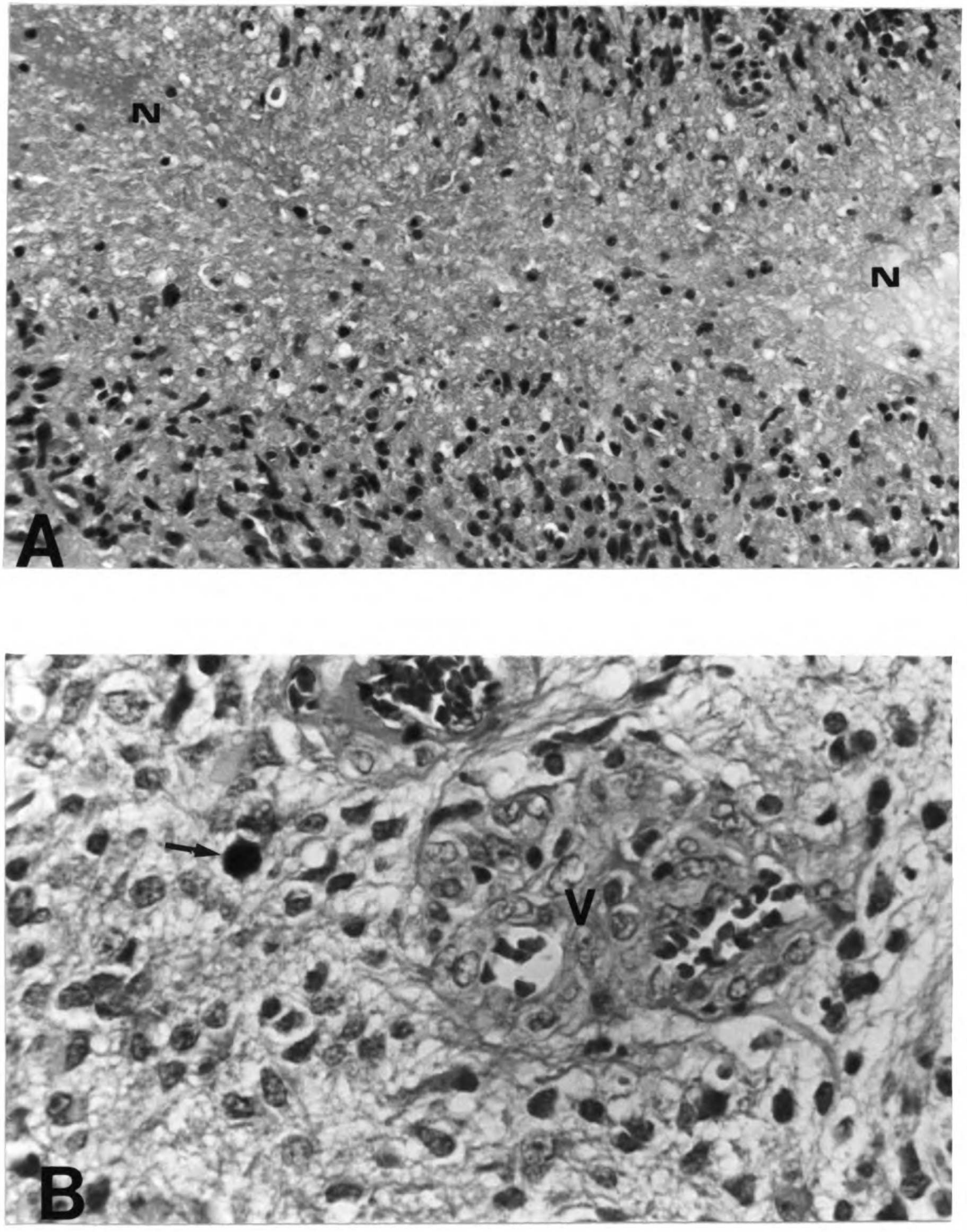


Figure 1 A - B

Fig. 2. (A) Glial morphology of SF-295 tumor cells at passage 3. Most of the cells in this field have slender, long cytoplasmic processes (arrows). Phase microscopy, x500. (B) All cells are positively identified by immunostains for GFAP. However, with progressive subcultivation, GFAP positive cells could no longer be identified in glioma-derived cell line SF-295. Indirect immunofluorescence x500.

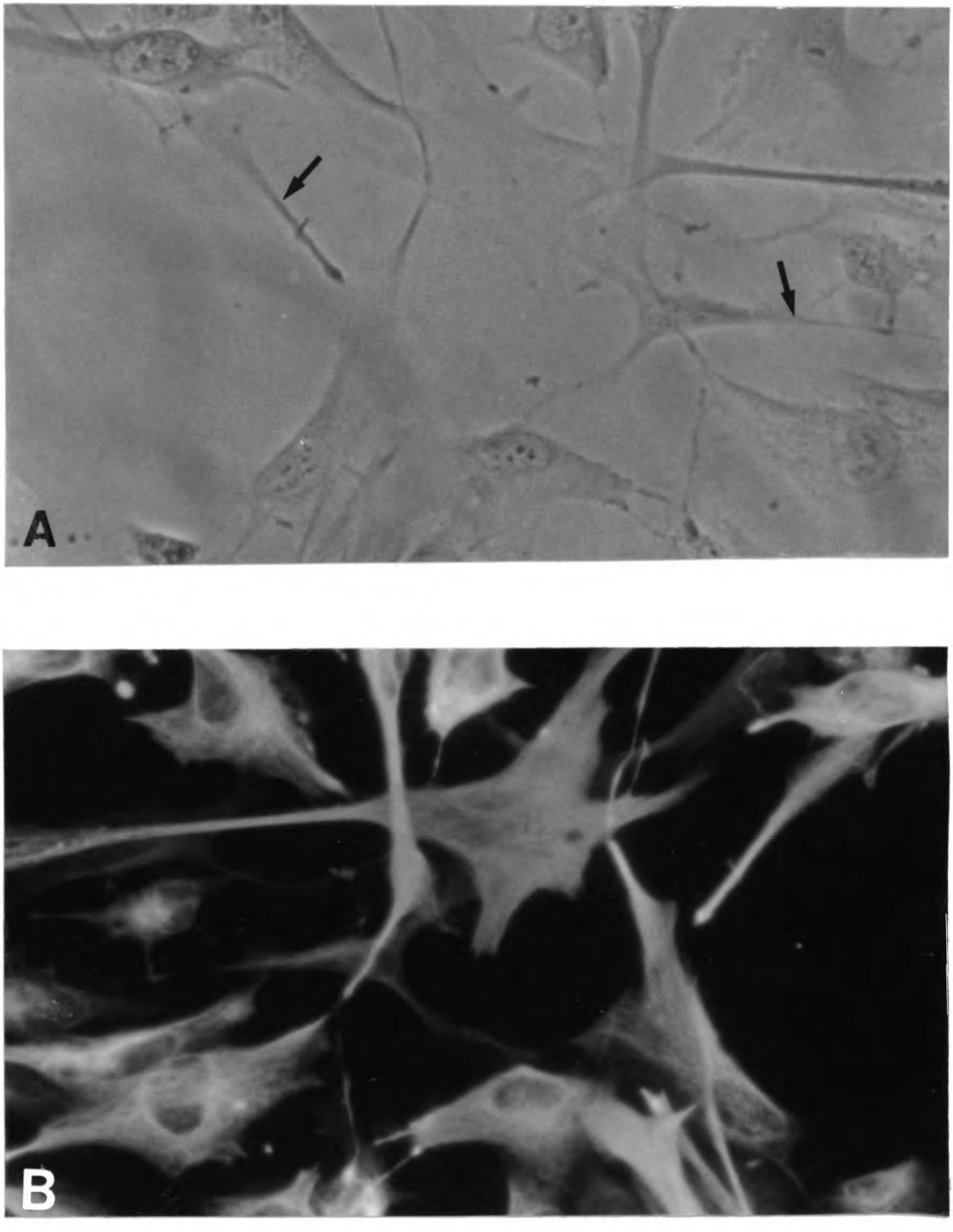


Fig 2 A - B

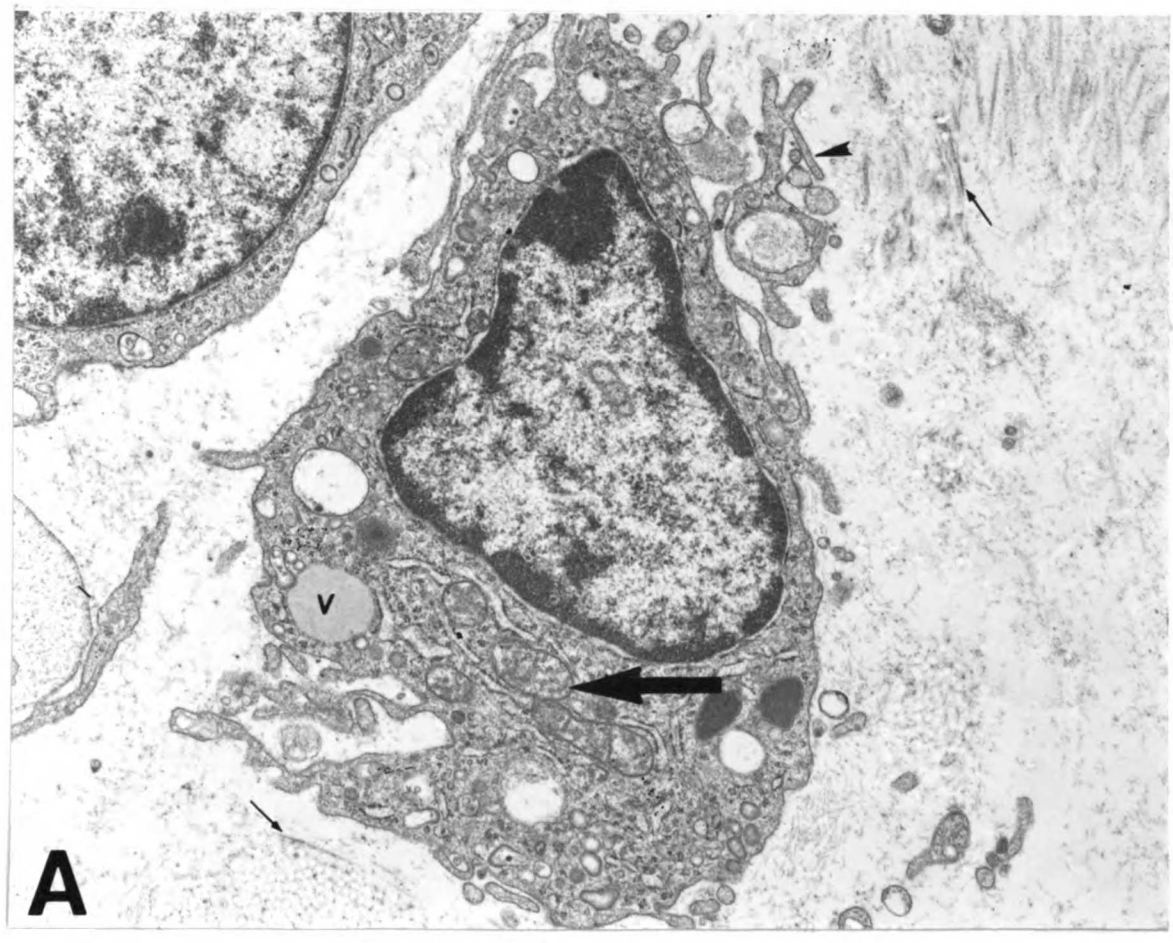


Fig. 3A. Ultrastructural features of glioma-derived cell lines. SF-126 tumor cells display microvilli (arrowheads), distended rough endoplasmic reticulum (large arrows), and vesicular bodies (V). Extracellular collagen formation is shown (small arrows). Electron microscopy x10,000.

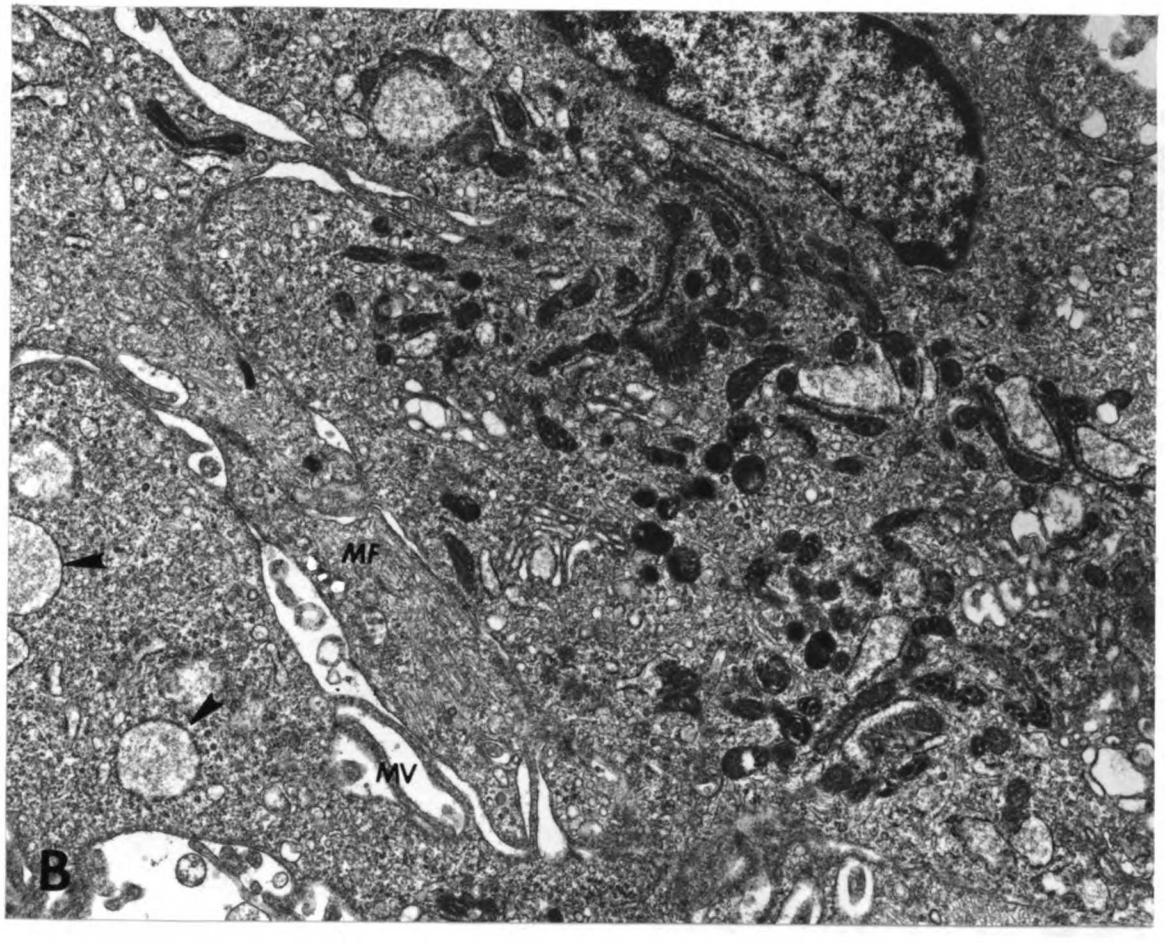


Fig 3B SF-268 tumor cells have distended rough endoplasmic reticulum (small arrows), microfilaments (MF), and prominent microvilli (MV). Electron microscopy x15,000.

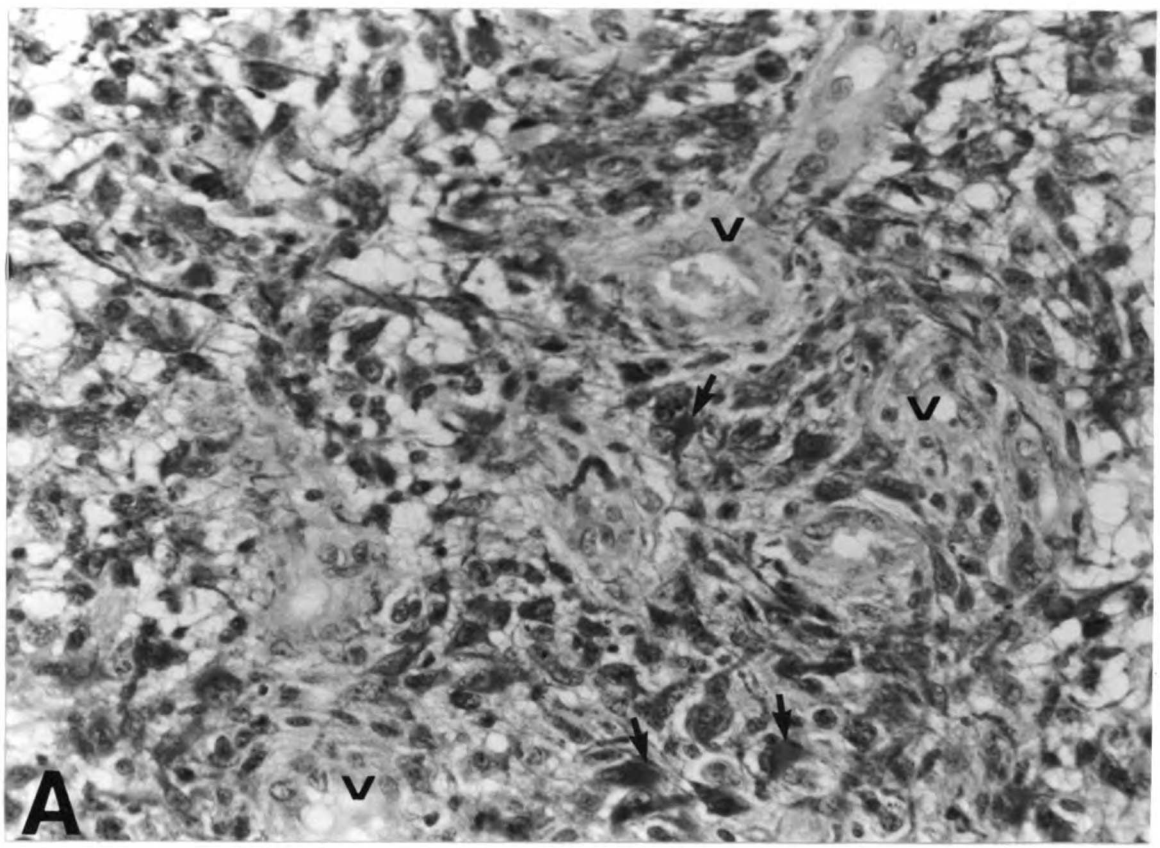
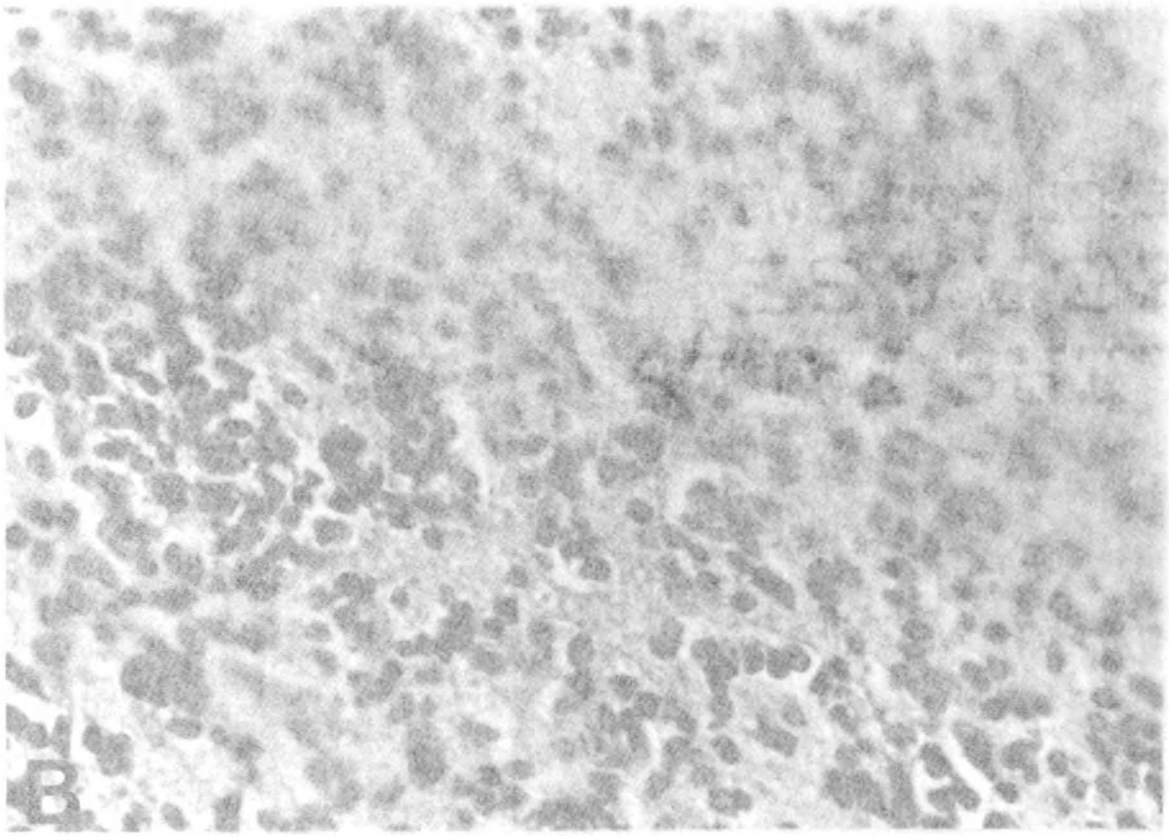


Fig. 4. Immunohistochemical analysis of malignant glioma specimens that gave rise to SF-295 (A), and SF-268 (B). (A) The glial element is richly positive in cells identified with GFAP antiserum (arrows). The vascular mesenchymal (V) regions are unstained.



[illegible] by the GLP antiserum. When [illegible] GLP positive cells in each passage [illegible]

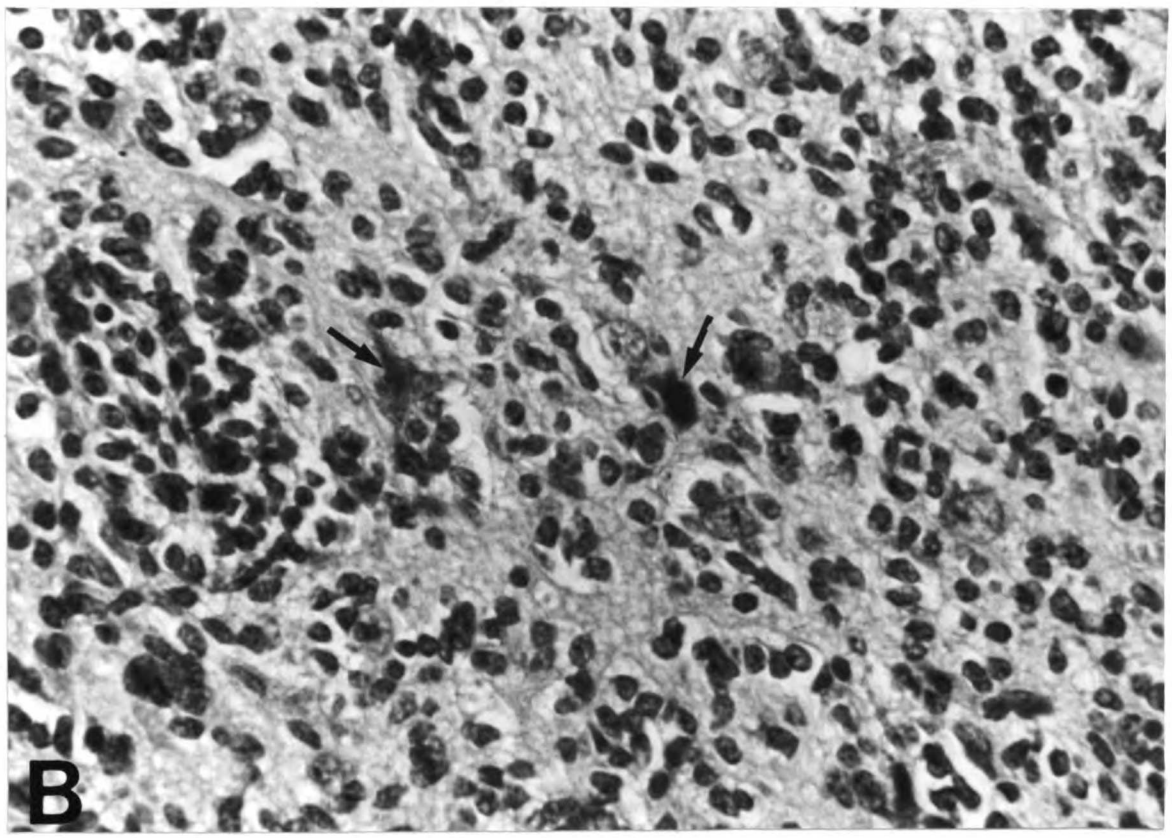


Fig 4B In this hypercellular field, only two cells are positively identified by the GFAP antiserum. When placed into culture, only SF-295 and GFAP positive cells in early passage culture. Light microscopy for both x400.

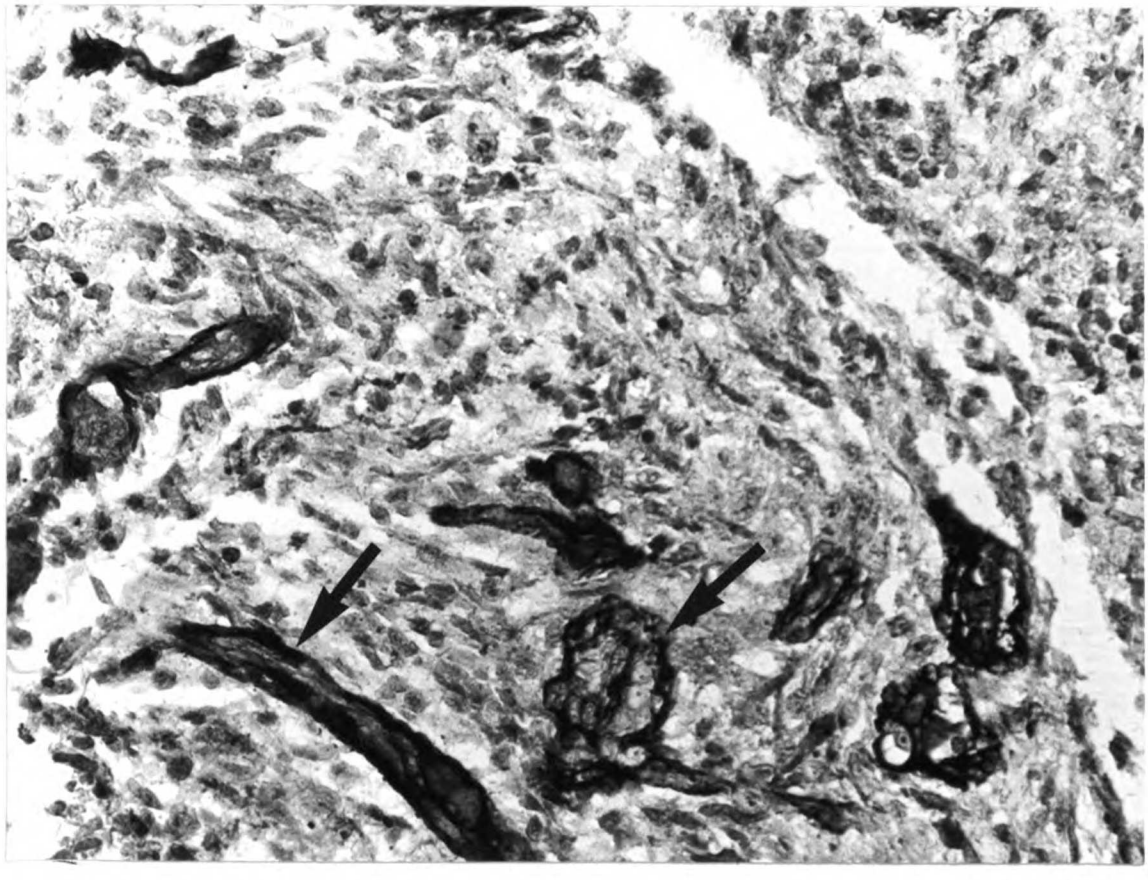


Fig. 5. Immunohistochemical analysis of malignant glioma specimen that gave rise to cell line SF-188. Only the vascular mesenchymal regions are positively immunostained for procollagen III (arrows). Light microscopy x300.

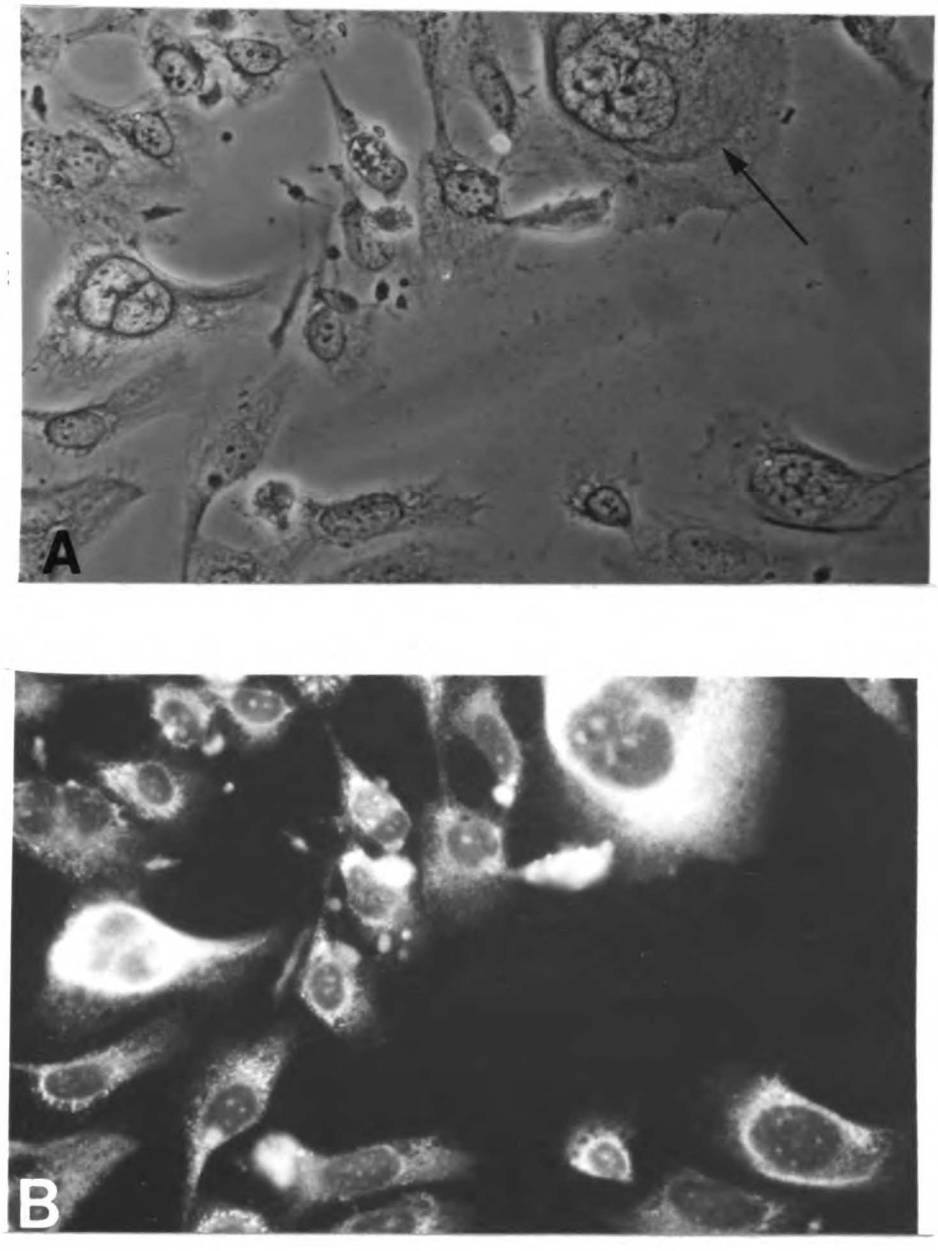


Fig. 6. Immunocytochemical analysis of glioma-derived cell lines. (A) SF-210 tumor cells at passage 8. The cells are flat and polygonal. A giant, multinucleated cell is shown (arrow). (B) SF-210 tumor cells immunostained for laminin. All cells are positively identified by the laminin antisera.

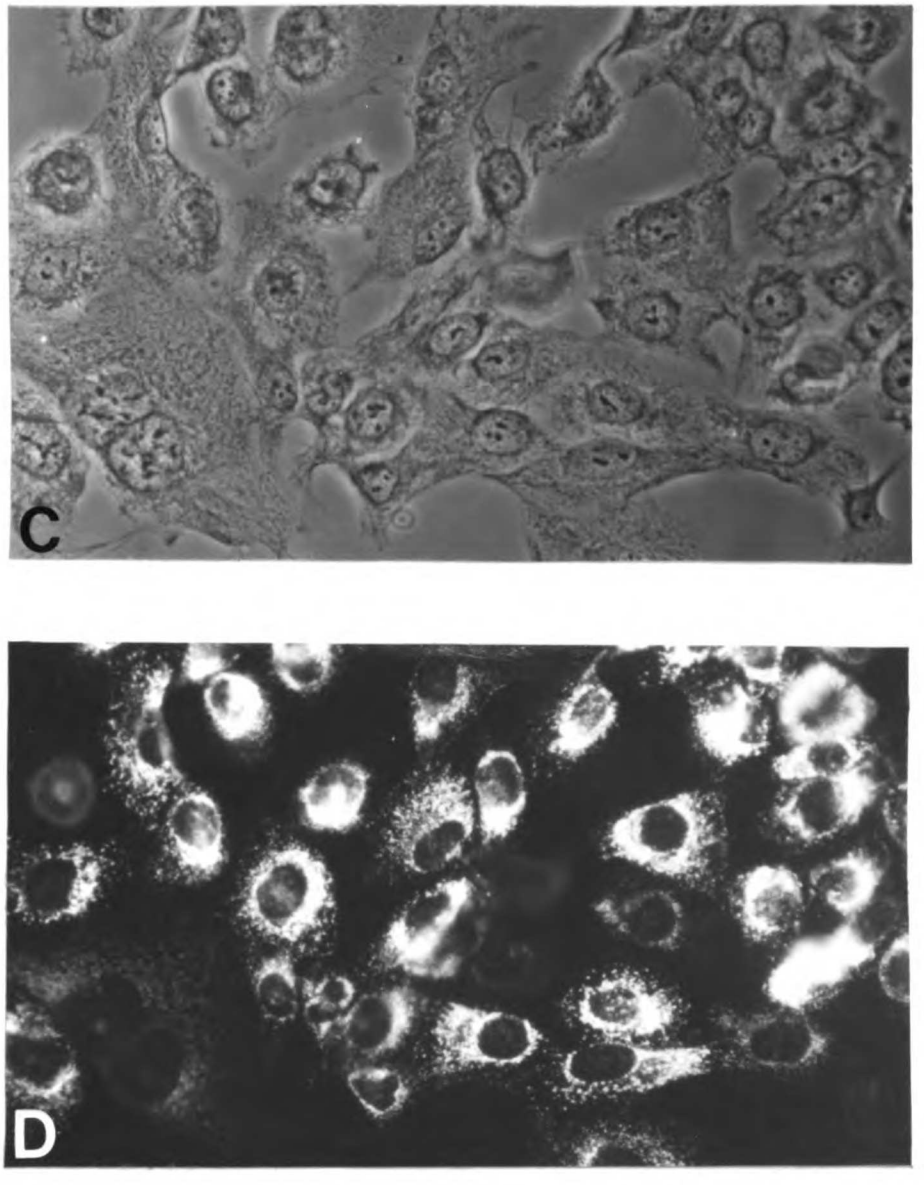


Fig 6C SF-126 tumor cells in culture, passage 57. The tumor cells are flat and polygonal with round nuclei and prominent nucleoli. (D) SF-126 tumor cells immunostained for procollagen III. All tumor cells display positive intracytoplasmic staining for procollagen III. (A) and (C) Phase microscopy x350. (B) and (D) Indirect immunofluorescence x350.

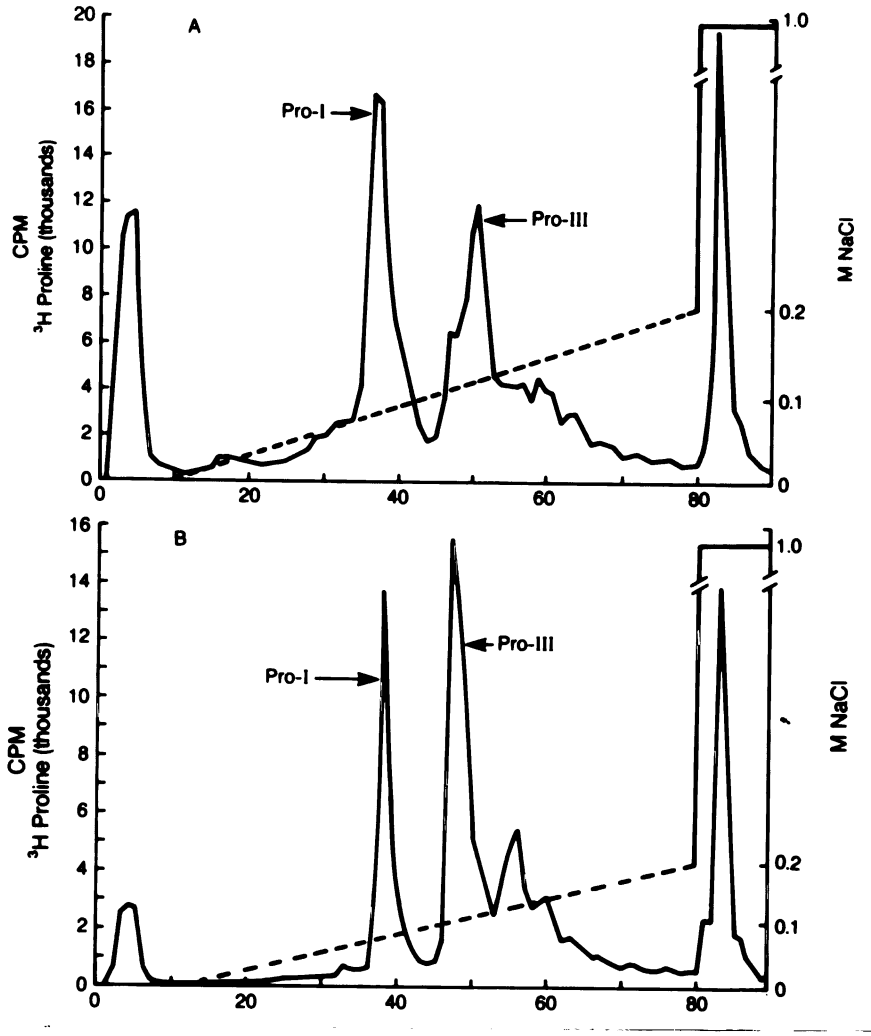


Fig. 7. Procollagen profiles of glioma-derived cell lines as identified by DEAE-cellulose chromatography. (A) The medium of SF-539, passage 55, demonstrates a tall peak for procollagen I and a smaller peak for procollagen III. (B) The medium of SF-126, passage 62, contains a radiolabeled proline peak for procollagen III that is greater than that for procollagen I.

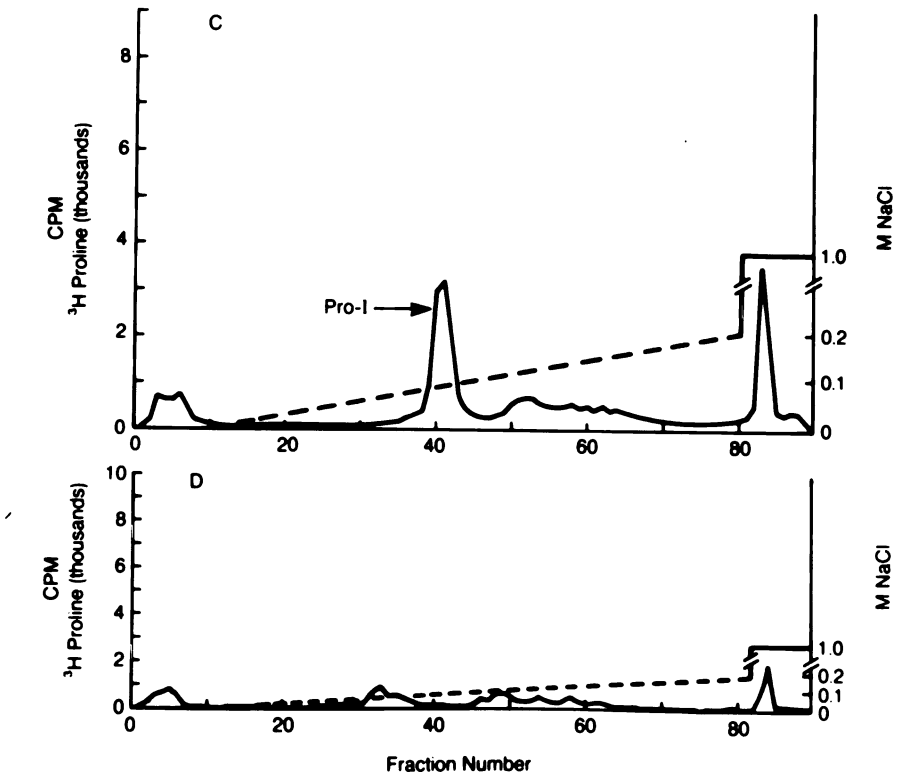


Fig 7(C) The medium of SF-210, passage 7, demonstrates a much smaller peak of activity for radiolabeled procollagen I. A procollagen III profile cannot be discerned. At higher passages, the procollagen I peak was lost from the medium of SF-210. (D) The medium of SF-268, passage 59, did not contain any demonstrable amounts of activity for either procollagens I or III. Absence of interstitial collagens by DEAE-cellulose chromatography was also documented for glioma-derived cell lines SF-188, SF-295, U 343 MG-A, and U 251 MG.

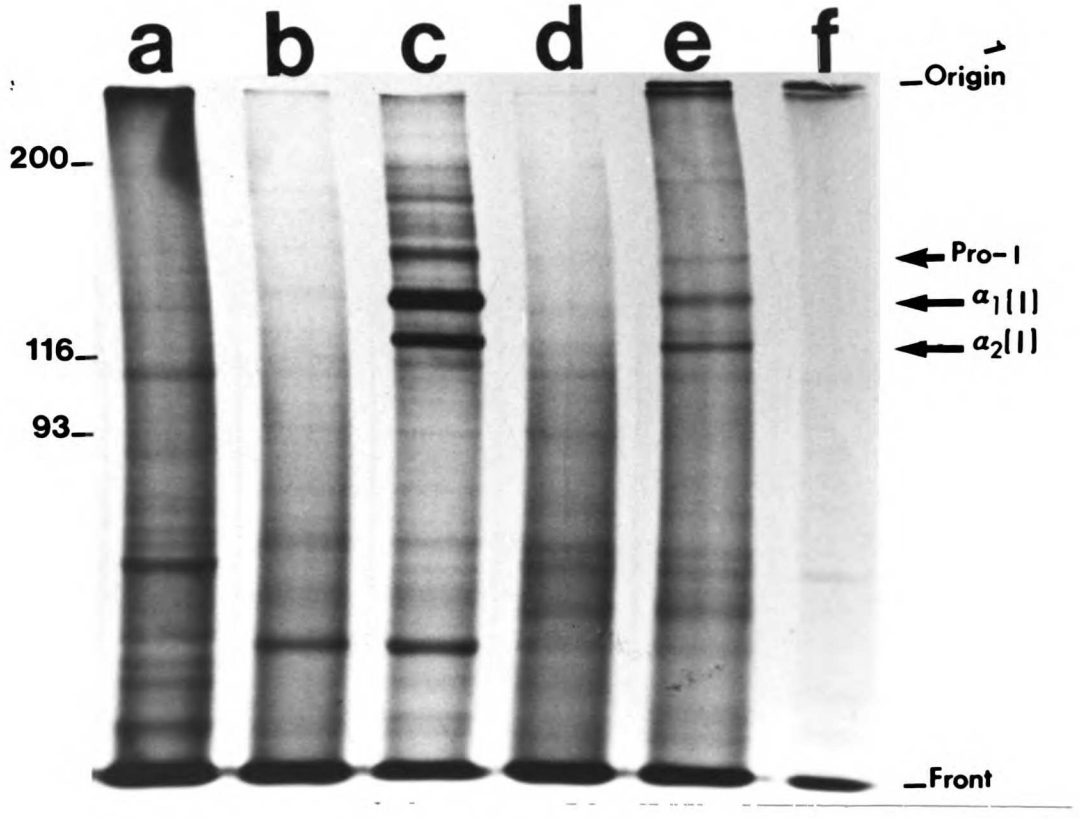


Fig. 8. SDS-PAGE analysis of ^{3}[H]proline-labeled and reduced proteins within the cell layers of the glioma-derived cell lines. Lane a: SF-268; lane b: SF-210; lane c: SF-126; lane d: SF-295; lane e: SF-539; lane f: SF-126 cell layer treated with collagenase, 10 U/ml, at 37 C for 4 hr (clostridium histolyticum, protease free; Calbiochem, San Diego, CA). Bands representing collagen type I (alpha$_1$ (I) and alpha2 (I)) are present only in cell lines SF-126 and SF-539. The procollagen I band is identified above the alpha$_1$ band. All bands are absent in collagenase treated cell layer of SF-126.

Table 1. Patient profile, tumor location and histopathological features of glioma-derived cell lines

Cell line	Patient Age, Sex	Tumor Location	Tumor Diagnosis	Histopathological Features				
				Hyper	Pleomorph	Necrosis	Pseudo	VMP
SF-126	50, F	L frontal	Glioblastoma	+	+	+	+	+
SF-188	8, M	R frontal	Glioblastoma	+	+	+	+	+
SF-210	72, F	L parietal	Glioblastoma	+	+	+	+	+
SF-268	24, F	R parietal	HAA	+	+	-	+	-
SF-295	67, F	L temporal	Glioblastoma	+	+	+	+	+
SF-539	34, F	R temporo-parietal	Gliosarcoma	+	+	+	+	S

HAA = highly anaplastic astrocytoma
Hyper = hypercellularity
Pleomorph = nuclear and cytoplasmic pleomorphism
Pseudo = pseudopalisading
VMP = vascular mesenchymal proliferation
S = sarcoma formation

Table 2. Growth and kinetic characteristics of glioma-derived cell lines

Cell line	Highest Passage	Doubling Time	Saturation Density*	Plating Efficiency		Chemoresponse		Morph	Tumorigenicity
				Monolayer	Agar	Monolayer	Agar		
SF-126	106	29 h	6×10^6	42	2.9	S	S	FB	-
SF-188	141	28 h	7×10^6	40	10	R	R	Epith	-
SF-210	102	28 h	5×10^6	34	0.65	R	R	Epith	-
SF-268	123	32 h	5×10^6	43	0.85	S	S	Fasc	-
SF-295	61	34 h	6×10^6	30	4.43	R	R	Glial	-
SF-539	85	37 h	5×10^6	23	0.2	R	R	FB	+

* = number of cells per 25 sq cm
S = cell line sensitive to BCNU
R = cell line resistant to BCNU
FB = fibroblastic
Epith = epithelial
Fasc = fascicular

Table 3. Ultrastructural features of six glioma-derived cell lines

Cell line	Prominent Nucleolus	MF's	IMF	Microvilli	Cell:Cell Junctions	Plasma Memb Interdigitation	Collagen Fibrils
SF-126	+	+	-	-	+	+	+
SF-188	+	+	-	+	+	-	-
SF-210	+	+	+	+	-	-	-
SF-268	+	+	+	+	+	-	-
SF-295	+	+	+	-	-	-	-
SF-539	+	+	+	+	-	+	+

MF = microfilament
IF = intermediate filament

Table 4. Immunocytochemical characterization of six glioma-derived cell lines

Cell line	Passage	GFAP	Glutamine Synthetase	VIII:RAg	Fibronectin	Laminin	Collagen Type IV	Procollagen III
SF-126	13	-	-	-	+++(100)	++(100)	+(50)	+(5)
	58	-	-	-	++(100)	++(100)	++(100)	+++(100)
SF-188	13	-	-	-	+(100)	+(100)	+(90)	+(5)
	68	-	-	-	+(100)	+(100)	++(100)	-
SF-210	10	-	-	-	++(100)	+(100)	++(90)	+(2)
	46	-	-	-	+(100)	+(100)	+(20)	-
SF-268	5	-	-	-	++(100)	+(100)	+(50)	+(10)
	49	-	-	-	-	+(5)	+(1)	-
SF-295	3	+++(50)	-	-	++(50)	++(50)	+(10)	-
	22	-	-	-	-	-	-	-
SF-539	1	++(12)	-	-	++(85)	++(85)	+(85)	++(40)
	42	-	-	-	++(100)	++(100)	+(80)	++(25)
U-251 MG	406	+++(85)	-	-	+(100)	++(100)	-	-
U-373 MG	118	++(50)	++(100)	-	+(100)	+(100)	-	-

Intensity of staining: - = negative; + = low; ++ = moderate; +++ = high.

Numbers in parentheses indicate percentage of cell staining positively.

GFAP = glial fibrillary acidic protein
VIII:RAg = factor-VIII-related antigen

CHAPTER 7

DISTRIBUTION OF EXTRACELLULAR MATRIX PROTEINS IN PRIMARY HUMAN BRAIN TUMORS: An Immunohistochemical Analysis

7.1 Introduction

The antigen expression data presented in Chapters 2 - 6 were derived largely from in vitro experimentation. The validity of such data is strengthened by correlating observations from tissue culture systems with the antigenic characteristics as they occur in vivo. In the present study, we examined a series of primary human brain tumors and normal brain specimens for the distribution of ECM macromolecules. The monoclonal antibodies and antisera used in this study were the same as those described for the monolayer culture system (Section 2.2.2). In addition, since it is becoming apparent that several soluble polypeptide factors, including the transforming growth factors, alter ECM deposition in normal and pathological states (110,213), we sought to determine the distribution and relative amounts of ECM macromolecules in normal human and in primary brain tumors

The ECM may be defined as the naturally occurring extracellular substrate upon which cells migrate, proliferate, and differentiate in vivo (87,88). The ECM functions as a biological adhesive that maintains the normal cytoarchitecture of different tissues and defines the key spatial relationships among dissimilar cell types (206). Outside the CNS, the ECM plays a fundamental role in modulating tumor cell invasion and metastasis (265,266) Although much has been learned about the biochemical composition and structure of the ECM in other organs, little is known of its structure and function in the CNS (30,32). We undertook this study to characterize the distribution of laminin, collagen type IV, fibronectin, and

procollagen type III in primary human brain tumors and to determine which cells may be involved in the production of these ECM proteins.

7.2 Materials and Methods

7.2.1 Monoclonal Antibodies and Antisera

A panel of monoclonal antibodies and antisera was used to localize ECM proteins in the tissue sections. Lyophilized antibody against laminin and fibronectin, raised in rabbits, was a gift from Dr. H. Kleinman (National Institute of Dental Research, NIH, Bethesda, MD). Monoclonal antibodies to procollagen type III and collagen type IV were donated by Dr. N. SundarRaj (University of Pittsburgh, Pennsylvania). Brain tumors of glial anlage were immunohistochemically identified by a rabbit anti-human antiserum to glial fibrillary acidic protein (GFAP), which was a gift from Dr. L. Eng (Stanford, CA). Cerebrovascular endothelial cells were stained by a rabbit anti-human Factor-VIII-related antiserum (Dako, Westbury, NY). The specificity of all antisera and monoclonal antibodies used in this study have been reported in detail elsewhere (225, Section 2.2.2).

7.2.2 Tumor Selection and Tissue Preparation

Thirty-eight tumors and 4 normal brain specimens were selected for study (Table 1). The gliomas were classified using criteria established at the University of California, San Francisco (UCSF) (147). A glioblastoma multiforme is a highly cellular glial neoplasm with nuclear and cytoplasmic pleomorphism and vascular endothelial proliferation. A highly anaplastic astrocytoma is a moderately to highly cellular glial neoplasm with at least two of the following characteristics: a high nuclear:cytoplasmic ratio, coarse nuclear chromatin, mitotic activity, and nuclear or cytoplasmic pleomorphism. A moderately anaplastic astrocytoma is a mildly to moderately cellular glial neoplasm with enlarged nuclei and a relatively uniform cytoplasm. GFAP was used as a marker to identify tumors of glial origin.

For comparison with the tumor specimens, samples of normal human brain were obtained from adult patients undergoing routine craniotomies for trauma or intractable seizures and from normal fetuses electively aborted at 12-20 weeks' gestation. Permission to obtain the fetal brain specimens was

granted by the Human Research Committee of the University of California, San Francisco.

All specimens were fixed for 24 hours at 0-4 C in Carnoy's solution (164) or in 10% neutral buffered formalin (167). The paraffin-embedded tissue blocks were cut into sections 5.5 um thick, mounted on glycerin-coated glass slides, deparaffinized, and rehydrated. Sections fixed in Carnoy's solution were pretreated with 0.05% collagenase (Sigma type I, C0130) in 0.05% $CaCl_2$, pH 7.4 (164) and those fixed in formalin were pretreated with 0.4% pepsin (Sigma) in 0.01 N HCl for 45 minutes at 37 C before immunostaining (167). Tissue sections immunostained for GFAP were not pretreated with proteolytic enzymes.

7.2.3 Immunohistochemical Staining

The intracytoplasmic and ECM proteins were immunostained with the PAP technique of Sternberger (257). For the primary antibodies raised in rabbits, PAP kit K548 (Dako) was used; for mouse monoclonal antibodies, PAP monoclonal kit K660 (Dako) was used. Primary antibodies (anti-GFAP, -fibronectin, -Factor-VIII-related antigen, and -laminin diluted 1:200; anti-procollagen type III and -type IV collagen diluted 1:100) were allowed to incubate overnight at 4 C. The slides were carefully rinsed in PBS after each step. The sections were then counterstained with hematoxylin and mounted with glycerol gelatin (Sigma). Immunostaining of ECM proteins in blood vessels in tumor sections served as an internal positive control. In control sections, the primary antibody was replaced with either non-immune rabbit serum (Dako) or ascites fluid (Cappel) at the same dilution as the primary antibody.

7.3 RESULTS

The difference in fixation technique did not affect the intensity of staining. However, preincubation of the specimens with the appropriate proteolytic enzyme was necessary to localize and enhance the various ECM reaction products. All 42 specimens were positively immunostained to various degrees by all of the antisera and antibodies to the ECM proteins. Staining was most consistent for laminin, type IV collagen, and fibronectin and was most variable for procollagen type III. Factor- VIII/vWF-related

antigen distinctly stained endothelial cells in all blood vessels; the subendothelial basement membrane and the perivascular regions stained less intensely.

7.3.1 Normal Brain

In the adult human brain specimens, laminin and type IV collagen were identically distributed in the basal lamina of cerebral parenchymal and leptomeningeal blood vessels (Fig. 1) and in the glial limitans externa. The procollagen type III reaction product was noted chiefly in the fibromuscular compartment of the larger arterioles and arteries of the leptomeninges and brain. Corpora amylacea in one specimen were positively identified by immunostains for fibronectin. In the fetal brain specimens, the distribution of all ECM macromolecules was similar to that of adult brain. In addition, the vascular and epithelial basement membranes of the choroid plexus stained positively and perivascular immunostaining for the various glycoproteins was observed even in the paraventricular germinal layer (Fig. 2).

7.3.2 Glioblastoma Multiforme

The most striking aspect of all 10 glioblastomas was the intratumoral vascularity, which was identified by antisera or antibodies to all ECM macromolecules (Fig. 3). Staining was particularly intense in thick-walled, hyperplastic blood vessels and in glomeruloid vascular formations. There was only occasional fragmentation of the endothelial basement membrane (Fig. 4). The glial limitans externa was also identified in all specimens and was similarly intact. Laminin and collagen type IV were similarly distributed along the endothelial basal laminae, but some less intense staining was seen beyond the basement membrane. Fibronectin was identified throughout all layers of blood vessels; some thick-walled vessels had a laminated appearance (Fig. 5). Procollagen type III was also immunolocalized to all layers of proliferated vessels. Immunostaining showed no evidence of ECM glycoproteins between glial tumor cells.

7.3.3 Gliosarcoma

Gliomatous regions of the three gliosarcomas were positively identified by immunostains for GFAP. Giant cells were negative for GFAP in two cases and positive in one. Sarcomatous regions were positively identified by immunostains for fibronectin, laminin, type IV collagen, and procollagen

type III in two tumors (Fig. 6). Granular, intracytoplasmic staining for laminin and fibronectin was present in the cytoplasm of some cells in the sarcomatous portion of one tumor.

7.3.4 Other Gliomas

The staining pattern for all ECM molecules in highly anaplastic gliomas was similar to that seen in glioblastomas multiforme. Vascular mesenchymal immunostaining was not as prominent. Both the glial limitans externa and the subendothelial basement membranes were intact. A moderately anaplastic astrocytoma was found to have a narrow, slit-like Virchow-Robin space that contained a moderately large arteriole with a lymphocytic infiltrate (Fig. 7). None of the gliomas, including the ependymomas and the oligodendrogliomas, stained positively for any of the ECM proteins except in perivascular regions.

7.3.5 Medulloblastoma

In both medulloblastomas, ECM proteins were found in a perivascular distribution. In one tumor in which a desmoplastic response had occurred, strands of fibrous connective tissue were positively identified by antisera or antibodies to all of the ECM proteins.

7.3.6 Meningioma

All ECM proteins were positively identified by immunostaining in all eight meningiomas. None of these tumors stained positively for GFAP. The intrinsic vascularity of the meningiomas was extremely well delineated by immunostains for the ECM proteins. In addition, intracytoplasmic staining of laminin and fibronectin was seen in one malignant and two transitional meningiomas (Fig 8). Intercellular spaces in whorl formations and psammoma bodies were positively identified by antisera to fibronectin in a meningotheliomatous meningioma.

7.4 Discussion

The extracellular space of the CNS has yet to be completely characterized. Whereas the parenchyma of the CNS appears to be filled with a relatively amorphous matrix that is largely free of collagens and other fibrous proteins (30,32,243), a well-defined ECM exists in the form of a true

basement membrane around all cerebral blood vessels and at the glial limitans externa (32). As a delimiting basement membrane, the glial limitans externa invests the entire cortical surface of the brain and separates astrocytic foot processes from pia-arachnoid cells. Thus, it forms an interface between cellular CNS elements derived embryologically from neuroepithelium and leptomeningeal elements presumably derived from the neural crest. In other tissues outside the CNS, a loss of coordination and an alteration in the interactions between mesenchymal cells and epithelial cells across a basement membrane are thought to be fundamental steps in the development and progression of cancer (174).

In most tissues, the ECM is composed of various types of collagen, noncollagenous glycoproteins such as laminin and fibronectin, glycosaminoglycans, and proteoglycans (98,128,279,300). Basement membranes are continuous ECMs consisting of type IV collagen, laminin, fibronectin, entactin, and heparan sulfate proteoglycan (128). Immunohistochemical studies of normal and pathological human CNS specimens have shown that ECM proteins are predominantly deposited at the junction between glial and mesenchymal elements (13,85,125,191,232).

Conventional histological stains such as reticulin and alcian blue do not accurately define the biochemical composition of the ECM because they recognize only large aggregate mixtures of ECM components. With monoclonal antibodies and antisera that identify individual ECM proteins, however, biochemically complex structures such as the glial limitans externa can be completely characterized (168). Another important advance in the immunohistochemical analysis of the ECM was the discovery that proteolytic enzymes can restore the antigenicity of formalin-fixed, paraffin- embedded tissues (60,106164). As a fixative, formalin links proteins by intermolecular bonds that render antigen binding sites temporarily inaccessible (228). Incubation of tissue sections with weak solutions of pepsin, trypsin, or pronase before immunostaining enhances staining of the ECM (60,164) without affecting cell preservation or increasing background staining. Incubation with proteolytic enzymes has also consistently enhanced the immunostaining of CNS specimens fixed in Carnoy's solution, even though it is not a cross-linking fixative (164,232).

Laminin has been immunolocalized to hyperplastic blood vessels and glomerular vascular formations in gliomas (80,164), in the cytoplasm of the

sarcomatous elements of some gliosarcomas (232), and in the vasculature and fibrillary extracellular spaces of some meningiomas (85,167). Immunostaining for laminin has shown that the glial limitans externa and the subendothelial basal laminae of blood vessels are almost always intact, even in the most anaplastic gliomas (85,167). In addition to this pattern of laminin immunoreactivity, in our study we were able to demonstrate a laminin reaction product in the cytoplasm of meningioma cells. The finding of laminin in the sarcomatous portion of gliosarcomas in vivo and in vitro (226) suggests that regardless of their histological origin, sarcoma cells synthesize rather than phagocytose laminin, as was previously hypothesized (232).

In the CNS, fibronectin has been immunolocalized to the gliomesenchymal junction in vivo (42,125,164,191,231). In addition to staining of all layers of both normal and tumor-associated blood vessels, the glial limitans externa, the leptomeninges, and meningioma cells in whorl formations, we demonstrated intense fibronectin immunoreactivity at the border between gliomatous and sarcomatous elements in a gliosarcoma and in the copora amylacea of a normal brain specimen.

The interstitial collagens, types I and III, have been immunolocalized to the leptomeninges, the fibromuscular coats of large cerebral blood vessels, and the glial limitans externa (13,243). Because of the ubiquity of the interstitial collagens in various tissues throughout the body, types I and III collagens tend to be weak immunogens (269). We therefore used a monoclonal antibody to procollagen III, directed to the aminopropeptide portion of type III collagen, to enhance the sensitivity of staining for type III collagen in the CNS. Procollagen III was found in blood vessel walls, in the leptomeninges, and in the sarcomatous element of the gliosarcoma.

Using an immunofluorescence analysis, Bellon, et al (13) localized type IV collagen to the subendothelial basement membrane of blood vessels in gliomas and meningiomas. The antibody to type IV collagen produced a linear staining pattern in capillaries and large vessels. Type IV collagen and laminin were localized to the same areas. Our results are similar to those of Bellon et al (13). In addition, we found that neuroepithelial derivatives (glia, neurons, glioma cells) never stained positively, even in the fetal brain specimens, and that the glial limitans externa identified by the monoclonal

antibody to type IV collagen remained intact even in the most malignant gliomas.

The integrity of basal laminae in the CNS, even in large malignant tumors, is in some ways contrary to the currently accepted paradigm of malignant cell behavior. Tumor invasiveness is a function of the ability of malignant tumor cells to transgress normal tissue barriers such as basement membranes (71,114). For example, at the transition from in situ to invasive carcinoma, local dissolution of the epithelial basement membrane can be observed microscopically and coincides with tumor cell invasion of the underlying stroma (71). Tumor cells that metastasize hematogenously must cross the endothelial basement membrane during entry into and egress from blood vessels. The success of malignant tumor cells in penetrating ECMs such as basement membranes, which are resilient mechanical barriers to invasion, depends in part on the ability of these cells to produce specific proteases (128,146). For poorly understood reasons, malignant gliomas rarely metastasize extracranially (36,236,252). In only 10-12% of cases does glioblastoma multiforme spread into cerebrospinal fluid pathways; in such cases, spread occurs as a result of ventricular or leptomeningeal involvement (113).

Many epithelial tumors are often associated with striking alterations in peritumoral ECM (283). When host stromal cells synthesize increased quantities of collagen and other ECM macromolecules in response to an adjacent invasive tumor, this process is called desmoplasia. Desmoplasia is a property peculiar to some epithelial malignancies such as breast and colon cancers, and certain neuroepithelial tumors such as medulloblastoma and glioblastoma multiforme. As demonstrated in this study, the medulloblastoma and glioblastoma are often highly enriched in stromal elements which have proliferated within or around the neoplasm and have laid down an exuberant ECM. In the Chapter 9 of this thesis, experimental data will be presented which will demonstrate some of the effects of soluble factors isolated from malignant glioma cell lines on normal mesenchymal cells.

The relative contributions that the tumor and different normal cell populations make toward the desmoplastic reaction in vivo are not known. However, there have been a number of in vitro studies in which the influence of tumor cells on normal stromal cell proliferation and ECM

production have been measured (11,112,173) Merrilees and Finlay (173) and Iozzo (112) have shown that a variety of tumor cells release polypeptides which act to alter and stimulate glycosaminoglycan and proteoglycan synthesis in normal mesenchymal cells in culture. One interpretation of these data is that the tumor cells "trick" the normal mesenchymal cells into depositing large quantities of extracellular proteins within the tumor matrix which serve to facilitate subsequent tumor cell proliferation and invasion.

The cellular controls by which tumor cells modulate collagen synthesis are also poorly understood. However, Bano et al (11) have shown that rat mammary adenocarcinoma cells secrete factors with molecular weights of 6 and 68 kD that stimulate net collagen production. And Roberts et al (213) have recenltly demonstrated that mesenchymal cells treated with TGF-beta can be selectively stimulated to incorporate proline for the synthesis of collagen. In this study, TGF-beta also induced local fibrosis and angiogenesis when injected subcutaneously into rats. In another study, TGF-beta was shown to increase fibronectin and collagen production among fibroblasts (110). Since TGF-beta has been isolated and purified from a number of tumors, it appears plausible that, through a paracrine phenomenon, TGF-beta may be causally related to the desmoplastic response seen in several tumors.

In summary, the immunohistochemical data obtained in this study suggest that collagenous and noncollagenous glycoproteins of the ECM in the normal CNS are found predominantly at sites of contact between neuroepithelial and mesenchymal or leptomeningeal elements. Which subclass of cells in the CNS synthesize and deposit the various ECM components in vivo remains an unanswered question; however, there is evidence that, at least in vitro, both immature astrocytes and normal adult leptomeningeal cells can synthesize basement membrane proteins (143,228). In addition, we have demonstrated that the cytoplasm of certain meningioma cells is positively identified by antibodies to laminin and fibronectin, which strongly implies that these tumor cells produce laminin and fibronectin. Finally, we have confirmed that the integrity of the glial limitans externa is often preserved, even in the most malignant gliomas. Further studies that focus on the distribution of other major components of the ECM such as the glycosaminoglycans and proteoglycans will be of great value in determining the role of this structure in the CNS under normal and pathological conditions.

(Part of the material in this Chapter has been previously published in The Canadian Journal of Neurological Sciences 14: 25-30, 1987. Permission to reproduce these data here was granted by the Canadian Congress of Neurological Scientists - see Appendix)

7.5 Figure Legends and Tables

Figure 1 Immunolocalization of type IV collagen in normal adult cerebral cortex. The thin-walled subendothelial basal laminae of the cortical capillaries are positively stained (large arrows). Neurons (small arrows) and intervening neuropil are unstained. Light microscopy, x 200.

Figure 2 Paraventricular germinal layer of human fetal brain, gestational age 18 weeks, stained for fibronectin. This densely cellular region demonstrates diffuse fibronectin positivity in all layers of the blood vessel walls (arrows). Light microscopy, x 200.

Figure 3 Immunostain for laminin clearly delineates the vascularity of a glioblastoma multiforme. The endothelial basement membrane in all large and small vessels is continuous and intact (arrows). Tumor cells are not stained. Light microscopy, x 75.

Figure 4 Immunostain for laminin in a glioblastoma multiforme identifies a thick-walled hyperplastic blood vessel (curved arrow) and a fragmented endothelial basement membrane (straight arrow) of a thin-walled blood vessel cut longitudinally. Light microscopy, x 500.

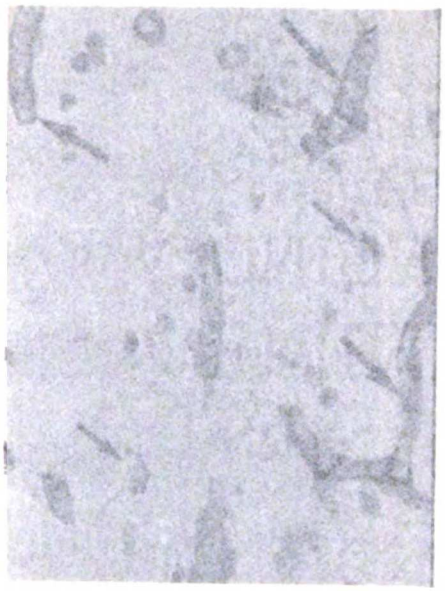


Figure 1

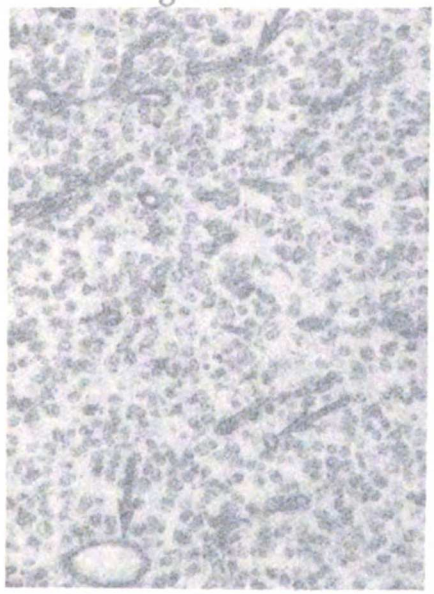


Figure 3

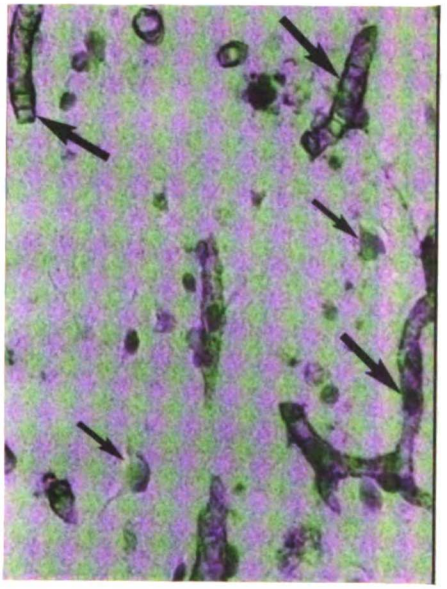


Figure 1

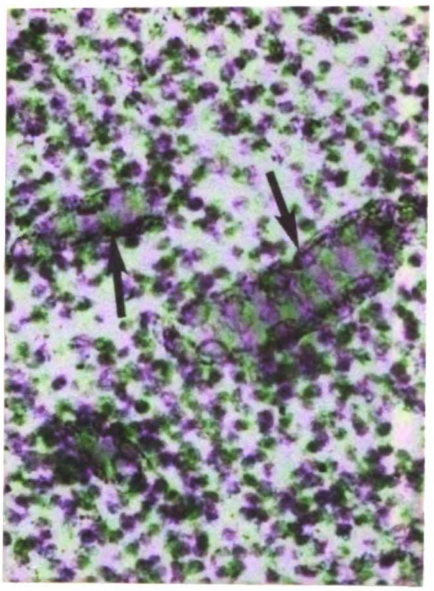


Figure 2

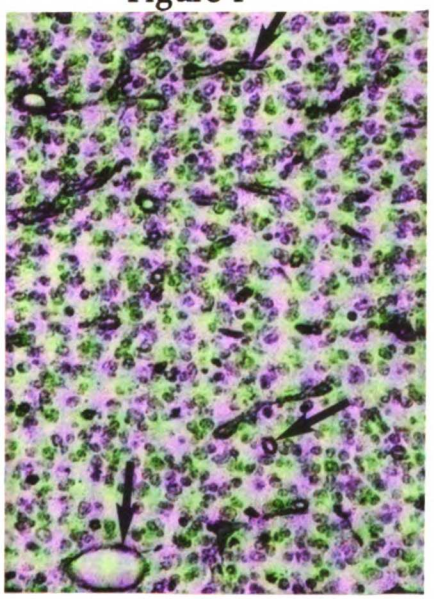


Figure 3

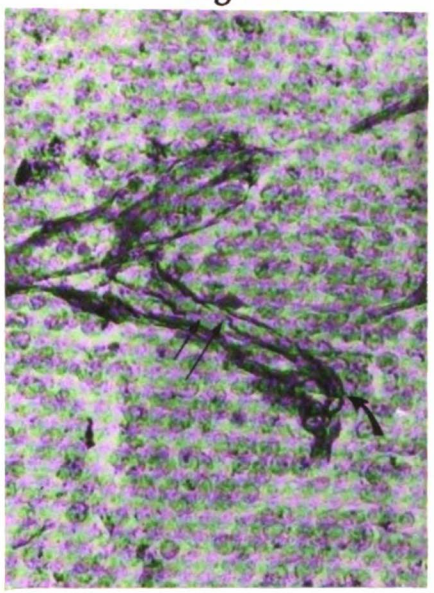


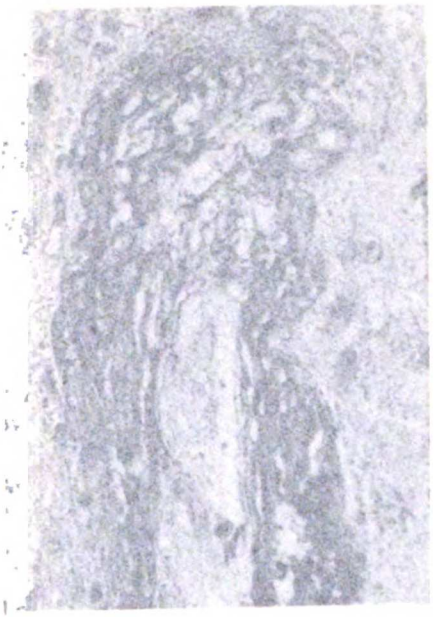
Figure 4

Figure 5 Immunostain for fibronectin in a glioblastoma. The entire thickness of a large, tangentially sectioned blood vessel is stained positively and is composed of several laminae. Light microscopy, x 200.

Figure 6 Immunostain for procollagen III in a gliosarcoma shows that sarcomatous regions (S) are separated from gliomatous regions (G) and stain positively for procollagen III. Light micrscopy, x 75.

Figure 7 Immunostain for laminin in a moderately anaplastic astrocytoma identifies the subendothelial basement membrane of a longitudinally sectioned blood vessel (large arrow) and the glial limitans externa (small arrow). The glial limitans externa is intact. The Virchow-Robin space contains several lymphocytes (arrowheads). The tumor cells and intratumoral regions are unstained. Light microscopy, x 200.

Figure 8 Meningiotheliomatous meningioma immunostained for laminin. The subendothelial basement membrane of the blood vessels (curved arrows) and the cytoplasm of the meningioma cells are positively identified in a whorl formation (straight arrows). Light microscopy, x 200.



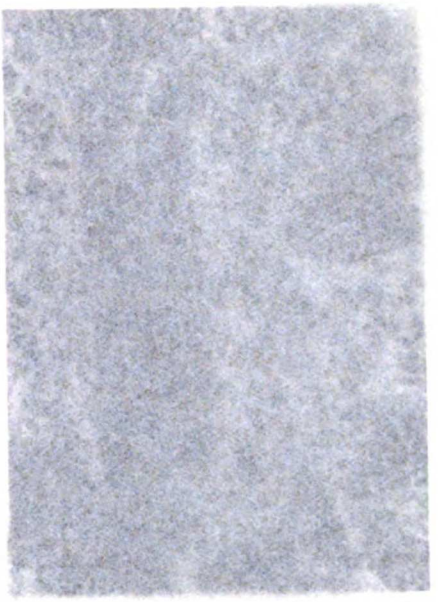


Figure 5

Figure 7

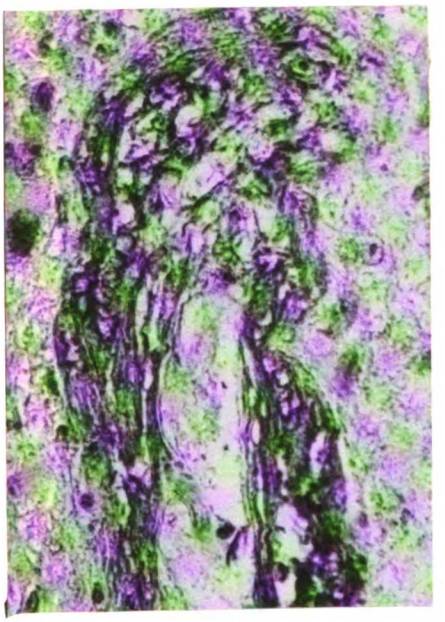


Figure 5

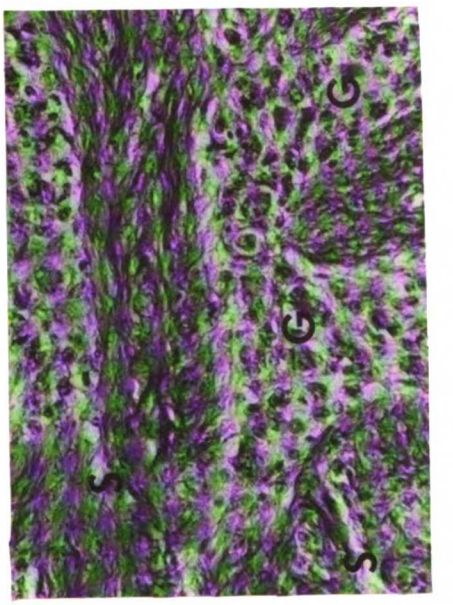


Figure 6

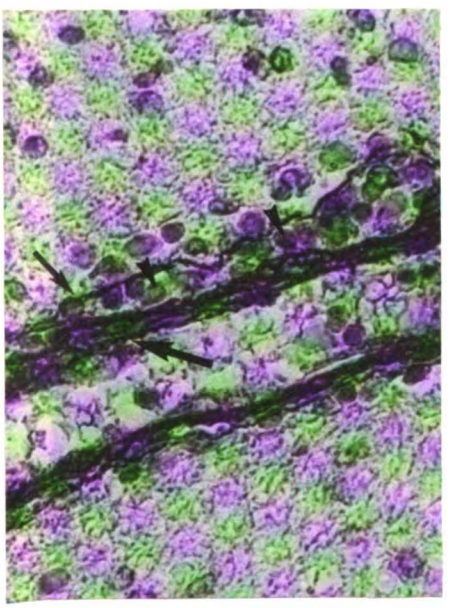


Figure 7

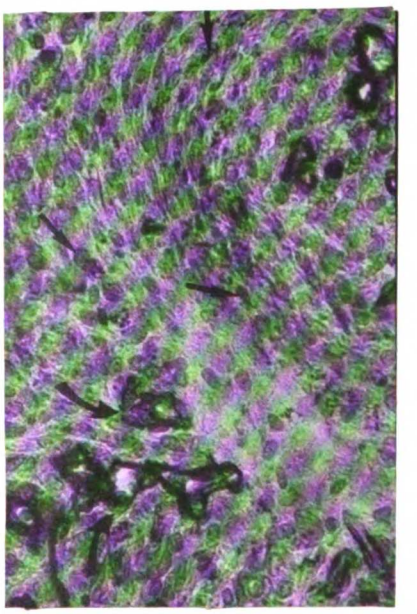


Figure 8

Table 1: CNS tumors and normal brain specimens examined for extracellular matrix protein expression.

Histopathological Diagnosis	No.
Glioblastoma multiforme	10
Highly anaplastic astrocytoma	6
Moderately anaplastic astrocytoma	3
Gemistocytic astrocytoma	2
Gliosarcoma	3
Oligodendroglioma	2
Ependymoma	2
Medulloblastoma	2
Meningioma	6
malignant	2
Normal human brain	
adult	2
fetal	2
Total	42

CHAPTER 8

MITOGENIC EFFECTS OF TRANSFORMING GROWTH FACTORS ON HUMAN MALIGNANT GLIOMA CELL LINES AND NORMAL HUMAN BRAIN CELLS IN CULTURE

8.1 Introduction

Growth factors are soluble polypeptides which act in part by their ability to bind to specific cell surface receptors in target cells. The binding of a particular growth factor to a receptor is thought to elicit a signal that transduces the plasma membrane causing intracellular events such as mitogenesis, increased protein secretion, and transformation to take place (160,258,280). Growth factors and their receptors, and their intracellular targets are now known to disrupt normal cell proliferation when the cells escape the mechanisms that normally regulate their activity (160). The hypothesis that growth factors, growth factor receptors, and their related biochemically active secondary and tertiary "messages" are involved in malignant cell growth is rapidly gaining substance

There have been no previous studies describing the effects of the TGF-alpha and -beta on normal human brain cells. According to the paracrine hypothesis as outlined in this thesis (Section 1.1), it is predicted that malignant glioma cells will secrete TGFs that will stimulate the proliferation and eventual transformation of previously normal, peritumoral leptomeningeal cells. In the present study, the mitogenic effects of purified human TGF-alpha and TGF-beta on normal human fetal and adult leptomeningeal cells were determined. In addition, the effects of the TGF-alpha and TGF-beta on normal human fetal glial cells and human malignant glioma cell lines were studied. Finally, since TGF-alpha is known to compete with the EGF-receptor (8,158,180,212,271), we analyzed the binding of radiolabeled EGF to a variety of normal brain-derived cells and malignant glioma cell lines in efforts to verify the presence of EGF-receptors on normal and malignant human brain cells. For all cell lines, a correlation was then

sought between the number of EGF-receptors and biological response to TGF-alpha.

8.2 Materials and Methods

8.2.1 Cell cultures

The cultures selected for use in this study have been completely characterized and are summarized in Table I. The cultures were maintained as described previously (Section 6.2.2). A-431 epidermoid carcinoma cells bearing high EGF-receptor numbers, and NRK (normal rat kidney fibroblast) cells, clone 14, were generous gifts from Dr. Lewis T. Williams, U.C.S.F..

8.2.2 Assay for growth promoting activity

To determine whether purified human transforming growth factors are mitogenic for NRK indicator cells, normal leptomeningeal cells, fetal human glial cells and malignant glioma cells in culture, approximately 10^3 cells were seeded into each of a 96-well flat bottom Nunclon microtiter plate (Nunc, Kanstrup, Denmark) and allowed to adhere overnite in MEM and 10% FCS. The serum-containing medium was then removed and replaced with serum-free medium and the attached cells were incubated for a further 24 hr before the addition of TGFs. TGF-alpha (recombinant human TGF-alpha, Rik Derynck, Genentech, South San Francisco) and TGF-beta (R&D Systems, Inc., Minneapolis, MN) were then added at various concentrations in 50 ul aliquots (250 ul total volume). Twenty-four hrs after factor addition, the wells were pulsed with 0.01 uCi of ^{3}H-thymidine (NEN Research Products, DuPont, Boston, MA), and incubated at 37 C for 18 hr. One hour before harvesting, 10 ul of an enzyme cocktail consisting of pronase and collagenase were added to each well. The cells were then harvested onto fiberglass filter paper with a multiple automated sample harveter (Skatron, Inc., Sterling, VA). The individual samples were then dried and placed in scintillation vials to which 3 ml of Betafluor (National Diagnostics, Somerville, NJ) was added. A scintillation counter (LS 8000, Beckman Instruments, San Jose) with a counting efficiency of 28% for the tritiated material was used to count all samples. Cells which achieved ^{3}H-thymidine incorporations greater than 200% or less than 75% of serum-free controls were considered to be stimulated or inhibited respectively.

8.2.3 EGF-Radioreceptor Analysis

The binding of [^{125}I] EGF to normal human leptomeningeal and fetal glial cells, and to malignant glioma cells in culture was performed as described previously (180,295). Clone A 431 of the human squamous cell carcinoma cell line (kindly provided by Dr. LT Williams) which carries a high number of EGF receptors/cell, was used as an internal control. Approximately 1 x 10^5 cells were seeded into each well of a 12-well Costar plate and allowed to adhere for 24 hours in MEM containing 10% FCS. On the day of the binding analysis, the medium was aspirated and the wells were washed once with 2 ml binding medium consisting of MEM, 1 mg/ml bovine serum albumin (BSA, type V, Sigma), and 20 mM HEPES, pH 7.4 at room temperature. The binding medium was then aspirated and 1 ml of binding medium containing [^{125}I] EGF (Collaborative Research, MA 10 uCi, 100,000 CPM) or [^{125}I]-EGF and various concentrations of purified, unlabeled EGF (receptor grade, Collaborative Research) was added and the cells were incubated in the binding mixture for 2 hr at 37 C. To demonstrate that TGF-alpha competes for the EGF receptor, in one experiment A-431 cells were incubated with various concentrations of unlabeled, recombinant human TGF-alpha in conjunction with the radiolabeled [^{125}I]-EGF. All binding reactions were stopped by washing 4 times with HBSS containing 1 mg/ml BSA at 4 C. The cells in each well were then solubilized with 1 ml 2% SDS for 10 min. The total amount of radioactivity bound to the cell monolayer was measured with a gamma counter. Nonspecific binding was determined by adding a 1000-fold excess (1 uM) of unlabeled EGF. Specific binding was calculated by subtracting the nonspecific binding from total binding. In selected cultures, the specific binding data was further analyzed with Scatchard plots.

8.3 Results

8.3.1 Effects of Transforming Growth Factors on Normal Cell Growth:

The effects of TGF-alpha and TGF-beta on the mitogenicity of NRK and normal human brain-derived cells are shown in Figures 1-3. TGF-alpha (10 ng/ml) stimulated the incorporation of ^{3}H-thymidine in NRK cells to a maximum of 220% of serum-free, untreated NRK cells; TGF-beta inhibited

the uptake of ^{3}H-thymidine in NRK cells at higher doses (1-10 ng/ml), but appeared to facilitate ^{3}H-thymidine uptake at lower doses (0.01-0.1 ng/ml) (Figure 1).

The effects of the purified transforming growth factors on the mitogenicity of normal cells derived from human brain and leptomeninges were highly variable. TGF-alpha greatly stimulated the uptake of ^{3}H-thymidine in certain adult leptomeningeal cell (SF-514P and SF-514G) and in fetal glial cell (SF-684) cultures (Figure 2). Other normal cell cultures (SF-183G, -673M) were unaffected by the addition of TGF-alpha. One fetal leptomeningeal cell culture (SF-622) was significantly inhibited by the addition of TGF-alpha.

TGF-beta appeared to stimulate the growth of only one normal cell culture (SF-514P) and only at low dose (0.01 ng/ml). All other normal cell cultures tested were either unaffected or partially inhibited by increasing doses of TGF-beta (Figure 3).

8.3.2 Effects of Transforming Growth Factors on Glioma Cell Lines

Purified TGF-alpha and TGF-beta did not cause marked stimulation or inhibition of ^{3}H-thymidine uptake in any glioma cell line tested (Figures 4 and 5). Most glioma cell lines were either mildly stimulated or stimulated not at all by the transforming growth factors. Only one cell line, SF-188, showed signs of inhibition when TGF-beta was added to the serum-free culture medium.

8.3.3 EGF Radioreceptor Analysis

The specific binding of [^{125}I] EGF to all normal cell cultures and malignant glioma cell lines is shown in table I. All normal cell cultures and malignant glioma cell lines had demonstrable specific binding of radiolabeled EGF to the cell surface; however, no cell line or culture bound [^{125}I] EGF to the same degree as A-431. Representative saturation binding curves for selected normal cell cultures and malignant glioma cell lines are shown in Figure 6. Normal cell cultures derived from SF-514, and malignant glioma cell lines SF-188 and U-343 MG-A demonstrated the greatest level of specific binding. Both normal cell cultures and malignant glioma cell lines demonstrated saturability of specific binding of [^{125}I] EGF.

Scatchard analysis of selected normal cell cultures and malignant glioma cell lines showed the presence of a single species of high affinity receptor with K_d of between 6.62×10^{-11} - 1.4×10^{-12} M (Figure 7). The number of EGF-receptors/cell varied from culture to culture and was between 1.0×10^4 - 1.0×10^5.

Purified TGF-alpha was found to inhibit the binding of [^{125}I] EGF to its receptor on A431 cells (Figure 8). Inhibition of binding of radiolabeled-EGF to its receptor was evident at 4 nM TGF-alpha.

8.4 Discussion

In 1976, Westermark (290) showed that EGF markedly stimulated the growth of normal human brain-derived ("glia-like") cells in culture. The mitogenic effect of EGF was clearly cell density dependent and was greater than that produced by either somatomedin B or fibroblast growth factor. However, since this study, there have been no further reports on how growth factors affect normal brain-derived cells, and in particular, how the transforming growth factors -- TGF-alpha and TGF-beta -- affect human glial and leptomeningeal mitogenesis. In our study, we have shown that there is a variable mitogenic response following treatment with the purified transforming growth factors among target normal glial and leptomeningeal cells.

The effects of TGF-beta on normal glial and leptomeningeal culture mitogenesis were largely inhibitory. Only one leptomeningeal culture (SF-514P) had increased [^{3}H]-thymidine incorporation when treated with low doses (0.01-0.1 ng/ml) of TGF-beta; all other normal cultures analyzed demonstrated no change in [^{3}H]-thymidine incorporation or were substantially inhibited by TGF-beta. TGF-beta has been shown to be mitogenic for a variety of fibroblastic cell types in monolayer culture (161,211,245). Curiously, in our study, NRK indicator cells were not stimulated but rather inhibited by increasing concentrations of TGF-beta. However, others (107,245) have reported the same finding with confluent monolayers of NRK cells. In contrast, normal and malignant epithelial cells in culture are usually growth inhibited when treated with TGF-beta

(124,211,245). It is interesting that, in our study, the normal glial and leptomeningeal cells, which are thought to be of neuroepithelial origin, were inhibited by TGF-beta.

TGF-alpha is a 7 kD single chain protein that shows a 30% homology with EGF and that acts through the EGF receptor (8,158,180,271). The ability of EGF and TGF-alpha to bind to the same receptor is presumably due to a similarity in conformation related to the homology of the disulfide bridges within each molecule (235). Despite many structural similarities between TGF-alpha and EGF, the two polypeptides remain antigenically distinct and act directly on certain indicator cells in different ways. In our study, there was variability of response of the cultures to TGF-alpha in that TGF-alpha was strongly mitogenic in 3 normal cell cultures (SF-514, SF-514G, and SF-684), had no effect in 2 (SF-183G, and SF-673M) and was inhibitory in 1 (SF-622). Variability of growth factor response among different cell lines and within clones of a given cell line has been reported previously for EGF (295). When combinations of TGF-alpha and TGF-beta were used in our mitogenic assay, the effects of both growth factors on cell proliferation were neither additive nor synergistic (data not shown).

Since binding of TGF-alpha to the EGF-receptor is a necessary condition for mitogenesis and cellular transformation (158,160,161), a major interest in growth factor research has been to increase our understanding of the structure and function of this receptor (108,280). Recently, the complete 1210 amino acid sequence of the human EGF receptor precursor was deduced from cDNA clones derived from placenta and from A431 carcinoma cells (280). The sequence suggests that the EGF receptor is a 170 kD species with an external EGF-binding domain at the amino terminal comprising about 621 amino acids (108). This external region is bound to the cytoplasmic domain through a single membrane-spanning segment of about 23 amino acids (108,280). It is intriguing that the transmembrane and intracytoplasmic sequences of the receptor molecule bear striking homology with the entire predicted v-erb B mRNA oncogene product (127,258,280).

EGF and TGF-alpha have been shown in previous studies to bind to the EGF-receptor on A-431 cells with high affinity (10^{-9} - 10^{-11} M) (72,141,256,258). Once bound, the receptor-ligand complex is thought to transmit a signal (in an as yet undetermined manner) along the receptor from the extracellular face across the transmembrane domain to the cytoplasmic

face. This signal leads to the activation of a tyrosine phosphokinase resulting in the phosphorylation of tyrosine residues on the cytoplasmic domain of the receptor (108,158,160,161,256,280). It is thought that tyrosine phosphorylation might be an important early step in the mitogenic process (280).

There have been few studies which have reported an EGF-radioreceptor analysis for normal brain cultures. Simpson et al (249) performed EGF-receptor studies on separated populations of primary astrocytes, oligodendrocytes and neurons derived from normal rat brain In this study, the density of EGF-receptors was found to be relatively high in astrocytes, intermediate in oligodendrocytes, and low in neurons. And Westermark (291) showed that cultures derived from adult normal human brain not only bound EGF but also responded mitogenically to EGF. In our study, all normal leptomeningeal and glial cell cultures specifically bound [^{125}I] EGF. In addition, Scatchard analysis of our normal cell cultures that were stimulated by TGF-alpha revealed the presence a large number (4.0×10^4 - 1.0×10^5) of high affinity (10^{-10} - 10^{-11} M) EGF-receptors. In contrast, normal cell cultures that were not stimulated by TGF-alpha had lower numbers of EGF-receptors/cell ($< 4.0 \times 10^4$). These data, at least for the normal cell cultures, imply that there may be a correlation between EGF-receptor number and mitogenic response to TGF-alpha.

There have been several reports describing the presence of EGF-receptors in human brain tumors (72,140,141,256,295). Libermann et al. (141) immunoprecipitated a 150 kD protein species directly from homogenates of glioblastoma multiformes and meningiomas using anti-sera specific for the EGF-receptor. The isolated protein in these specimens could be autophosphorylated and the level of incorporation of [^{32}P]-phosphate appeared to correlate with the amount of EGF-receptor. Tissue specimens from adult normal human brain were not found to contain any demonstrable amount of the EGF-receptor when analyzed in the same fashion. In another study (140), 4 of 10 primary human brain tumors of glial origin which expressed high levels of EGF-receptors were found to have amplified EGF receptor genes. Steck et al. (256) have shown that <u>in vitro</u>, the majority of glioma cells will bind [^{125}I] EGF at a receptor level of 40-80,000/cell. And Westphal et al. (295) have reported the isolation of an EGF-receptor from a human glioma-derived cell line which has different properties from that derived from A-431 cells. However, with respect to malignant gliomas, either

in vivo or in vitro, there does not appear to be any simple correlation between EGF-receptor number and biological activity such as mitogenesis or invasiveness (229). In our study, TGF-alpha did not significantly stimulate the growth of any of the glioma cell lines examined, yet all had bound [^{125}I]-EGF specifically -- some to a high level.

Part of the explanation for the poor correlation between EGF-receptor number and mitogenesis has been ascribed to the "down-regulation" phenomenon of the receptor (14,89). In this process, receptor:ligand complexes are thought to be internalized at a rate which exceeds that of the replenishing of the receptor at the cell surface. While this might serve as one explanation, it is clear that further studies, including post-receptor analyses, are needed to fully determine the reason why there is a lack of correlation between EGF-receptors and biological activity in most malignant glioma cultures.

In summary, we have determined that TGF-alpha can be stimulatory to normal leptomeningeal and glial cells in culture, and that the amount of stimulation in the normal cells appears to correlate with an increase in EGF-receptor number. In contrast, although all glioma cell lines bound EGF, none was stimulated to grow by TGF-alpha. And finally, in our system, we were able to confirm the results reported by others that TGF-alpha competes with radiolabeled EGF for the EGF receptor.

8.5 Figure Legends and Tables

Figure 1: Effects of TGF-alpha and TGF-beta on NRK cell mitogenicity. TGF-alpha stimulated the uptake of ^{3}H-thymidine in a dose dependent fashion up to a maximum of 220% of serum-free control at 10 ng/ml. TGF-beta inhibited the uptake of ^{3}H-thymidine at higher doses.

Figure 2: Effects of TGF-alpha on the mitogenicity of normal human brain-derived cells. TGF-alpha increased ^{3}H-thymidine uptake in 2 adult leptomeningeal cell cultures (SF-514P and SF-514G) and in one fetal glial cell culture (SF-684). The other normal cell cultures analyzed were either not stimulated (SF-183G and SF-673M) or were significantly inhibited by the addition of TGF-alpha.

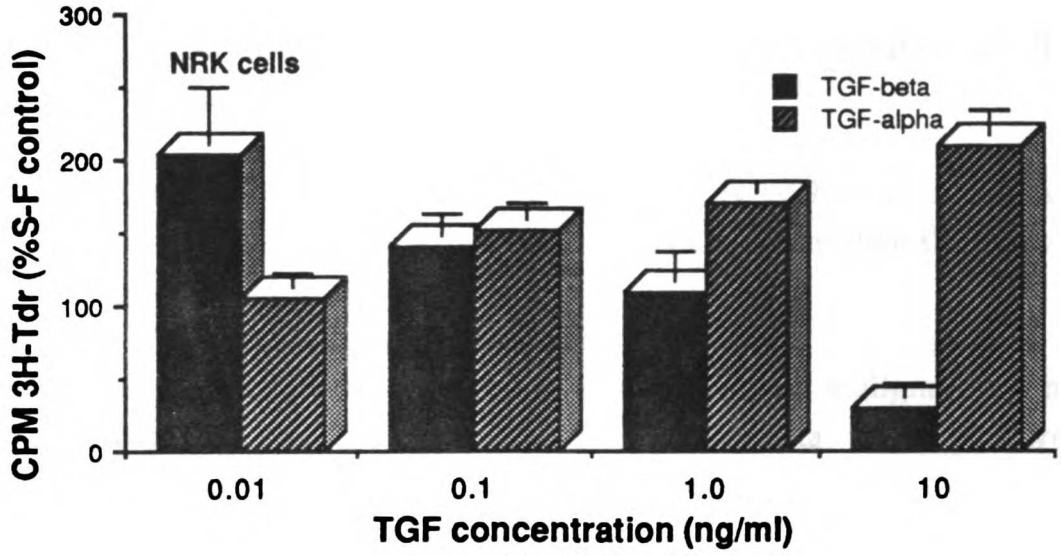


FIGURE 1

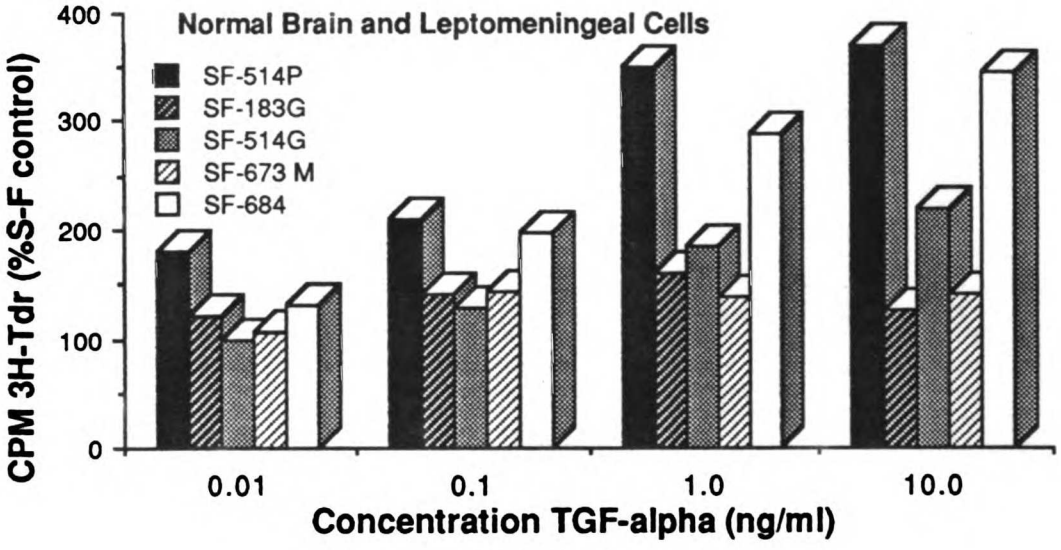


FIGURE 2

Figure 3: Effects of TGF-beta on normal human leptomeningeal and glial cultures. TGF-beta did not significantly affect the incorporation of ^{3}H-thymidine in any normal cell culture examined.

Figure 4: Effects of TGF-alpha on the mitogenicity of human malignant glioma cell lines. TGF-alpha by itself did not appear to stimulate the growth of any of the glioma cell lines.

Figure 5: Effects of TGF-beta on the mitogenicity of human malignant glioma cell lines. No glioma cell line was stimulated by TGF-beta. SF-188 showed signs of inhibition of ^{3}H-thymidine incorporation when TGF-beta was added to the assays system.

Figure 6: Representative [^{125}I] EGF saturation binding curves for selected leptomeningeal and glioma cells in culture. Normal leptomeningeal cell culture SF-514P and malignant glioma U 343 MG-A demonstrated the highest degree of specific [^{125}I] EGF binding.

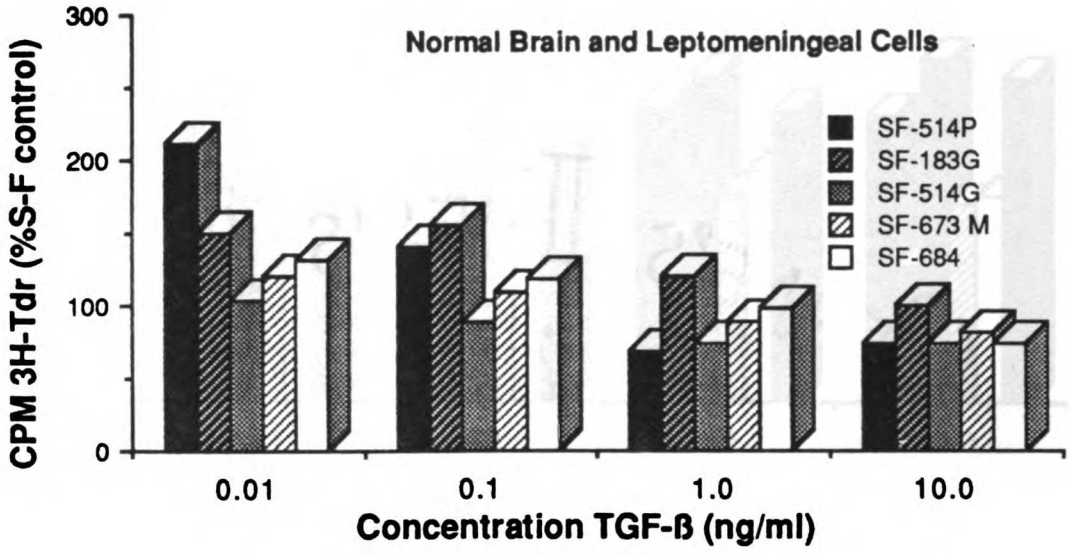


FIGURE 3

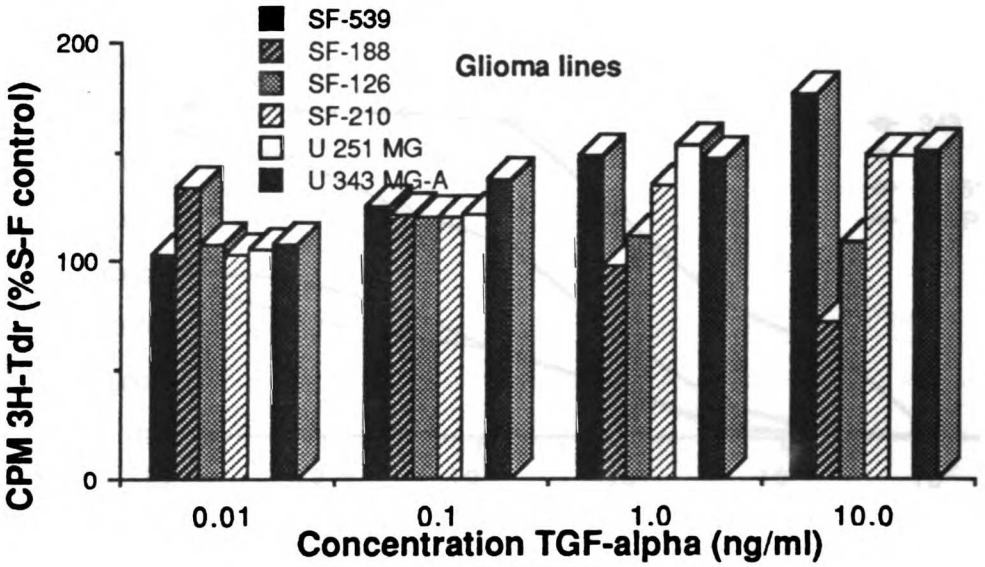


FIGURE 4

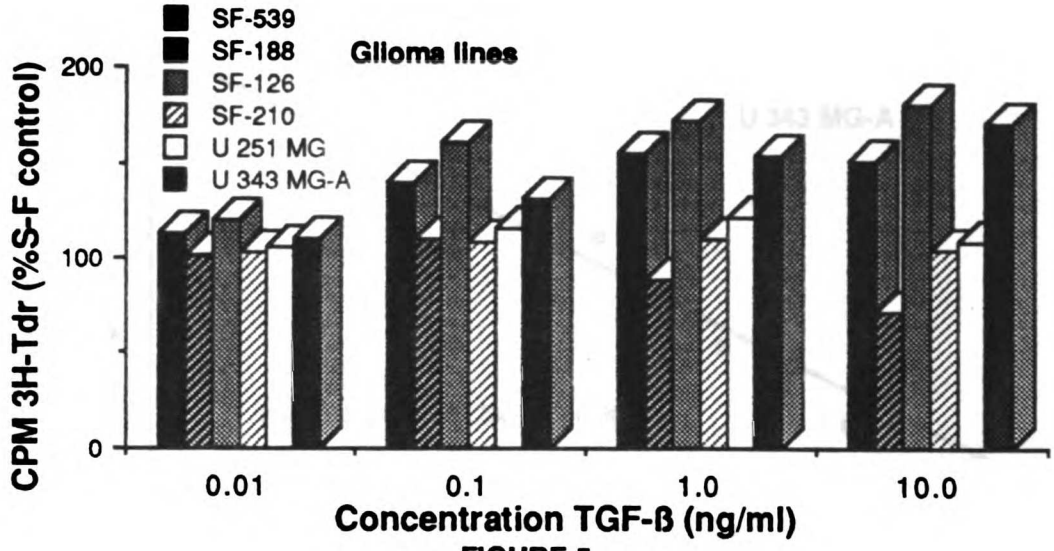


FIGURE 5

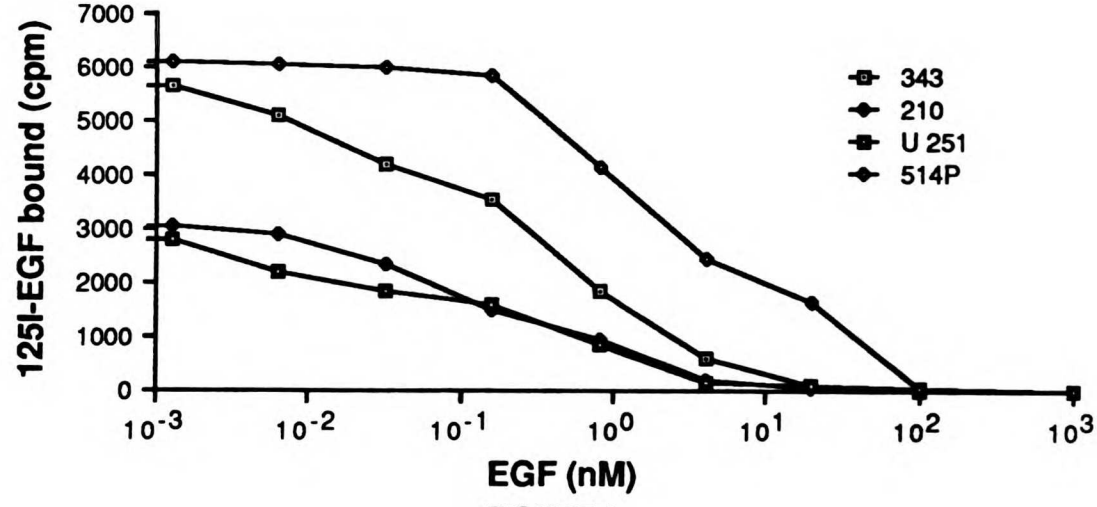


FIGURE 6

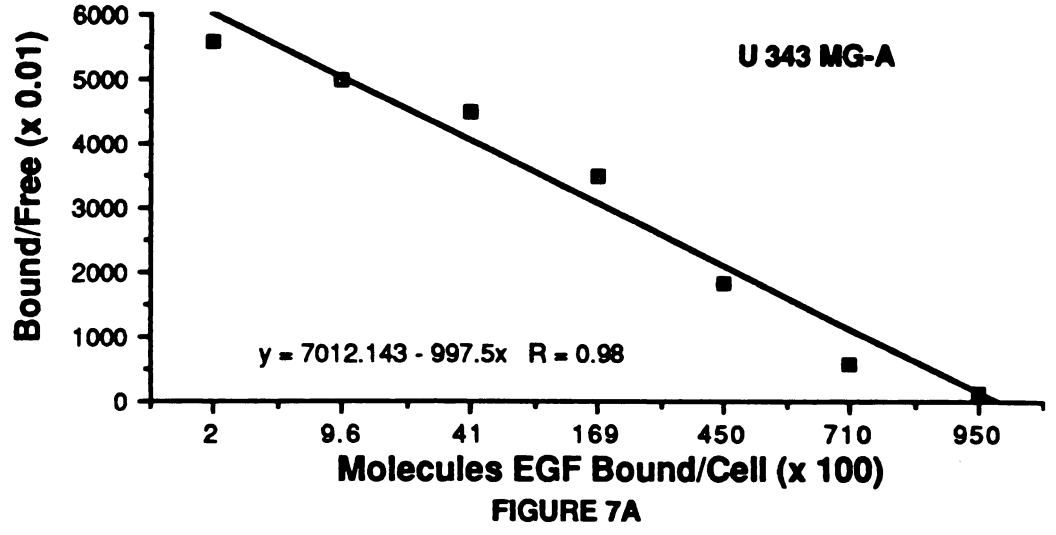


FIGURE 7A

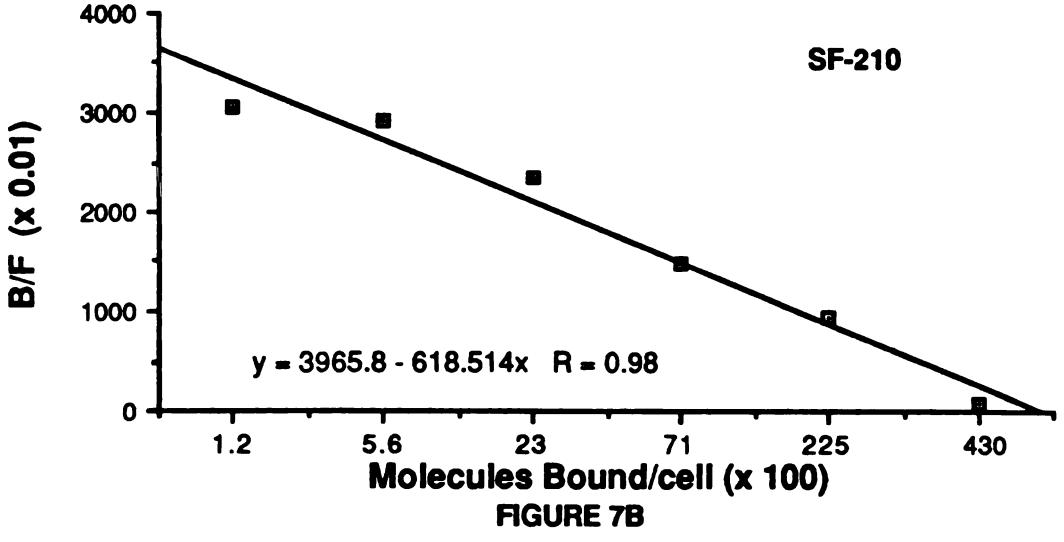


FIGURE 7B

Figure 7: Scatchard analysis of EGF binding in U 343 MG-A and SF-210. A straight line function is observed in both glioma cell lines suggesting a single species of receptor. For U 343 MG-A, the K_d is 1.0×10^{-11}; and for SF-210, the K_d is 6.8×10^{-11} suggesting that only high affinity receptors are present.

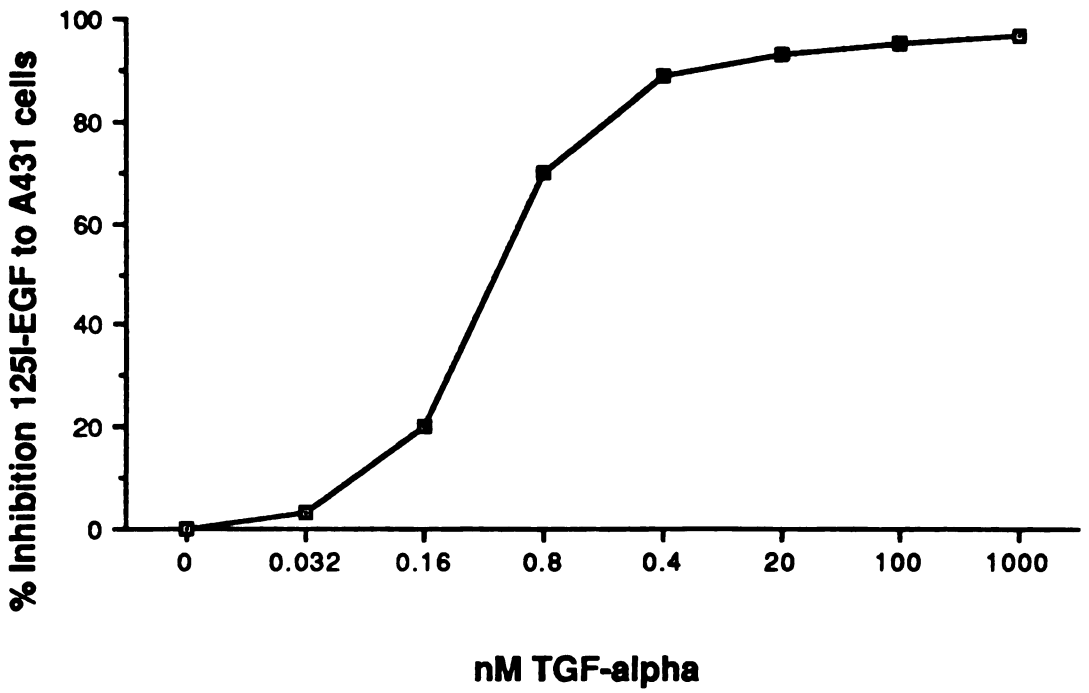


Figure 8: Binding of TGF-alpha to the EGF-receptor is verified in this experiment depicting competitive binding of TGF-alpha with [125]I EGF for the EGF receptor on A431 cells.

TABLE I:

Cell cultures used in mitogenic and EGF radioreceptor assays:

Cell Culture	Derivation:	^{125}I-EGF Specific binding(cpm)
SF-514P	Adult human leptomeningeal	6165
SF-514G	Adult human brain (gray matter)	5984
SF-183G	Adult human brain (gray matter)	3540
SF-622M	Fetal human leptomeningeal	920
SF-673M	Fetal human leptomeningeal	3480
SF-684	Fetal human brain	4500
SF-126	Human Glioblastoma Multiforme	3861
SF-188	Human Glioblastoma Multiforme	4674
SF-210	Human Glioblastoma Multiforme	3524
SF-539	Human Gliosarcoma	2875
U-251 MG	Human Glioblastoma Multiforme	3644
U-343 MG-A	Human Anaplastic Astrocytoma	5800
NRK	Normal Rat Kidney Fibroblasts	Not determined
A 431	Epidermoid Carcinoma	40,000

CHAPTER 9

ISOLATION AND CHARACTERIZATION OF TRANSFORMING GROWTH FACTORS FROM HUMAN MALIGNANT GLIOMAS

9.1 Introduction:

Polypeptide growth factors that are able to confer the transformed phenotype on normal cells have been termed TGFs (154). The transformed phenotype is operationally defined by the loss of density-dependent inhibition of cell growth in monolayer culture, overgrowth in monolayer culture, characteristic change in cellular morphology, and acquisition of anchorage-independent growth (212). Untransformed nonneoplastic cells will not form progressively growing colonies in soft agar, whereas the property of irreversible anchorage-indepndent growth of cells in culture has a particularly high correlation with neoplastic growth in vivo (212). Therefore, the growth of normal cells in soft-agar bio-assay systems, in particular, can be an important and quantitative measurement of the relative amounts of TGFs present in a given sample (10).

TGFs can be found in almost all neoplastic and certain nonneoplastic tissues from all species thus far studied (212). For example, TGFs have been partially purified from several human tumor cell lines, from solid mouse, rat and human tumors, from the conditioned medium of chemically transformed rat and mouse cells, and from a variety of nonneoplastic human and rodent tissues including human placenta, kidney, lung, mouse embryos, human urine, serum, and platelets (8,10,43,70,80,89,107,154,160,161,210,213,278).

The TGFs belong to a family of heat- and acid-stable peptides that require intrachain disulfide bonds for activity (271). Generally, TGFs have molecular weights ranging from 6 kD - 30 kD depending on their source and

the normal indicator cell line used to screen for colony formation in soft agar. To date, TGFs have been classified into two main types -- TGF-alpha and TGF-beta -- according to certain biological and physical properties (210): TGF-alpha is a 6500 kD species which, by itself, induces a limited extent of anchorage-independent growth of NRK cells (8). TGF-alpha competes with the EGF for binding to the cellular 170 kD EGF-receptor and induces tyrosine residue phosphorylation and down regulation of the EGF-receptor, but fails to cross-react with antibodies generated against EGF (229,271). By cDNA sequence analysis, TGF-alpha has recently been shown to exist as a much larger precursor molecule (circa 20^{+} kD) which, like the EGF precursor, has transmembane and intracellular domains (29).

TGF-beta is a 25,000 kD disulfide bonded double chain species which does not induce anchorage-independent growth if added alone to NRK cells but acts to synergize with TGF-alpha (or EGF) to potentiate the transforming action of these two factors (8). Certain indicator cell lines, however, such as AKR-2B cells, human diploid fibroblasts and EGF-receptorless NR-6 cells are stimulated to form colonies by TGF-beta in the absence of EGF (245). TGF-beta does not bind to the EGF receptor but appears to bind to its own specific receptor of 280 kD with high affinity (81,159). Because TGF-beta has been detected in a variety of non-neoplastic cells such as platelets and placenta (10,80), the possibility exists that it has a role to play in normal physiologial cell growth (10). However, by itself TGF-beta is not a mitogen for most cell types (81,107,209). It has been shown by some (81) that TGF-beta increases the number of EGF-receptors on the cell surface of NRK cells thus providing a partial explanation for the increased mitogenic and transforming response of NRK cells to combinations of TGF-alpha and TGF-beta. The TGF-beta cDNA encodes a polypeptide of 391 amino acids (57), and the TGF-beta mRNA has been found in at least one glioblastoma multiforme cell line A-172. Recently described properties of TGF-beta include its ability to stimulate mesenchymal cell growth, collagen synthesis and ECM deposition (213). And Cheifetz et al (37) have now identified 3 different species of TGF-beta (TGF-beta1,2, and 3) through extensive HPLC purifications from porcine and human platelets.

We undertook the present study to determine: 1)If human malignant gliomas secrete TGFs into their serum-free conditioned medium and, more importantly 2) If the glioma-derived TGFs could cause the phenotypic transformation of normal human leptomeningeal cells. The latter

determination was important to test the hypothesis described previously on the pathogenesis of the gliosarcoma. Separation and purfication of the glioma-derived TGFs within the serum-free conditioned medium proceeded in a step-wise fashion beginning with acid extraction followed by molecular sieve chromatography and reverse-phase HPLC. We report our findings from a series of glioma cell lines in which three were identified as secretors of TGFs. The glioma-derived TGFs from two of these three cell lines were then purified to near homogeneity as described below.

9.2 Materials and Methods

9.2.1 Cell Cultures

Cell cultures were maintained at 37 C in 75 cm^2 plastic tissue culture flasks (Falcon no. 3024) in Eagle's MEM with 10% fetal calf serum. Eight human malignant glioma-derived cell lines were used in initial studies -- SF-126, -188, -210, -268,-539, -295, U-343 MG-A, and U-251 MG -- and these cell lines have been previously well characterized (Chapters 5 - 6).

9.2.2 Source of Transforming Growth Factors: Collection of Glioma Conditioned Medium

Serum-free conditioned medium by the malignant glioma cell lines served as the source of transforming growth factors. Glioma cells were grown in 850-cm2 plastic roller bottles (Corning, NY) with 100-150 ml of Eagle's MEM supplemented with 10% fetal calf serum and 50 ug/ml gentamicin. Cultures were incubated at 37 C and 0.2 RPM under a 95%:5% air/CO_2 atmosphere. When the cell layer reached 80% confluency, the cultures were incubated for two consecutive 3-h periods with two 50-ml of serum-free MEM per bottle to eliminate serum proteins (158,271). These two collections were discarded. Five consecutive 150-ml volumes of serum-free MEM were subsequently collected per roller bottle over an 8-day period and kept as "conditioned medium". After each individual collection, the conditioned medium was filter-sterilized by passage through a 45-um mesh filter (Amicon) before storage at -70 C. Aliquots of glioma cell conditioned medium were then analyzed for activity in the mitogenic and soft agar assays as outlined below. The protein concentration of the glioma conditioned

medium before and after purification for the TGFs was determined using the Bio-Rad protein determination kit (Bio-Rad, Richmond CA).

9.2.3 Growth Promoting Assay

Aliquots of the above harvested glioma conditioned medium with known amounts of protein were analyzed for their ability to stimulate [^{3}H] thymidine incorporation among NRK indicator cells and fetal human leptomeningeal cells (SF-673 M). The assay for testing growth promoting activity has been described in Section 8.2.2. Glioma-derived conditioned medium which enhanced [^{3}H] thymidine incorporation among normal cells to levels greater than 200% or more of serum-free controls was considered stimulatory.

9.2.4 Soft Agar Assay

Soft agar assays for testing anchorage-independent growth-stimulating activity in the glioma conditioned medium and purified glioma TGF samples were conducted as described previously (Section 5.2.3) with slight modification. Briefly, 1-5 x 10^3 normal leptomeningeal or NRK cells were suspended in 1 ml of 0.3% Agar (purified Grade, Fisher Scientific, Fair Lawn, NJ) supplemented with 10% fetal calf serum in the absence or presence of aliquots of glioma-conditioned medium and layered over 1 ml of a similarly prepared 0.5% agar-medium basal layer in 35 x 10 mm^2 plastic tissue culture dishes (Lux, Miles Labs, Naperville, IL). All cultures were incubated at 37 C in a humidified 5% CO_2/95% air atmosphere without further feeding for 10-14 days before staining of the colonies with INT. Colonies greater than 30-40 um were scored as positive and counted. All experiments were performed in triplicate.

9.2.5 Concentration of Glioma Conditioned Medium

Conditioned medium from glioma cell lines that was active in the soft agar assays was concentrated using three different techniques:

1. The conditioned medium from SF-210 (4 L) was lyophilized to dryness using a VirTis multi-port lyophilizing unit (Virtis, Gardiner, NY) (270).

2. The conditioned medium from U-343 MG-A (8 L) was concentrated using a hollow fiber concentrator (CH2, Cartridge S1Y1, M_r cut-off 3000) at room temperature (158).

3. The conditioned medium from U-251 MG (6 L) was concentrated by passing the entire sample over a column (1.5 x 90 cm, Bio-Rad) of glass beads (Controlled-Pore Glass Beads, CPG00120, 200-400 mesh, Electro-Nucleonics, Inc., Fairfield, NJ) pre-equilibrated in 10 mM sodium phosphate buffer, pH 8.0 at 4 C. The conditioned medium was filtered through the column of glass beads at flow rates of 100 ml/hr with a peristaltic pump. Adherent proteins and activity were then eluted from the column using a 20% ethylene glycol solution in 10 mM sodium phosphate buffer, pH 8.0, containing 1 M sodium chloride at 4 C at a flow rate of 50 ml/ hr (2).

9.2.6 Acid-Extraction of the Concentrated Glioma Conditioned Medium

Lyophilized conditioned medium from SF-210 was resuspended in 1 M acetic acid and dialyzed for 48 hr in Spectrapor 3 dialysis tubing (Spectrum Medical Industries) at 4 C against a total of 100 volumes of 1 M acetic acid (158,271). Concentrated glioma medium from U-343 MG-A and U-251 MG was placed directly in Spectrapor 3 dialysis tubing and dialyzed for 48 hr as described above. The dialysate in all cases was then lyophilized to dryness and then dissolved in 1 M acetic acid before application to gel filtration columns as described below. Portions of the lyophilized acid-extracted medium were resuspended in 5 mM acetic acid, 50 mM NaCl before being tested in the previously described mitogenic and soft agar assays.

9.2.7 Molecular Sieve Chromatography

The lyophilized acid-extracted dialysates were then applied to columns (1.5 x 90 cm) of Bio-Gel P-60 200-400 mesh (BioRad) equilibrated with 1 M acetic acid (158,180,271). The columns had been previously calibrated with ovalbumin (M_r = 44,000), myoglobin (M_r = 17,000), cytochrome C (M_r = 14,000), insulin (M_r = 5,700) and Vit B12 (M_r = 1,350). The columns were eluted at 22 C with 1 M acetic acid at a constant flow of 12 ml/hr and 2 ml fractions were collected. Fractions were monitored either by absorbance at A_{280} or by individual protein determinations. Aliquots (10-20 ul) from alternate fractions were dried at room temperature in a Speed Vac Concentrator (Savant) for mitogenic, soft agar and EGF-radioreceptor studies.

Aliquots from every fifth fraction containing sufficient protein were analyzed by SDS-PAGE as described below.

9.2.8 EGF-Radioreceptor Assay

Active fractions from molecular sieve and high pressure liquid chromatography were assayed for their ability to bind to A431 human epidermoid carcinoma cells as described previously (Section 8.2.3). Samples to be assayed were lyophilized as described above, and dissolved in 250 ul binding medium containing 10% fetal calf serum.

9.2.9 Reverse Phase High Pressure Liquid Chromatography

Further purification of the glioma-derived TGFs from SF-210 and U-343 MG-A was achieved with reverse-phase HPLC (158,208,271). Fractions capable of supporting anchorage independent growth from molecular sieve chromatography were pooled, lyophilized and redissolved in 0.05% TFA in water. Samples were filtrated through a Millex GV (Millipore) membrane filter and injected into a Rainin Rabbit HPX HPLC system controlled by a Macintosh computer. An Isco V4 absorbance detector and a RP-18 12 micron, 21.4 mm ID x 25 cm column (Dynamax - Macro) with guard module were used. Sample absorbance was measured at A_{220}. The mobile phase consisted of a 0.1% TFA/water and 0.1% TFA/acetonitrile gradient at a flow rate of 12.5 ml/min. One ml fractions were collected in disposable borosilicate tubes. Aliquots were lyophilized for subsequent soft agar and EGF-radioreceptor assays. In addition, protein profiles of the active fractions were determined by SDS-PAGE as described below.

9.2.10 SDS-Polyacrylamide Gel Electrophoresis

Electrophoresis of whole conditioned medium and appropriate fractions from molecular sieve and high pressure liquid chromatography was performed on polyacrylamide slab gels as described previously (Section 2.2.6). A 15-20% resolving gel was used for protein separation (158,161); a 4.5% stacking gel was used for protein loading. Gels were run for approximately 2 hr at 12.5 mA. After electrophoresis, gels were fixed with 50% methanol in water and silver-stained (Bio-Rad).

9.2.11 Physicochemical Treatments of Purified Glioma Transforming Growth Factors:

Characterization of the purified glioma transforming growth factors was achieved by physicochemically treating 0.1 ug aliquots (dissolved in 5 mM acetic acid and 50 mM NaCl) of the active fractions from HPLC before performing soft agar assays.

To test trypsin sensitivity, the glioma factors were incubated for 2 hr at 37 C with trypsin (100 ug/ml) alone or with trypsin (100 ug/ml) plus soybean trypsin inhihibitor (200 ug/ml) (Worthington, Locale). Trypsin and soybean trypsin inhibitor were preincubated together in a small volume of MEM for 30 min at 37 C. Aliquots with trypsin only received soybean trypsin inhibitor (200 ug/ml) after the 2 hr incubation.

Heat stability of the growth factors was tested by heating the glioma factors in a 60 C water bath for 30 min, or by boiling the glioma factors for 3 min.

Sensitivity of the glioma factors to reducing agents was determined by exposing the samples to 50 mM DTT (BioRad) at 37 C for 16 hr. To remove DTT prior to analysis, the samples were dialyzed in Spectrapor 3 dialysis tubing against 5 mM acetic acid and 50 mM NaCl prior to testing in the soft agar system.

Acid stability of the glioma growth factors was assessed by dialyzing aliquots in Spectrapor 3 tubing for 20 hr at 4 C against 0.1 M acetic acid (pH 3.0 - 2 changes of 1000-fold volume). After filtration these samples were dialyzed again against 5 mM acetic acid and 50 mM NaCl for 12 hr before testing by soft agar analysis. Stability of the glioma transforming growth factors to a basic milieu was tested in a similar fashion except that ammonium hydroxide (25 mM, pH 12.0) was used as the dialyzing solution.

EGF- and TGF-alpha-like activity within the glioma transforming growth factors was assessed by analyzing aliquots in the EGF-radioreceptor studies, by adding TGF-beta (100 ng/ml) to the samples, and by incubating the samples overnight in the presence of 100 ug/ml of anti-TGF-alpha monoclonal antibody (29,219). In the latter case, control samples consisted of adding anti-TGF-alpha monoclonal antibody (100 ug/ml) alone.

Because of the high affinity for all angiogenic factors for heparin, FGF-like activity within the glioma transforming growth factors was determined by first passing aliquots of purified factor over a column (1.5 x 10 cm, 30 ml) of

heparin-Sepharose (Pharmacia, Uppsala, Sweden) equilibrated with 0.6 M NaCl. 0.01 M Tris-HCl, pH 7.5. After a wash of about 5 column volumes, the glioma-derived samples were eluted with a gradient of 100 ml of 0.6-3.0 M NaCl in 0.01 M Tris-HCl, pH 7.5 at a flow rate of 20 ml/hr at 4 C. Wash and elution samples were then dialyzed exhaustively against 5 mM acetic acid and 50 mM NaCl and then tested in the soft agar assays.

PDGF-like activity was analyzed by incubating the glioma-derived factor aliquots in anti-PDGF monoclonal antibody (Collaborative Research) overnight at 37 C before analysis in the soft agar system. Controls consisted of incubating indicator cells in anti-PDGF monoclonal antibody by itself.

9.2.12 Immunochemical Localization of TGFalpha in Human Malignant Brain Tumors

To determine the cytologic distribution of TGF-alpha in human brain tumors, a limited histochemical analysis was performed using a well-characterized anti-TGF-alpha monoclonal antibody (29,57,58,56).

Specimens from normal adult and fetal human brain and from a series of brain tumors of varying malignancy were prepared for immunohistochemistry either by quick freezing the specimen to -70 C or by fixing the tissue specimens within 2 hr of surgery. The specimens were fixed overnite in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phophate buffer, pH 7.5. The specimens were then paraffin-embedded and cut into sections 6 um thick. Frozen sections were similarly cut into 6 um sections. The staining procedure was performed as described previously (Section 4.2.4). For immunohistochemistry, the TGF-alpha monoclonal antibody was used at a dilution of 1:300 in PBS.

9.2.13 Synthesis of Messenger RNAs for Transforming Growth Factor alpha by Human Brain Tumors

A) Isolation of the TGF-alpha cDNA insert

The TGF-alpha probe was obtained by radiolabeling the cDNA insert contained in pTGF-C17N (29) which comprises the complete coding sequence. The plasmid (pSPG5) which carries the gene for ampicillin resistance and contains the TGF-alpha insert was a generous gift of Dr. Rik Derynck, Genentech, South San Francisco. E coli strain 294 was used for transfecting

bacterial cells with the circular DNA plasmid pSPG5 (44,47). Briefly, logarithmically growing 294 cells in L-Broth were pelleted at 5000 RPM in a Sorvall Centrifuge for 5 min at 4 C. The bacterial cell pellet was resuspended in 5 ml of 50 mM $CaCl_2$ and incubated for 30 min at 4 C. The cells were then pelleted again at 5000 RPM for 5 min and resuspended in 1 ml of 50 mM $CaCl_2$. Dilutions of the pSPG5 were then added to the $CaCl_2$-treated 294 cells and the mixtures were incubated for 30 min at 4 C. The mixtures were heat-shocked at 42 C for 2 min, and then incubated for 45 min at 37 C in L-Broth. The mixture was then plated onto L-broth agar plates containing 50 ug/ml ampicillin and incubated overnite at 37 C.

A colony from the ampicillin containing L-broth agar plates was then picked and grown overnite as a 1 liter suspension culture in L-broth containing ampicillin (50 ug/ml). Plasmid DNA was then isolated by the method of Birnboim and Doly (23) The cells were pelleted at 5000 RPM for 5 min in a Sorvall at 4 C. The bacterial pellet was resuspended in 20 ml solution containing 50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA and 5 mg/ml lysozyme (Calbiochem, La Jolla, CA), and incubated for 5 minutes at room temperature. Forty ml of a freshly prepared solution containing 0.2 N NaOH and 1% SDS were added to the mixture which was gently shaken several times before incubating for 10 min at 4 C. Thirty ml of an ice-cold 3 M potassium acetate solution were then added to the tube which was again inverted sharply several times before incubation on ice for 10 min. The mixture was then centrifuged at 12,000 RPM in the Sorvall for 20 min at 4 C. The supernatant containing the plasmid was transferred to Corex tubes to which 0.6 volumes of isopropanol was added. Following a brief mix, the solution was kept on ice for 15 min. Plasmid DNA was then recovered by centrifugation in the Sorvall at 10,000 RPM for 20 min at 4 C. The supernatant was discarded. The pellet was washed in 70% ethanol at room temperature, and the nucleic acid was dried briefly in a vacuum dessicator. The pellet was then dissolved in Tris-EDTA solution (pH 7.4) before the plasmid DNA was purified by centrifugation in cesium chloride-ethidium bromide density gradients described below.

Closed circular DNA was then purified by cesium chloride (1 g/ml - Sigma, St Louis) -ethidium bromide (approximately 0.5 mg/ml final concentration - Bio Rad) centrifugation. Centrifugation was at 40,000 RPM for 36-48 hr at 20 C in a 50 Ti rotor. Circular plasmid DNA was obtained by

recovering the lowest band visualized before the RNA pellet. Ethidium bromide was eliminated by extraction with 1-butanol saturated with NaCl. The recovered, purified plasmid was then dialyzed against Tris-EDTA (pH 8.0) for 24 hr, with 3 changes of 1000x volume.

The plasmid (200 ug) was then placed in a cocktail (total volume 400 ul) containing 10x EcoR1 buffer (New England Bio Labs, Beverly, MA), EcoR1 (New England Bio Labs) and water, and incubated for 2 hr at 37 C. The plasmid DNA was then loaded into the center well of a 1% low-melting-temperature agarose gel and electrophoresed at room temperature for 4 hr at 60 amps. The band representing the cDNA insert for TGF-alpha cut by EcoR1 was then excised from the agarose gel, and placed in 5 volumes of Tris-EDTA (pH 8.0). The agarose was melted for 5 minutes at 65 C, and the insert was recovered by sequential extractions with equal volumes of phenol, phenol:chloroform (1:1), and chloroform. The purified cDNA insert was then recovered by ethanol precipitation and then radiolabeled with ^{32}P (radiolabeled dCTP, New England Nuclear) by the nick-translation procedure as described by Taylor (263) before Northern hybridization experiments were performed (infra vide).

B) RNA Extraction from Glioma Cells and Human Brain Tumor Specimens

RNA was extracted from SF-210, U 251 MG, and U 343 MG-A glioma cells which were grown in roller bottles and harvested by trypsinization at early confluency (35). The cell pellets were washed thorougly in PBS before they and a series of primary human brain tumors that were quick frozen to -70 C were homogenized in 4-6 vol of 5 M guanidinium isothiocyanate (Bethesda Research Laboratories) in 5% beta-mercaptoethanol (Sigma). Approximately 0.5 g/ml of DNA-grade cesium chloride was added to the homogenates. Sarcosyl (Sigma) was then added to a final concentration of 4%. The homogenized mixture was then layered carefully onto a 1.5 ml bed of a saturated 5.7 M cesium chloride solution in plastic Beckman ultracentrifuge tubes. The samples were then centrifuged at 32,000 RPM for 16 hr. The RNA pellets were dissolved in 200 ul of 10 mM Tris, 5mM EDTA, and 1% SDS, pH 7.4, and then heated at 65 C for 10 minutes in Eppendorf tubes. The RNA was then extracted with an equal volume of 4:1 chloroform: butanol. Purified RNA was then ethanol precipitated by adding 0.1 vol 3 M sodium acetate and 2.5 vol 95% ethanol and incubating at 20 min at -70 C.

Following microfugation for 10 min, the pellets were washed with 70% ethanol, dried in a Speed Vac Concentrator, and dissolved in 0.1% diethlypyrocarbonate-treated water. The purity of the RNA preparations was assessed by spectrophotometric analyses of aliquots read at OD 230, 260, and 280.

C) Northern Hybridization

Total RNA (15-20 ug) from the glioma samples was electrophoresed into formaldehyde/1.25% agarose gels and blotted onto nitrocellulose paper (267). The nitrocellulose filters were hybridized overnite at 41 C with the ^{32}P-labeled cDNA TGF-alpha probe in 50% formamide, 3x SSC, 1x Denhardt's solution, 50 mM Hepes (pH 7.0), 0.2 mg/ml salmon sperm DNA (New England Bio Labs), and 0.15 mg/ml yeast RNA (New England Bio Labs). Typically hybridization was performed using 1-2 x 10^6 cpm of ^{32}P nicktranslated DNA/ml solution. The filters were then washed at room temperature for 1 hr in 2 x SSC, 1 x Denhardt's solution; for 90 min in 0.1 x SSC, 0.1% SDS at 50 C; and finally for 15 min in 0.1 x SSC.

9.3 Results

9.3.1 Mitogenic Assay

Conditioned medium harvested from all glioma cell lines was mitogenic for both NRK indicator cells and fetal human leptomeningeal cells (673 M) (table I). For most glioma cell lines, the increased [^{3}H] thymidine uptake was dose dependent (Figure 1 and 2).

Conditioned medium from three glioma cell lines – SF-210, U-343 MG-A, U-251 MG -- that was acid-extracted before testing in the mitogenic assay was similarly stimulatory for NRK and fetal leptomeningeal cell cultures although the degree of stimulation was not as great as for directly harvested conditioned medium (Figure 3).

9.3.2 Soft Agar Assay

In preliminary experiments there was no significant growth of NRK cells in soft agar under control conditions in which growth factors or conditioned medium were not placed in the assay system. No NRK colonies

were formed in agar under control conditions at seeding densities as high as 1 x 10^4 NRK cells/dish.

The conditioned medium from three (SF-210, U-343 MG-A, U 251 MG) of eight glioma cell lines stimulated the growth of NRK cells in soft agar (Figure 4 and 5). When the conditioned medium from these three cell lines was acid-extracted and then tested for ability to stimulate NRK anchorage independent growth, all three cell lines stimulated soft agar colony formation to a degree greater than that of directly harvested conditioned medium (Figure 6).

Acid-extracted conditioned medium from SF-210 stimulated the formation of large colonies by SF-673 M fetal leptomeningeal cells in agar (Figure 7). The acid-extracted conditioned medium from U-251 MG, or U-343 MG-A did not cause normal human leptomeningeal cells to grow in soft agar (data not shown).

The effects of TGF-alpha on NRK colony formation are shown in Figure 8. TGF-alpha by itself caused NRK indicator cells to grow in soft agar. The addition of TGF-beta (1.0 ng/ml) to TGF-alpha in the agar assay system resulted in a significant increase in colony number (Figure 8). TGF-beta by itself did not cause NRK indicator cells to grow in soft agar.

9.3.3 Concentration of glioma conditioned medium

The conditioned medium from SF-210 (4 L) was lyophilized to dryness and resuspended in 100 ml of 1 M acetic acid prior to further purification procedures. This resulted in a 40-fold reduction in volume.

The conditioned medium from U-343 MG-A (8 L) was concentrated 100-fold using the hollow fiber concentrator from Amicon.

Proteins from U 251 MG with molecular weight range 1,000-30,000 were concentrated using the Controlled-Pore Glass beads from Electro-Nucleonics. Elution of the adherent proteins was achieved as shown in Figure 9. The fractions which were able to stimulate NRK growth in soft agar were then pooled, lyophilized and purified as for the other glioma cell lines.

Aliquots of concentrated conditioned medium from several glioma-derived cell lines were loaded into separate lanes and electrophoresed in 10% SDS-polyacrylamide gels (Figure 10). The conditioned medium contained a multitude of separate proteins; commonly occurring proteins shared amongst the glioma-derived cell lines were found at 150 kD and 54 kD.

9.3.4 Molecular Sieve Chromatography

The majority of the protein within the acid-extracted conditioned medium from glioma cell lines SF-210, U-251 MG, and U-343 MG-A was found in the void volume of the molecular sieve column chromatograms (Figures 11,14, &16).

Bio Gel P-60 column fractions (23-43) from SF-210 contained a peak of colony-stimulating activity with an apparent molecular weight range of 15-20 kD. When the active fractions were pooled, lyophilized and resuspended in 5 mM acetic acid and 25 mM NaCl, a dose-dependent stimulation of [^{3}H] thymidine incorporation and colony formation among NRK cells was found (Figure 12). A similar dose-dependent stimulation of human leptomeningeal cell growth (SF-673 M) in soft agar was seen (data not shown) with ng aliquots from the active fractions. When the pooled material from the active Bio Gel P-60 fractions was electrophoresed and silver stained, a series of protein bands was visualized at molecular weights 14-21 kD (Figure 13).

The Bio Gel P-60 fractions from U 343 MG-A with retained transforming growth factor-like activity had a molecular weight range of 6 - 15 kD (Figure 14). The pooled, NRK soft agar-stimulating fractions were comprised of a heterogeneous collection of proteins by SDS-PAGE (Figure 15). Aliquots from the pooled active fractions were similarly mitogenic when tested on NRK cells in the [^{3}H] thymidine assay (data not shown).

TGF-like activity from U 251 MG was separated on a Bio Gel P-30 molecular sieve column. The active fractions had an apparent molecular weight range of 12 - 30 kD (Figure 16), and stimulated the uptake of [^{3}H]-thymidine in a dose dependent fashion in NRK cells (data not shown).

The active fractions from all three glioma cell lines were then tested for their ability to compete with [^{125}I] EGF for the EGF-receptor on A-431 epidermoid carcinoma cells. Only the pooled material from the active fractions of U 343 MG-A was found to effectively compete with the radiolabeled EGF for the EGF receptor (Figure 17). There was no inhibition of [^{125}I] EGF binding when active fractions from SF-210 were used, and only a modest amount of inhibition when similarly active fractions from U 251 MG were used.

9.3.5 Reverse Phase High Pressure Liquid Chromatography

The proteins present in the active fractions of SF-210 and U 343 MG-A from molecular sieve chromatography were further separated by HPLC.

For SF-210, a second run HPLC produced a series of protein peaks, one of which contained TGF-like activity (Figure 18). NRK colony formation in soft agar was maximum at 27% acetonitrile. When aliquots of the purified factor were lyophilized, resuspended in buffer and electrophoresed in 17.5% polyacrylamide gels, a single molecular weight species of 20 kD was found (Figure 19). Aliquots from the active fractions by HPLC were similarly found to increase colony formation among NRK indicator cells and SF-673 M leptomeningeal cells in a dose-dependent fashion (Figure 20).

Fractions capable of inducing NRK soft agar growth were similarly found with U-343 MG-A by HPLC. A broad peak of TGF-like activity was found to be maximum at 32% acetonitrile (Figure 21). By SDS-PAGE analysis, the pooled active fractions from HPLC were still heterogeneous in protein composition (Figure 22) with significant protein bands found at 6, 12, and 15 kD. Active fractions from HPLC stimulated colony formation among NRK indicator cells but not leptomeningeal cells in soft agar (Figure 23).

9.3.6 Physicochemical Characterization of Glioma Transforming Growth Factors

The essential properties of the glioma TGFs from SF-210 and U 343 MG-A were determined through a series of physicochemical studies, the results of which are shown in Tables II and III. The TGFs from SF-210 and U 343 MG-A are proteins which are stable to acid and heat, but labile to base and reducing agents.

The potency of the TGF(s) isolated from U 343 MG-A is greatly increased in the presence of TGF-beta (10 ng/ml) but not EGF (10 ng/ml). Passage of the purified TGF over a column of heparin-Sepharose does not significantly alter NRK colony formation. And monoclonal antibodies to TGF-alpha completely eliminate NRK colony formation. These data when considered with the ability of the partially purified factor from Bio-Gel P60 to compete with the EGF-receptor strongly imply that at least one of the proteins secreted and isolated from U 343 MG-A with transforming potential is in fact TGF-alpha.

In contradistinction, the purified transforming growth factor from SF-210 is not potentiated by the addition of TGF-beta or EGF, and is not inhibited by TGF-alpha monoclonal antibodies. Furthermore, this 20 kD soluble polypeptide causes the anchorage independent growth of fetal human leptomeningeal cells (SF-673 M) in soft agar (Figure 24). No other factors including PDGF, EGF, TGF-alpha, TGF-beta, or bFGF at appropriate concentrations stimulated 673 M colony formation in soft agar. Finally, the partially purified factor does not bind to the EGF receptor. Thus it appears that the malignant glioma cell line SF-210 secretes a novel TGF with neither TGF-alpha- nor TGF-beta-like activity which alone stimulates human leptomeningeal-derived cells to grow anchorage independently in soft agar.

The TGF purification schemes for SF-210 and U 343 MG-A are shown in Tables IV and V. Following protein separation by HPLC on uBondpak c 18 columns, the TGFs isolated from SF-210 and U 343 MG-A were purified 160 fold and 200 fold respectively.

.c3.9.3.7 Immunochemical Localization of TGF-alpha in Human Malignant Brain Tumors:

The immunohistochemical localization of TGF-alpha in 20 human normal brain and brain tumor specimens is summarized in Table VI.

Two glioblastoma multiformes were found to have positive intracytoplasmic staining for TGF-alpha (Figure 25). However, not all malignant cells were stained equally well, and many tumor cells were unstained.

All five moderately anaplastic astrocytomas stained postively for TGF-alpha (Figure 26). A greater percentage of tumor cells were positively identified than in the glioblastomas. The perivascular regions and capillary endothelial cells were unstained.

Positive staining was appreciated in 1/2 highly anaplastic astrocytomas. No positive stainin was seen in 1 meningioma, 1 hemangioblastoma, 1 Schwannoma, and 2 normal adult human brain specimens.

Scattered primitive neuroblasts were positive within human fetal brain specimens, 16-22 weeks gestational age (Figure 27).

Positive staining for TGF-alpha at the dermal-epidermal junction within human fetal material served as a positve control in all cases (J. Wilcox, personal communication).

9.3.8 Expression of TGFalpha mRNA in Human Malignant Gliomas

On a preparative agarose DNA gel, the TGF-alpha cDNA insert (approximately 1250 bases) was identified (Figure 28). The insert was recovered and purified (Figure 29) in sufficient quantity to be radiolabeled and used in Northern hybridizations.

The presence of TGF-alpha mRNA was examined in a variety of malignant glioma cell lines and human malignant brain tumor specimens. TGF-alpha mRNA of about 4.5 - 4.8 kilobases was clearly present within the total RNA preparation of U 343 MG-A (Figure 30). A message for TGF-alpha was not found in either U 251 MG or SF-210.

TGF-alpha mRNA was not detected by Northern hybridization in 3 malignant gliomas, 1 ependymoma and 3 specimens of normal human brain.

9.4 Discussion

This study provides the first direct experimental evidence that human malignant gliomas secrete soluble polypeptides with TGF-like activity. In addition, the data presented here support the paracrine hypothesis concerning the pathogenesis of the gliosarcoma as stated in Section1.1: That malignant glioma cells secrete TGFs which cause the phenotypic transformation of previously normal mesenchymal cells.

The autocrine hypothesis of carcinogenesis maintains that a malignant cell both produces and utilizes the same growth factor and thus escapes the usual checks and balances imposed on the growth of normal cells (14,99,117). Despite its widespread appeal, the autocrine hypothesis has been exceedingly difficult to prove (99) largely because most tumor cell lines either secrete growth factors or possess growth factor-receptors but rarely both. For this reason, interest has recently been focused on the possibility that tumor-derived growth factors might in addition (or instead) stimulate the proliferation of peritumor fibroblasts and vascular cells in order to provide the necessary vasculature to support relentless tumor growth (73,89,245).

Many epithelial tumor are often associated with striking amounts of peritumoral mesenchymal tissue and ECM (283). This process has been termed desmoplasia and is a property peculiar to some epithelial

malignancies such as breast and colon cancers, and certain neuroepithelial tumors such as the medulloblastoma and the glioblastoma multiforme. In terms of the gliosarcoma, the most widely held hypothesis regarding its pathogenesis is that the continuous, unbridled stimulation of leptomeningeal or perivascular mesenchymal cells by the malignant glioma leads to malignant transformation and sarcomatous formation.

To test this hypothesis in this study, well-characterized human malignant glioma cell lines were used as sources of TGFs. While the histogenetic origins of many of the cell types contained within a glioblastoma multiforme are uncertain, two of the cell lines (U 251-MG and U 343 MG-A) with TGF-like activity express GFAP over serial passages indicating that they at least are of a glial origin. Although the glial origin of the third cell line with TGF-like activity (SF-210) cannot be definitively proved at this time, it is unlikely that SF-210 is derived from a mesenchymal precursor as interstitial collagens are not produced by this cell line (Chapter 6).

The choice of normal human leptomeningeal cells as targets for the glioma TGFs is appropriate for three reasons: 1) Leptomeningeal cells are contiguous to astrocytes in the CNS and separated from them by the glial limitans externa alone. 2) When a gliosarcoma is found in the CNS, the sarcomatous portion often takes origin from the leptomeninges (101), and 3) Well-characterized leptomeningeal cells are readily available for in vitro experimentation. Normal human astrocytes would have provided an attractive alternatve target cell-type for direct testing of the autocrine hypothesis for malignant gliomas. However, one of the major findings of this thesis is that at present normal human astrocytes cannot be cultivated in highly purified forms (Chapter 3).

Since TGF's are secreted by transformed cells in culture at such low concentrations, the rapid and efficient concentration of large volumes of tumor-derived conditioned medium has been of prime importance in the biochemical and biological analysis of TGFs (158,271). In this study, glioma conditioned medium was concentrated by standard lyophilization techniques, by controlled-pore glass column chromatography, and by ultrafiltration. Of these, ultrafiltration through a hollow-fiber filter apparatus proved to be the most efficient technique. While the volumes of conditioned medium used in our study were modest (4 - 8 L/ glioma cell line) in comparison to what others have reported (20 - 100 L/tumor cell line), nevertheless our yield of

partially purified TGFs by HPLC was similar to that previously described (0.1 - 10 ug/liter) (9,19,154,158).

The biologic assay upon which the amount of TGF-like activity in a tumor sample or cell line is based is the growth of normal indicator cells in an anchorage-independent system. To date, a variety of epithelial and fibroblastic cell lines have been used to study the effects of TGFs on soft agar colony formation, but most studies have utilized NRK cells (clone 14) and AKR-2B cells as the common indicator cells because of their exquisite sensitivities to TGFs (212,229). Despite the general acceptance of the utility of certain normal indicator cell lines in TGF bio-assays, some caveats are needed. First, Salomon et al (229) have recently shown that normal mouse or rat mammary epithelial cells are better target cells for TGFs isolated from conditioned medium from breast carcinoma cell lines than are fibroblasts. Thus, it is becoming apparent that TGFs produced by one tumor cell type may be able to stimulate the anchorage-independent growth of a particular indicator cell type yet be completely unable to facilitate the soft agar growth of another. Second, Rizzino et al (209) have clearly shown that the use of a single indicator cell line may be fraught with difficulties in any TGF analysis because of potential growth factor synergism. In their study, FGF and PDGF were found to potentiate the soft agar growth of NRK indicator cells depending on whether plasma-supplemented, serum-supplemented or serum-free medium was used. And third, there continues to be considerable lab-to-lab variability in terms of TGF-effect on the agar growth of normal indicator cells, including NRK cells. For example, it has been reported by some (58) that TGF-alpha by itself will cause NRK growth in soft agar, whereas others (8,158) have stated that TGF-alpha (or EGF) will only cause NRK soft agar growth in the presence of TGF-beta. Despite these definite limitations and shortcomings, the ability for cells to grow in soft agar probably remains the best <u>in vitro</u> assay for neoplastic transformation (180,212).

In our study, three of eight well-characterized human malignant glioma cell lines produced soluble factors which resulted in the anchorage-independent growth of NRK cells in soft agar. The TGF-like factors from the conditioned medium of two of the three gliomas were partially purified and characterized as described below. Todaro et al (271) were the first to show that growth stimulatory factors released by human melanoma,

rhabdomyosarcoma, and bronchogenic carcinoma cell lines could induce the anchorage-independent growth of normal human fibroblasts. These normal human fibroblasts were capable of achieving a 4.2% cloning efficiency in soft agar when exposed to the tumor-derived TGFs.

The TGF isolated from U 343 MG-A by HPLC is acid- and heat-stable with a molecular weight range of 12 kD - 20 kD. Transforming activity is destroyed completely under basic conditions (pH 12.0), with reducing agents, and following incubation with anti-TGF-alpha monoclonal antibodies. In addition, NRK colony formation is potentiated when TGF-beta (10 ng/ml) is added to the the partially purified TGF from this malignant glioma cell line. Finally, aliquots of this partially purified TGF from the gel filtration column were found to compete directly with radiolabeled [^{125}I]-EGF for the EGF-receptor on A431 epidermoid carcinoma cells. Taken together, these physicochemical data imply that U 343 MG-A glioma cells secrete a TGF-alpha-like growth factor. We cannot definitively state at this point that TGF-alpha is the only molecular species purified from U 343 MG-A glioma cell conditioned medium by HPLC because, by SDS-PAGE, 3 protein bands were identified at molecular weights 6 kD, 12 kD and 15 kD. However, Bringman et al (29) have recently shown that TGF-alpha is synthesized as a much larger 160 residue long precursor which is a glycosylated and palmitoylated transmembrane protein. Cleavage of this large precursor TGF-alpha molecule at various domains results in the detection in tumor-derived conditioned medium of several molecular species (5 kD, 12.5 kD, 15 kD, 18 kD and 20 kD) many with retained TGF-alpha activity. It is therefore conceivable that the protein bands that we observed on SDS-PAGE from the HPLC-purified U 343 MG-A conditioned medium actually represent heterogeneous forms of TGF-alpha.

It is unlikely that the partially purified U 343 MG-A TGF-alpha-like molecular complex contained FGF or PDGF. Since FGF (and all the angiogenic factors) has high affinity for heparin (64,244), we passed the U 343 MG-A purified complex over a column of heparin-Sepharose and found that the NRK transforming activity did not bind to the column. And since well-characterized antibodies to PDGF exist, we incubated aliquots of anti-PDGF monoclonal antibodies with the purified U 343 MG-A TGF and found similarly that activity was not altered. Thus it appears that when conditioned medium from the malignant glioma cell line U 343 MG-A is processed

according to TGF-purification protocols, a conventional TGF-alpha-like molecule can be isolated.

The purified TGF isolated from SF-210 is similarly acid- and heat-stable and shows lability towards base and reducing agents. In contrast to the TGF-alpha-like factor isolated from U 343 MG-A, the SF-210 TGF is not potentiated by addition of TGF-beta to the NRK soft agar system, is not inhibited by monoclonal antibodies to TGF-alpha, and does not compete with radiolabeled-EGF for the EGF receptor. Furthermore, the SF-210 TGF-activity does not bind to a heparin-Sepharose column and is not eliminated by incubation with monospecific antibodies to PDGF. By SDS-PAGE, the HPLC-purified SF-210 TGF is resolved as a single protein band at 20 kD whose migration is not altered by reducing agents. These results imply that the purified SF-210 TGF is neither TGF-alpha nor TGF-beta. Furthermore, this purified TGF does not appear to resemble other conventional growth factors by molecular weight or physicochemical criteria.

Recently a third class of TGFs (TGF-gamma) has been described which induce the clonogenic growth of NRK cells in the absence of EGF (103,278,284,285). Compounds with TGF-gamma-like activity neither bind to the EGF-receptor nor potentiate TGF-alpha (103,278,284). Interestingly, TGF-gamma has been found in high concentration in a neuroblastoma (209). And Tsushima et al have identified a 15 kD species from human anterior pituitary glands with TGF-gamma-like activity. However, until such time as the final purification of these TGF-gamma factors is achieved, the term TGF-gamma must understandably be used to describe a heterogeneous collection of soluble polypeptides whose behaviour differs considerably from conventional TGFs.

The TGF we have isolated from SF-210 appears to be a novel growth factor with TGF-gamma-like activity. In addition to its physicochemical properties described above, the SF-210 TGF was capable of inducing human fetal leptomeningeal cells (SF-673 M) to form colonies in soft agar. Neither the partially purified TGFs from U-343 MG-A and U 251 MG, nor the purified growth factors (TGF-alpha, TGF-beta, EGF, PDGF, bFGF) by themselves could stimulate SF-673 M leptomeningeal cells to grow anchorage independently. The phenotypic transformation of previously normal human leptomeningeal cells by a novel human glioma-derived TGF as demonstrated here provides direct experimental data to support the hypothesis of

gliosarcoma formation as outlined previously. Interestingly, SF-673 M was the only normal human leptomeningeal cell culture which formed colonies in soft agar in response to the TGFs out of 12 different normal cell cultures analyzed. Thus, SF-673 M, a fetal leptomeningeal cell culture, becomes an important tool with which to study the biochemical properties and mechanism of action of the novel TGF secreted by SF-210. The fact that SF-673 M is a fetal culture raises important questions concerning the expression of specific growth factor receptors on fetal cells which may not be present in the adult state. And finally, since only the partially-purified TGF from SF-210 could induce normal leptomeningeal colony formation, the possibility exists that unique brain-derived TGFs may play important roles in the genesis of certain neoplasms peculiar to the CNS.

While the data presented here have helped answer some fundamental questions concerning the pathogenesis of the gliosarcoma, it is clear that the results are only preliminary and further experimentation is needed. First, the partially characterized TGFs from U-343 MG-A and from SF-210 in particular need to be purified completely. The most efficient way to purify these TGFs would be by performing an amino acid sequence analysis of the amino-terminal of each and by comparing this sequence with those of other known growth factors. If, in the case of SF-210, a novel amino-terminal sequence is found, then either antibodies could be raised against it, or ribonucleic acid probes could be synthesized which encode for it, so that the entire gene sequence could be determined by molecular biological techniques. Identification of the TGF cDNA would provide a means by which large quantities of purified TGF could be synthesized by recombinant techniques. Second, all the TGF data reported here were derived from in vitro experiments. Because of the obvious limitations and artifacts intrinsic to all in vitro work, it will be of fundamental importance to determine the role of TGFs in human brain tumors in vivo. To this end, an attempt was made to study the distribution of TGF-alpha in human brain tumors in situ.

In our immunohistochemical analysis of 20 human brain specimens, TGF-alpha was immunolocalized to malignant cells within a series of malignant glial neoplasms. Interestingly, there was a trend toward increased immunoreactivity among the more differentiated glial neoplasms. All the moderately anaplastic astrocytomas expressed TGF-alpha to a high degree when compared to highly anaplastic astrocytomas and glioblastoma

multiforme. While the meaning of this pattern is unclear, one possible explanation may be that in the early phase of glial neoplasia (as is presumably the case with moderately anaplastic astrocytomas), the malignant glioma cells are obligate secretors of relatively high quantities of TGF-alpha in order to escape growth-inhibitory-control factors and to become autonomous with respect to the restrictions of the normal cell cycle. With progressive neoplasia, the malignant glioma cells may require less TGF-alpha in order to replicate. It is well known that, in order to replicate, most poorly differentiated tumor cell lines require less in the way of exogenous growth factors than do highly differentiated tumor cell lines and normal cells in culture (99,290). Of interest, other brain tumor-types including meningiomas, hemangioblastoma, and schwannomas were not positively identified with the TGF-alpha antibody in this study, but specimens from fetal brain were lightly but reproducibly immunostained. TGF-alpha has now been localized to a variety fetal tissues (235). Its presence in embryogenesis but not in normal adult states suggests that the gene for TGF-alpha may be activated in fetal states but repressed in normal adult states. Tumorigenesis may proceed with derepression of the gene for TGF-alpha.

The localization of TGF-alpha within human brain tumor specimens was further assessed by an analysis of the presence of mRNA coding for TGF-alpha within a small series of human brain tumors. None of the brain tumors from which adequate RNA could be harvested was found to contain the message for TGF-alpha. However, an RNA-preparation from U-343 MG-A was found to contain a 4.8 kilobase message for TGF-alpha. In their analysis of mRNAs for TGFs in human tumors, Derynck et al. (56) found that the message for TGF-alpha existed primarily in epithelial but not mesenchymal or hematogenous tumors. The one glioblastoma multiforme tumor specimen examined in their study did not contain the message for TGF-alpha. Clearly a larger sample of glial tumors must be analyzed before the relative presence or absence of TGF-alpha can be determined definitively.

We have presented data which support the hypothesis that human malignant glioma cell lines secrete TGFs that can cause the phenotypic transformation of previously normal mesenchymal cells. Although this work has been performed largely in in vitro systems and is as such preliminary, it provides the first direct evidence incriminating TGFs in the pathogenesis of human brain tumors. Once the glioma-derived TGFs are

9.5 Figure legends and Tables

Figure 1: Mitogenic effect of glioma-derived conditioned medium on NRK cells. All 4 glioma cell lines shown here were stimulated to increase [^{3}H]-thymidine incorporation with increasing concentration of protein. Conditioned medium from U-251 MG had the greatest effect on NRK cells and increased radio-labeled thymidine uptake to 700% that of serum-free control NRK cells.

Figure 2: Mitogenic effect of selected glioma-derived conditioned medium on fetal human leptomeningeal cells (SF-673 M). A dose-dependent increase in [^{3}H]-thymidine incorporation is seen with increasing protein concentration for each cell line. The stimulatory effects were greatest for SF-188 (600% serum-free control at 10 ug/ml) and least for U 251 MG (210% serum-free control at 10 ug/ml).

Figure 3: Effect of acid extracted conditioned medium from selected glioma cell lines on the uptake of [^{3}H]-thymidine for NRK cells. A dose-dependent increase in radiolabeled thymidine uptake is seen for all cell lines, but the magnitude of [^{3}H]-thymidine incorporation is less than that observed with freshly prepared conditioned medium (cf Fig 1).

Figure 4: Growth of NRK cells in soft agar when incubated in glioma-derived conditioned medium. Of the eight glioma-derived cell lines tested, only three (SF-210, U-343 MG-A, U 251 MG) had conditioned medium which resulted in NRK colony formation in soft agar. A dose-dependent increase in colony formation is seen with the greatest colony formation seen for NRK cells exposed to conditioned medium from SF-210.

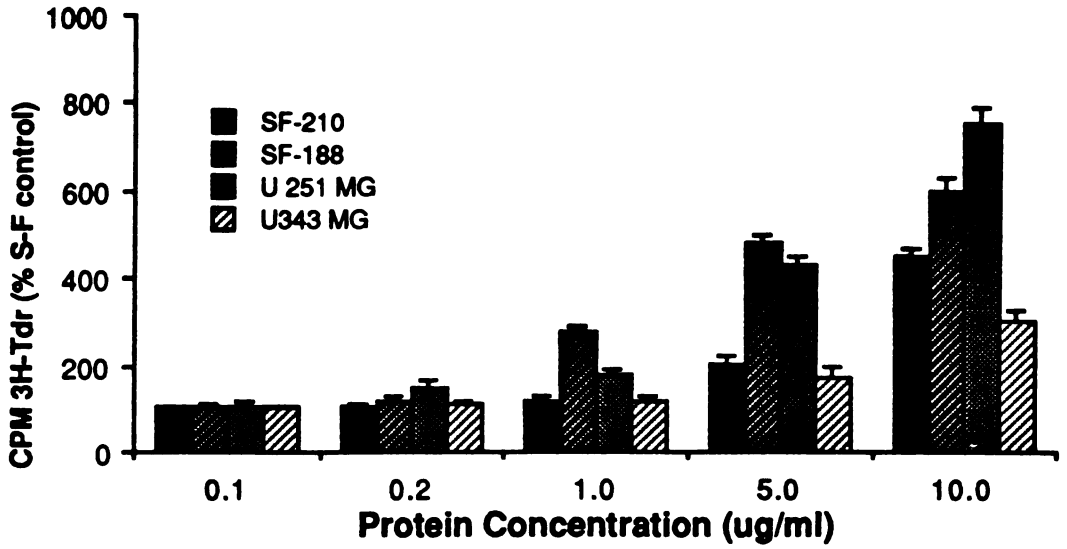


FIGURE 1

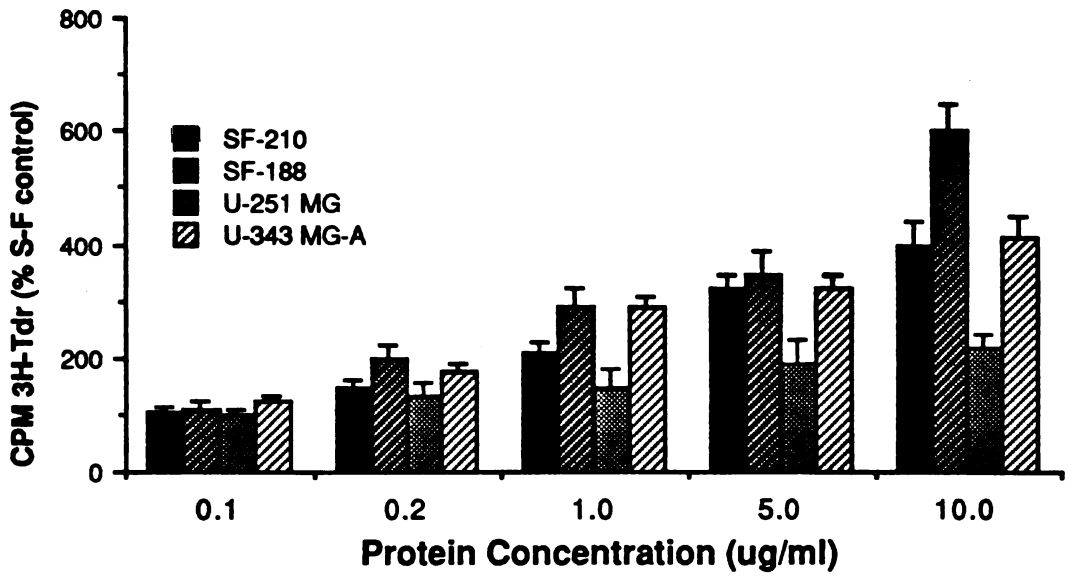


FIGURE 2

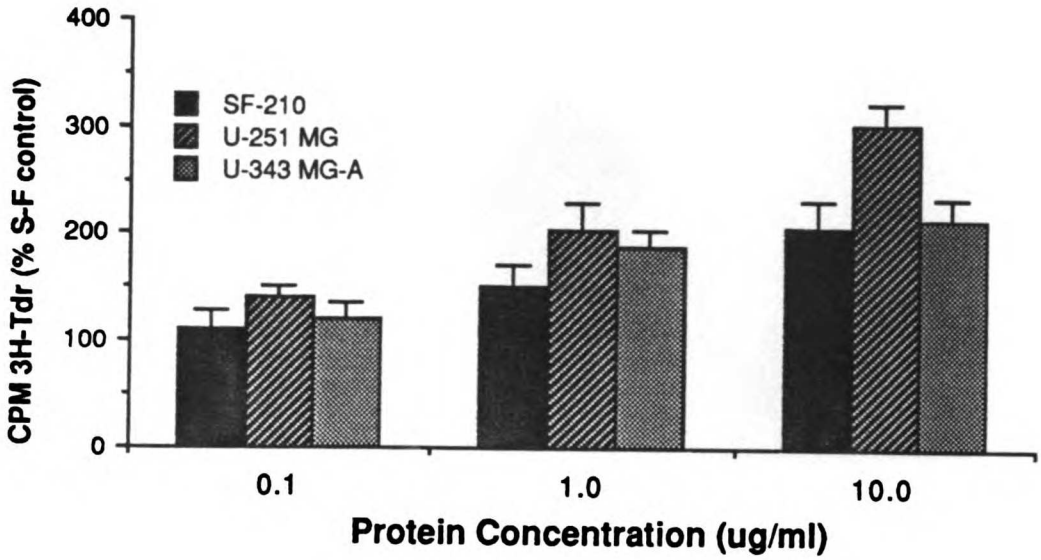


FIGURE 3

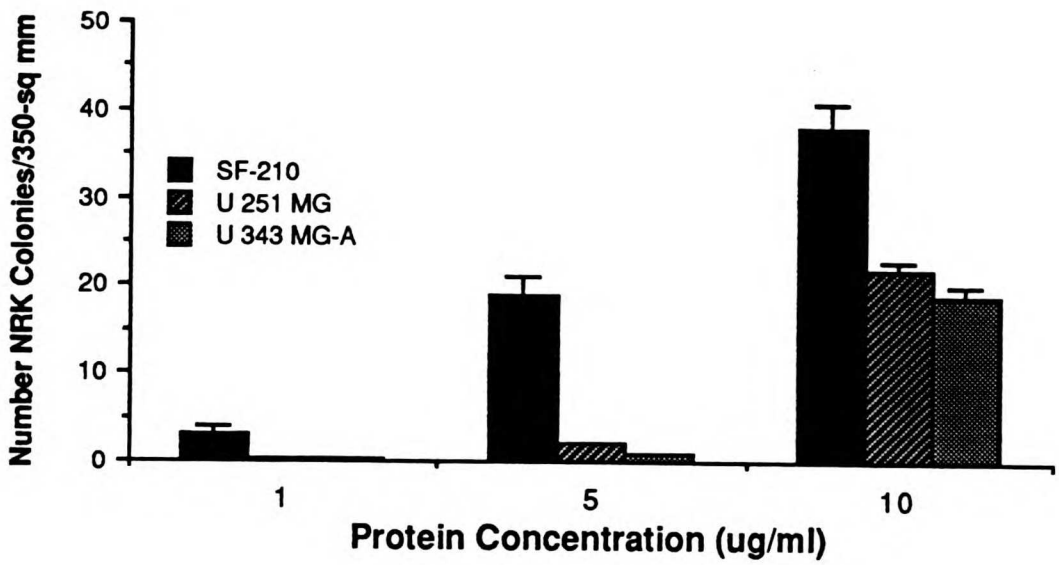


FIGURE 4

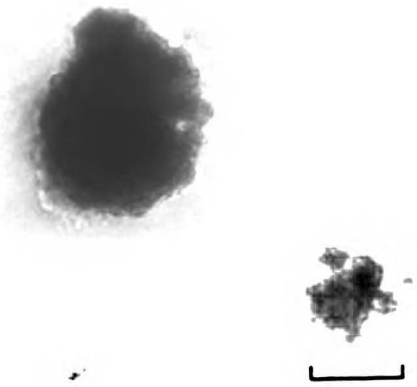


Figure 5: Characteristic large, NRK colony forming in soft agar assay system. Only colonies containing more than 40 cells or greater than 40 um were scored as positive. Line = 40 um.

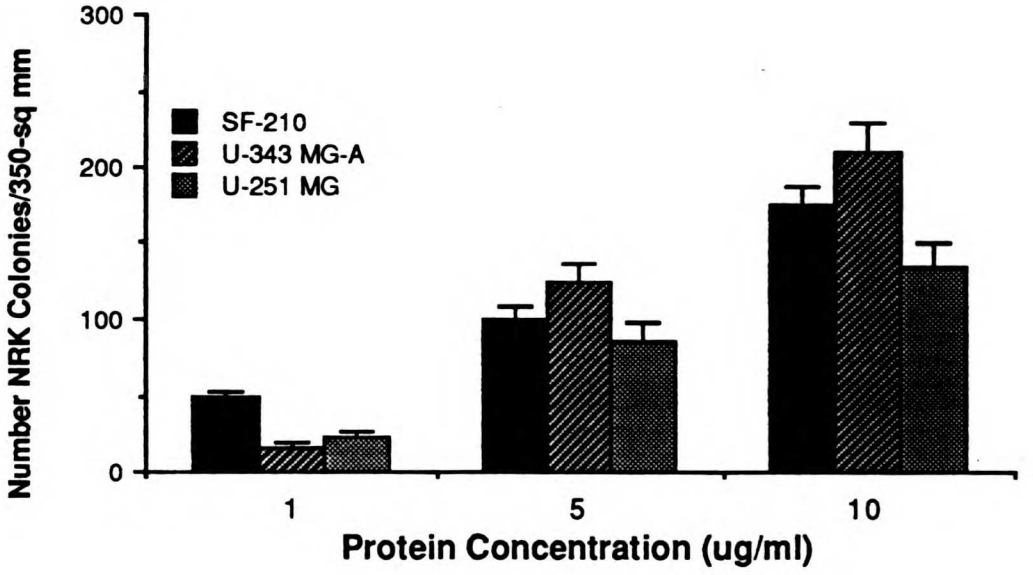


FIGURE 6

Figure 6: Incubation of NRK cells in glioma-derived acid-extracted conditioned medium results in significant colony formation. Colony formation was observed with SF-210, U 343 MG-A and U 251 MG. The greatest number of NRK colonies (205) was seen following incubation with U 343 MG-A conditioned medium at 10 ug/ml.

Figure 7: Growth of normal human leptomeningeal cells in soft agar. From a total of 12 normal human leptomeningeal cell cultures tested, only SF-673 M cells grew in soft agar when exposed to acid-extracted conditioned medium from SF-210. The results of four normal human leptomeningeal cultures are shown. The growth of SF-673 M leptomeningeal cells in soft agar was dose dependent with a maximum response of 60 colonies at a protein concentration of 10 ug/ml.

Figure 8: Effects of purified TGF-alpha on NRK cell growth in soft agar. A dose-dependent increase in NRK colony formation is seen with concentrations of TGF-alpha between 1-10 ng/ml. When TGF-beta (1.0 ng/ml) is added to the bio-assay system, a synergistic response and marked increase is seen with respect to NRK colony formation.

Figure 9: Controlled-Pore Glass Chromatography used to bind proteins from the conditioned medium of U-251 MG with molecular weights 4 kD to 33 kD. Six liters of conditioned medium were passed over the column (1.5 cm x 90 cm) at a flow rate of 100 ml/hr. After the column was washed with phosphate buffer (pH 8.0), adherent proteins were eluted from the column with 20% ethylene glycol in 10 mM sodium phosphate buffer, pH 8.0, containing 1 M sodium chloride. Fractions of 1 ml were collected and tested for absorbance at A_{280} and for NRK colony stimulation. Active fractions (20-37) were collected, pooled and used in further purification steps.

Figure 10: Silver-stained 10% SDS-polyacrylamide gel showing distribution and number of proteins found in the conditioned medium of glioma-derived cell lines. Lane C SF-126; Lane D SF-210; Lane E SF-188; Lane F SF-539; Lane G U 343 MG-A; Lane H U 251 MG. Commonly occurring protein bands shared amongst the glioma-derived cell lines are found at 150 Kd and 50 Kd (arrowheads). Protein standards (Lanes A - B) are as shown.

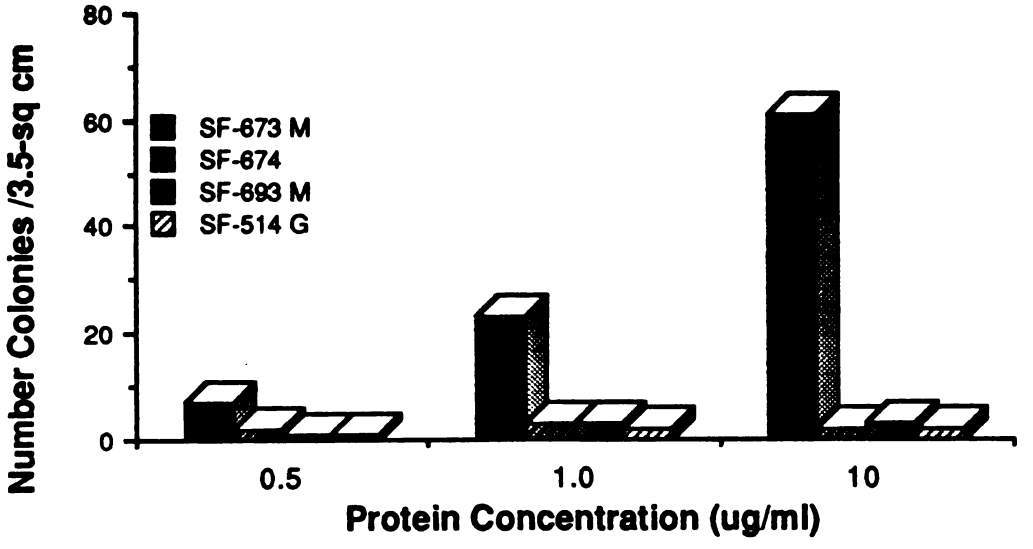


FIGURE 7

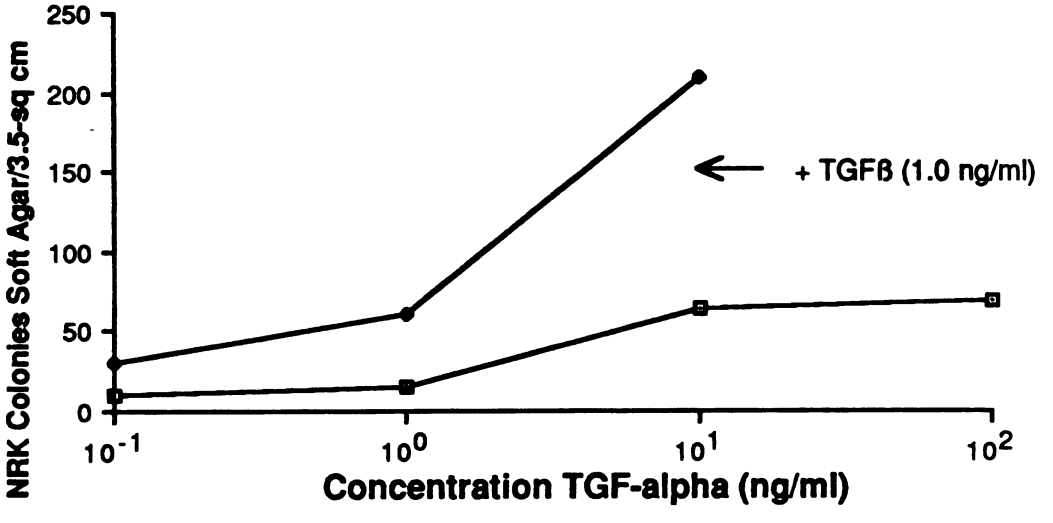
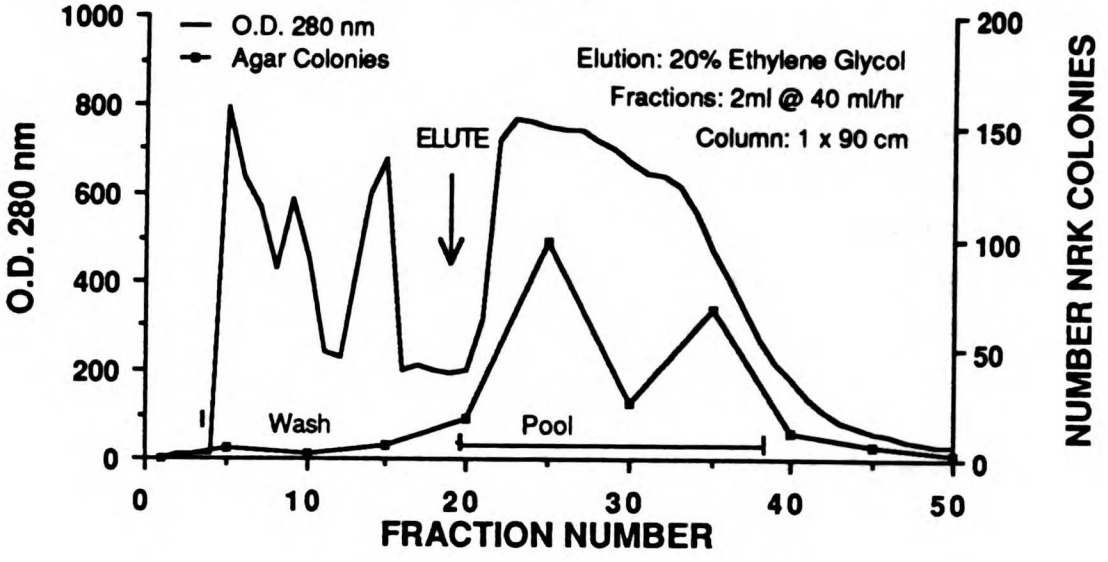


FIGURE 8

FIGURE 9



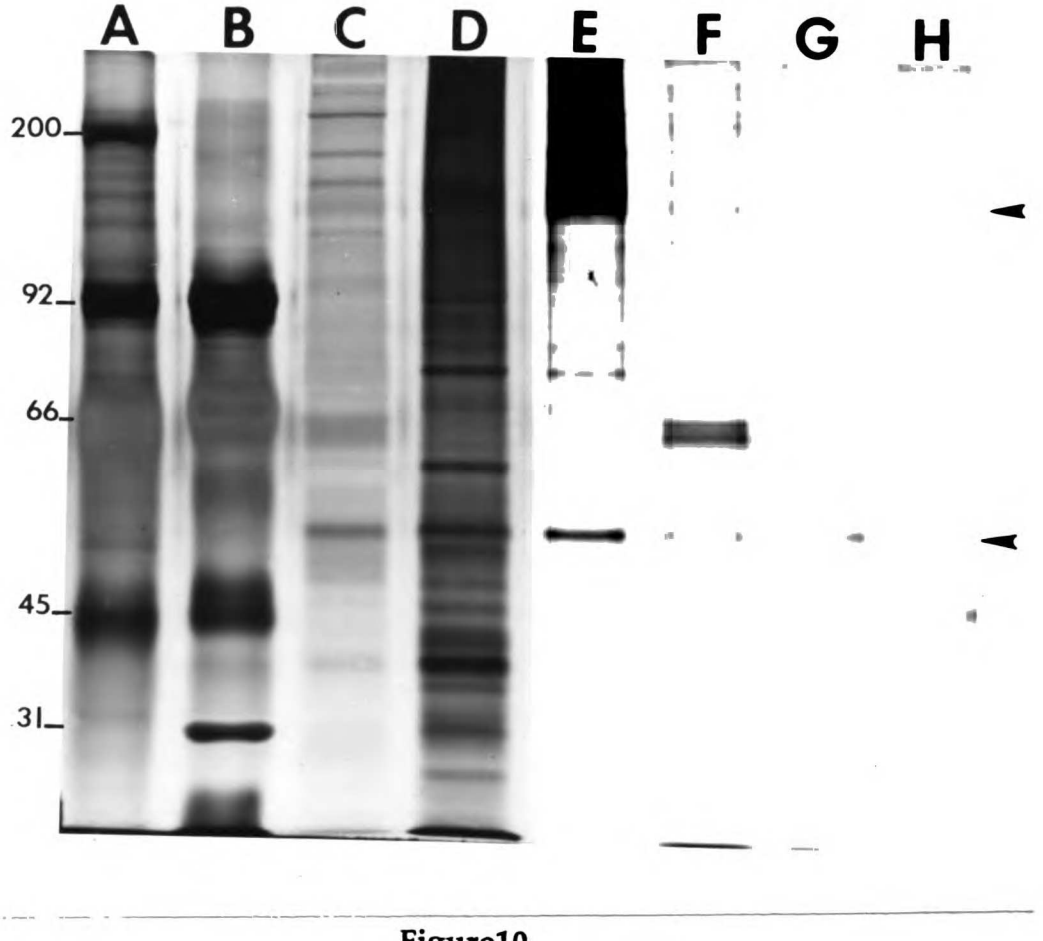


Figure10

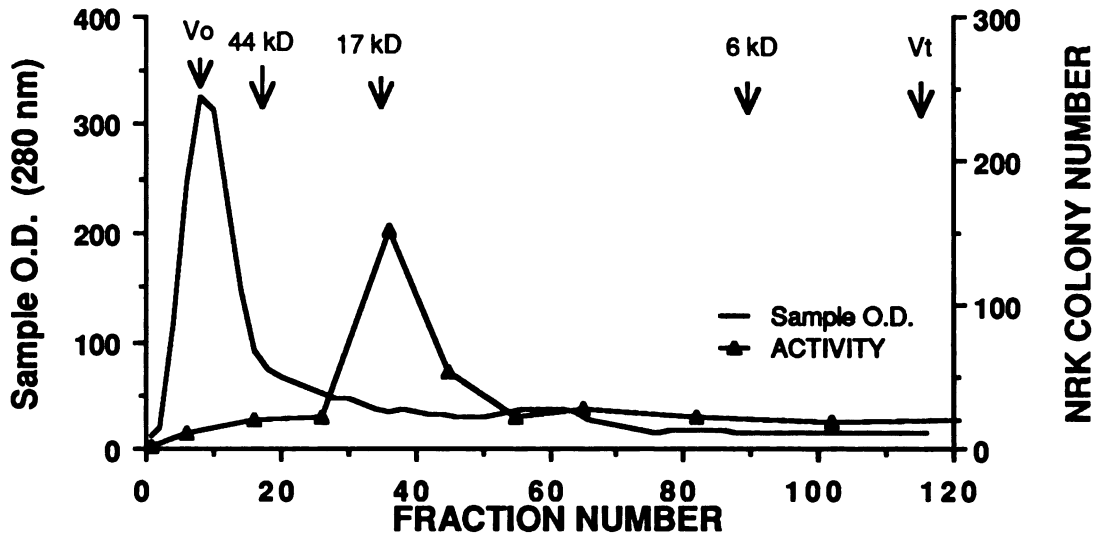
Figure 11: BioGel P-60 gel filtration column for purification of SF-210 glioma-derived conditioned medium. Acid extracted conditioned medium from SF-210 (4 L) was passed over a column (1.5 x 90 cm) of BioGel P60 200-400 mesh pre-equilibrated with 1 M acetic acid. Fractions 22-42 contained a single peak of NRK colony stimulating activity at an apparent molecular weight of 17 kD. The bulk of the proteins contained within the acid extracted sample were found in the void volume. Molecular weight standards are ovalbumin (44 kD), myoglobin (17 kD), insulin (6 kD).

Figure 12: Effect of active aliquots from BioGel P60 column chromatography on NRK colony formation and [^{3}H]-thymidine uptake. With increasing concentration of protein, there is progressive increase in both NRK colony formation (open squares) and thymidine incorporation (filled diamonds).

Figure 13: Silver-stained, 17.5% SDS-polyacrylamide gel containing aliquots of fractions from Bio-Gel P-60 gel filtration column of SF-210. Progressive fractionation of samples into smaller molecular weights is seen with increasing fraction number. At fraction 35, approximately 8 protein bands are visualized between molecular weight ranges of 14 kD and 23 kD. The proteins contained within fractions 23-43 were pooled, lyophilized and processed for HPLC. For comparison purposes, samples taken directly from whole conditioned medium (lane 1) and acid extracted conditioned medium (lane 2) are shown. Molecular weight standards are as shown.

Figure 14: Molecular sieve chromatography (Bio-Gel P60) of acid extracted conditioned medium from U-343 MG-A. A broad peak of activity is seen between fractions 60-100 with an apparent molecular weight of 9 kD. The bulk of the protein was found in the void volume. Molecular weight standards are as shown. Active fractions were pooled, lyophilized and processed for further purification with HPLC.

FIGURE 11



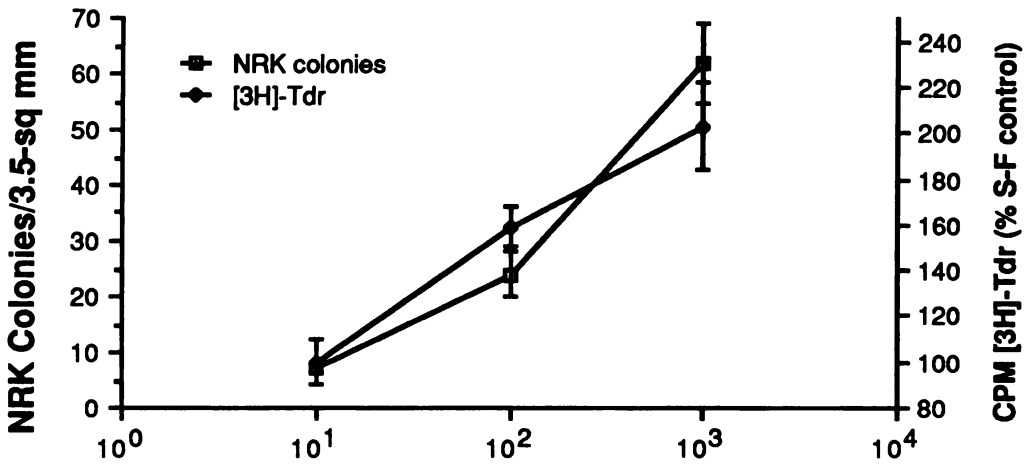


FIGURE 12

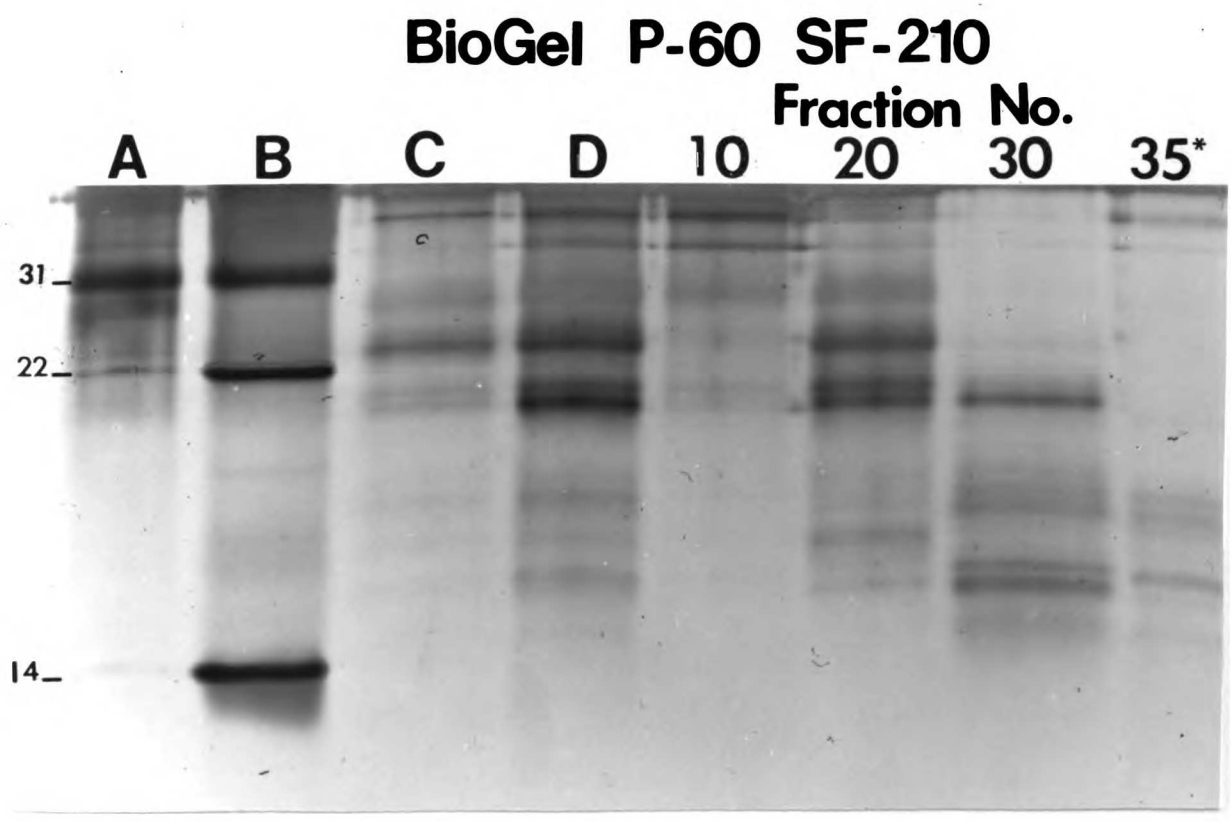


Figure 13

FIGURE 14

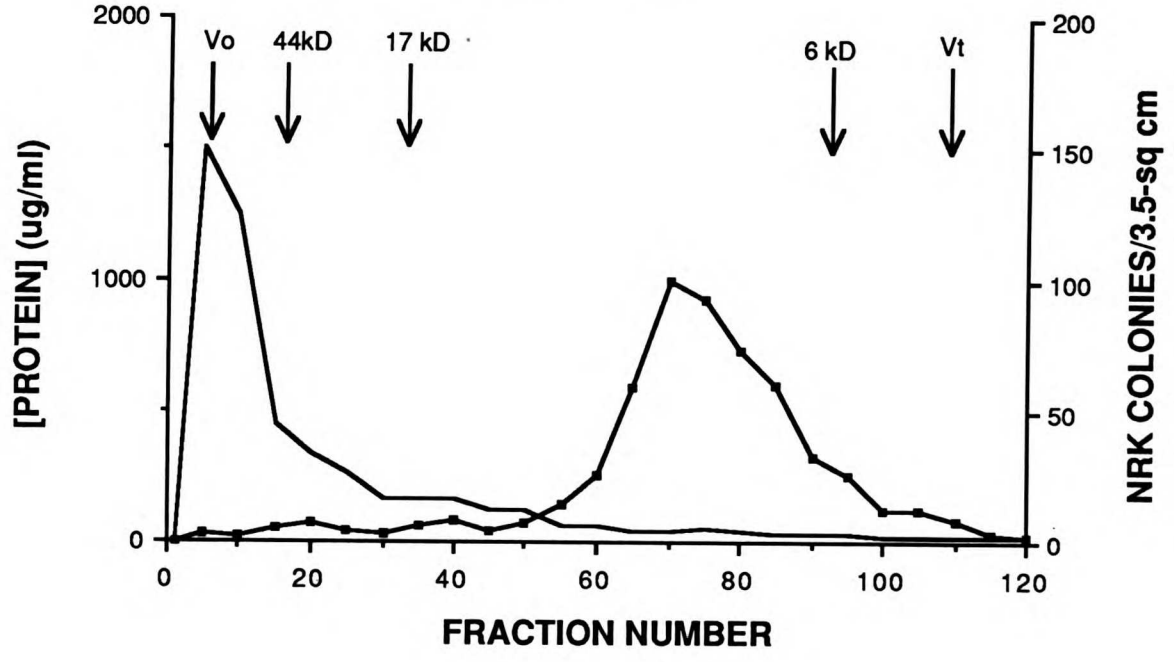


Figure 15: Silver-stained, 17.5% SDS-polyacrylamide gel containing fraction aliquots from Bio-Gel P60 gel filtration column from U 343 MG-A. With increasing fraction number, there is progressive diminution of size of proteins within each fraction. The fractions containing activity were found between 6-12 kD. Active fractions were pooled, lyophilized and processed for HPLC as described.

Figure 16: Molecular sieve chromatography (Bio-Gel P30) of acid extracted conditioned medium from U 251 MG. A broad peak of NRK colony stimulating activity is seen near the void volume with an apparent molecular weight of 12-30 kD. Fractions were similarly monitored for A_{280}.

Figure 17: Effects of glioma-derived conditioned medium on competition for the EGF-receptor on A431 epidermoid carcinoma cells. Partially purified transforming factors from the active fractions of the Bio-Gel molecular sieve columns from U 251 MG, U-343 MG-A and SF-210 were incubated with radiolabeled EGF, and the percent inhibition of EGF binding to the EGF-receptor was measured. Only the TGF from U 343 MG-A competed directly and effectively for the EGF-receptor. The TGF from SF-210 competed not at all, and from U 251 MG only partially.

Figure 18: rpHPLC for TGF from SF-210. The active fractions from Bio-Gel P60 column were lyophilized, and resuspended in 0.05% TFA in water. The sample was applied to the HPLC system and an acetonitrile gradient was used to separate the proteins. Protein absorbance is shown as A_{220}; NRK colony stimulating activity came out as a single peak at acetonitrile concentration of 28%. The active fractions were then pooled, lyophilized in a speed vac apparatus, and tested for physicochemical properties.

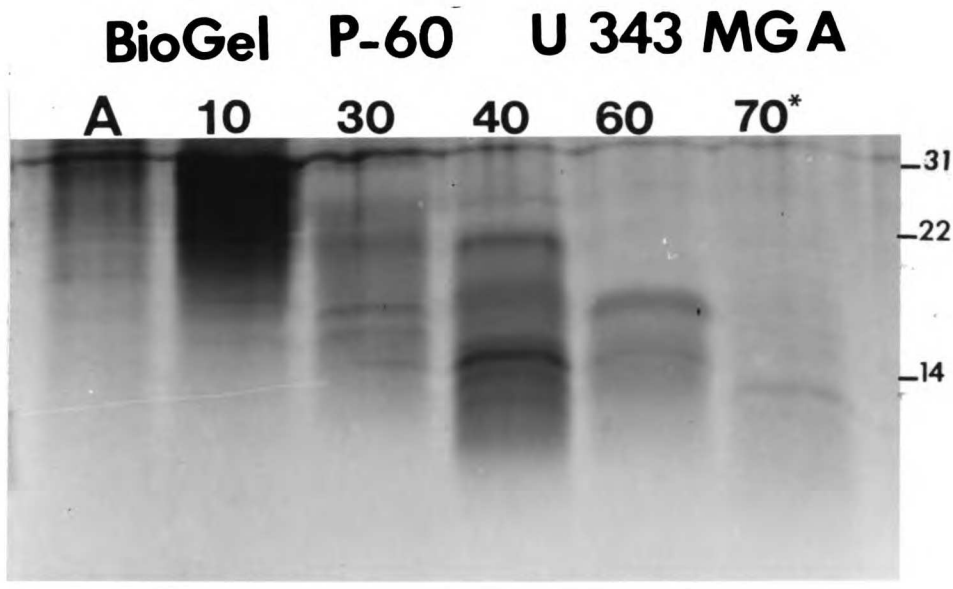
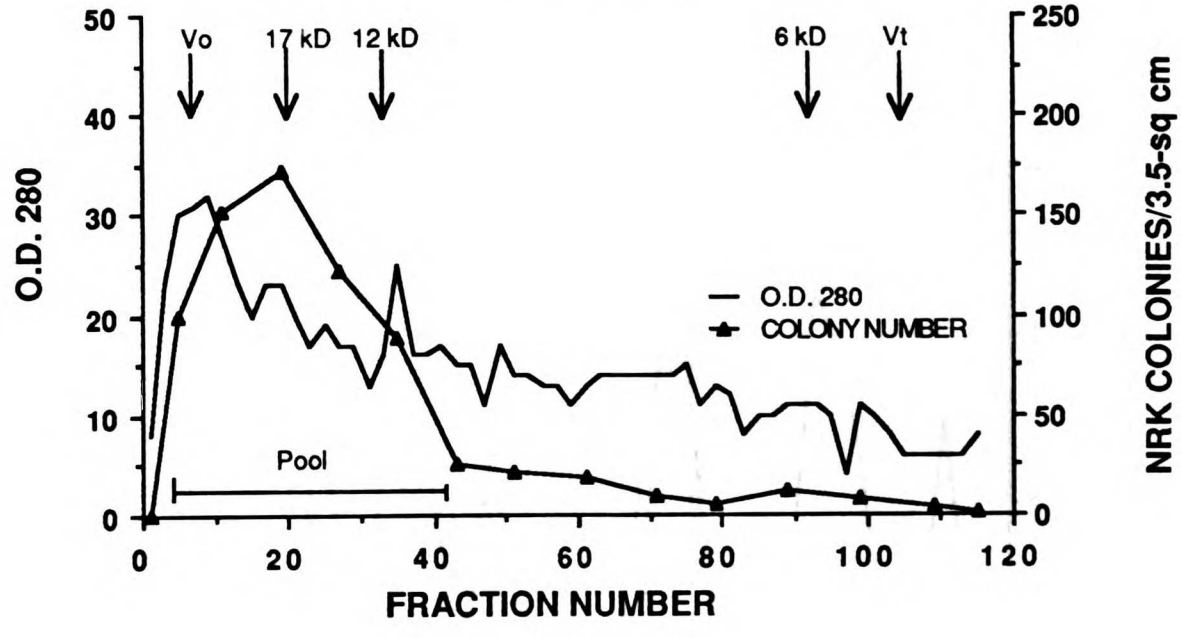


FIGURE 15

FIGURE 16



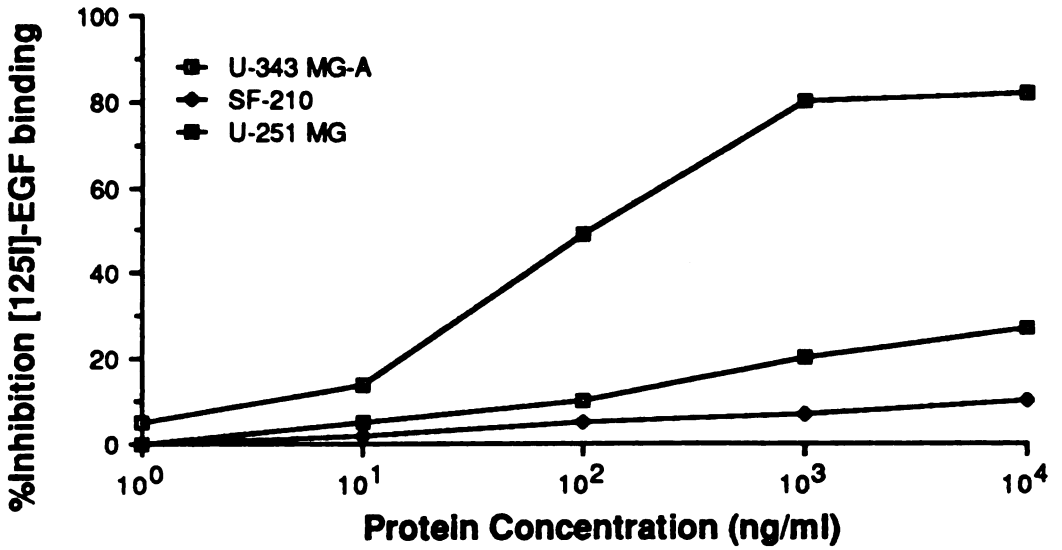


Figure 17

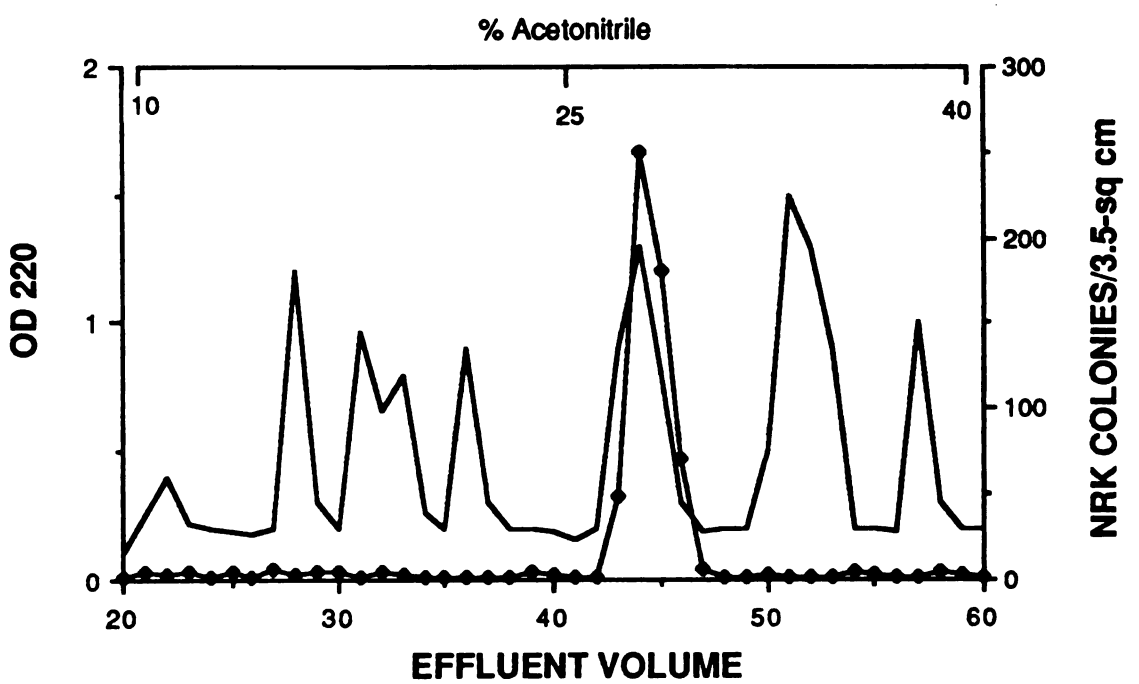


Figure 18

Figure 19: Silver-stained 17.5% SDS-polyacrylamide gel of the active fractions from HPLC for glioma-derived SF-210 TGF. The active fractions contained a single protein species with a molecular weight of 20 kD (lane 3). The protein was not altered in molecular weight under reducing conditions (lane 4). Molecular weight standards are as shown (lanes 1 and 2).

Figure 20: Effects of purified SF-210 TGF from HPLC on NRK and normal human leptomeningeal cell colony formation. With increasing protein concentrations (ng/ml) there is progressive increase in colony number reaching a plateau at approximately 1 ug/ml. NRK cells were more efficient than SF-673 M cells at forming colonies at a given concentration of protein.

Figure 21: rpHPLC separation of proteins from U-343 MG-A Bio-Gel P60 active fractions. By HPLC, a broad peak of NRK colony stimulating activity is found at approximately 32% acetonitrile. Proteins were measured at A $_{220}$. Active fractions were pooled, lyophilized and utilized in subsequent physicochemical studies and in SDS-PAGE.

Figure 22: Silver-stained 17.5% SDS-polyacrylamide gel of the active fractions from HPLC for glioma-derived U-343 MG-A TGF. Three protein species are found at 6, 12 and 15 kD. Molecular weight standards are as shown.

Figure 23: Effects of purified U-343 MG-A TGF from HPLC on NRK and normal human leptomeningeal colony formation. Only NRK cells formed significantly large and numerous colonies in response to increasing concentration of protein.

Figure 24: Effects of growth factors on SF-673 M colony formation. Only the purified TGF from SF-210 caused SF-673M colony formation. All other growth factors and growth factor combinations did not result in significant colony formation among SF-673 M cells. All growth factors were added at a concentration of 10 ng/ml.

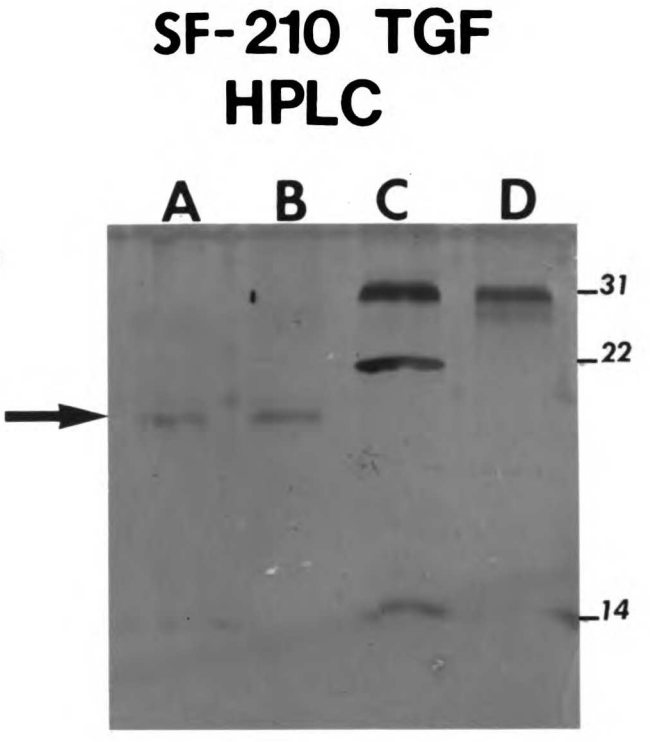


Figure 19

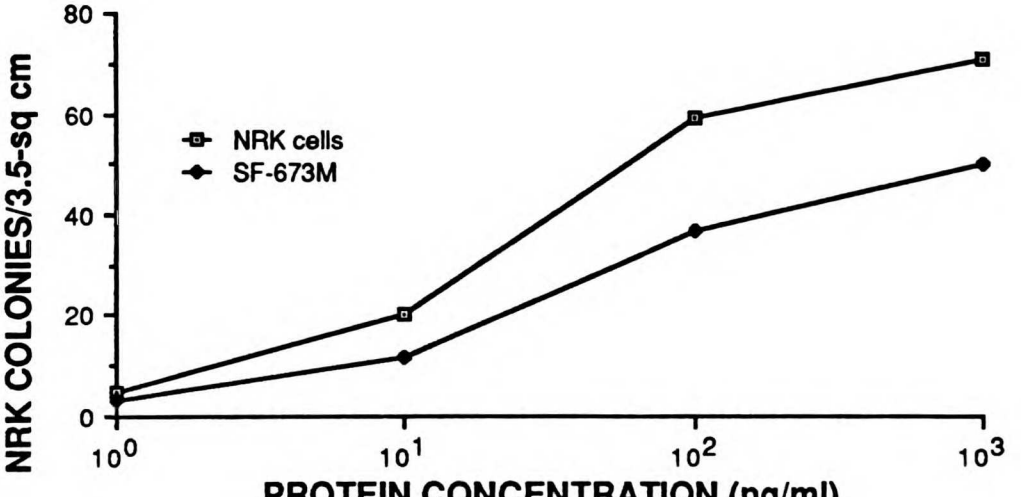


FIGURE 20

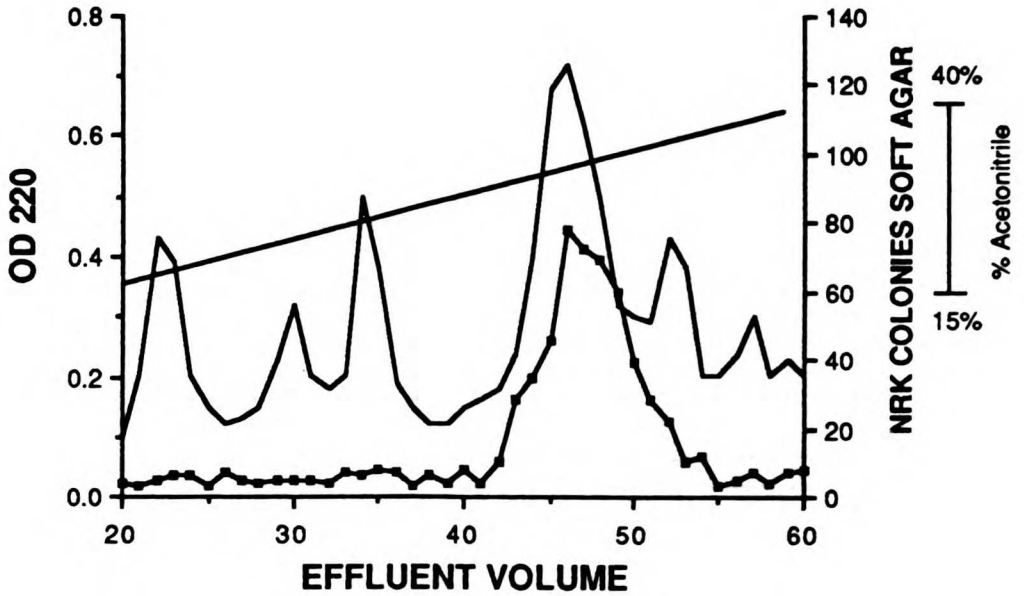


FIGURE 21

U 343 MG TGF

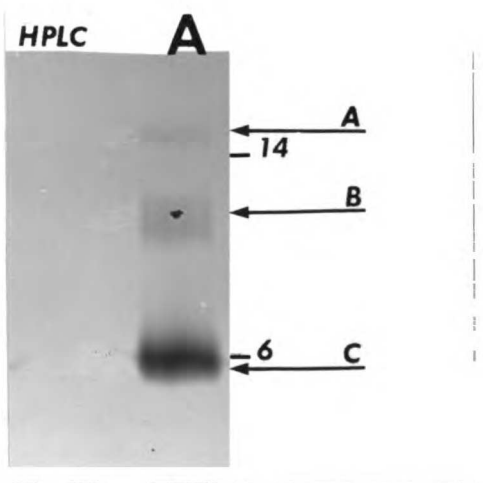


Figure 22

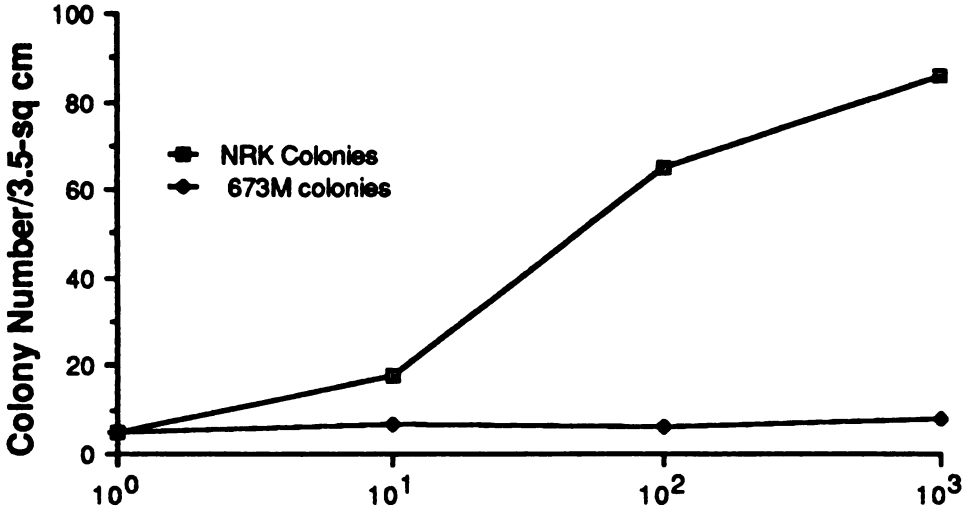
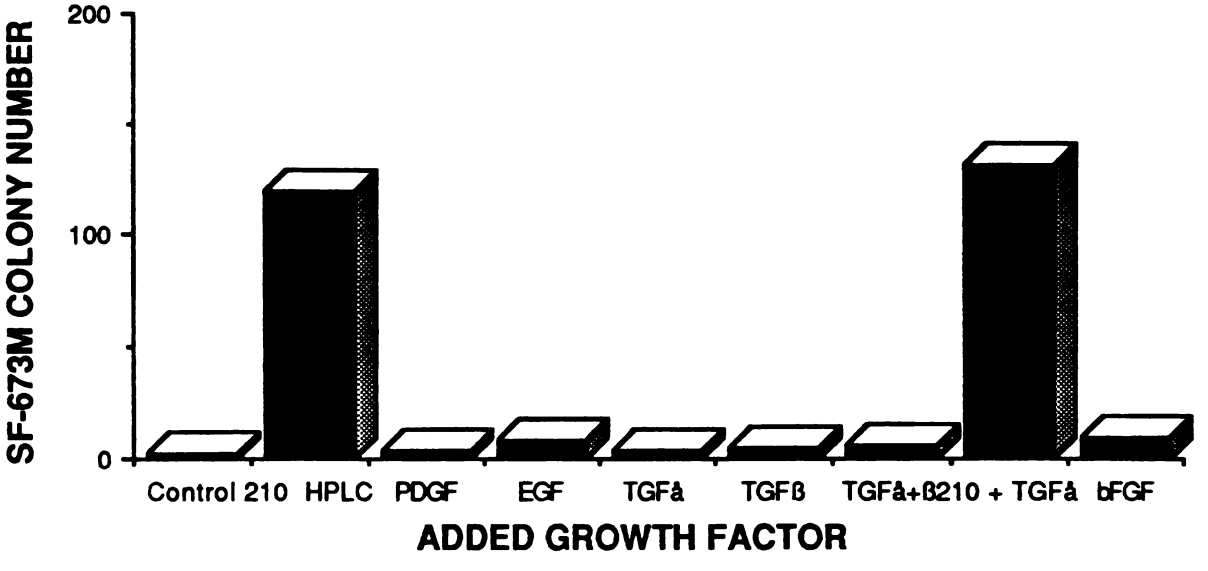


FIGURE 23

FIGURE 24



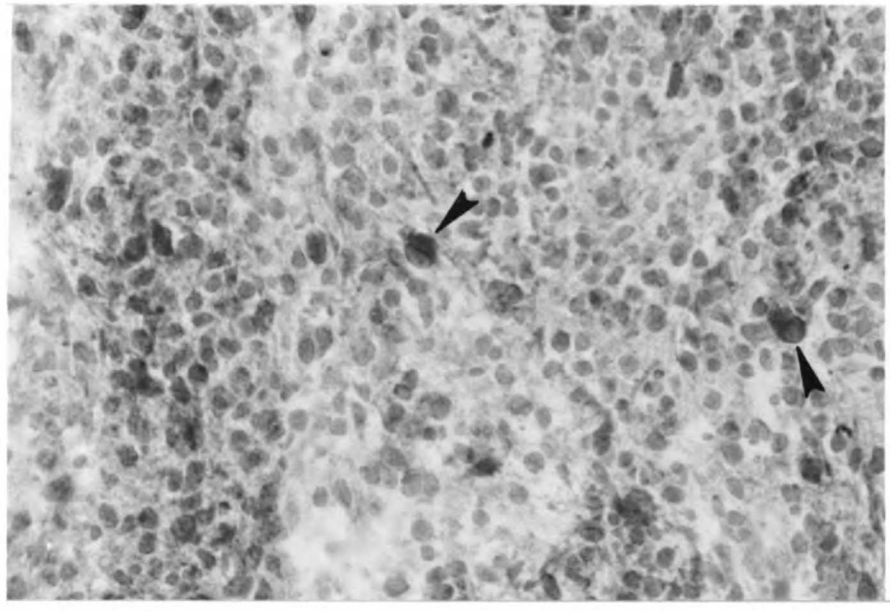
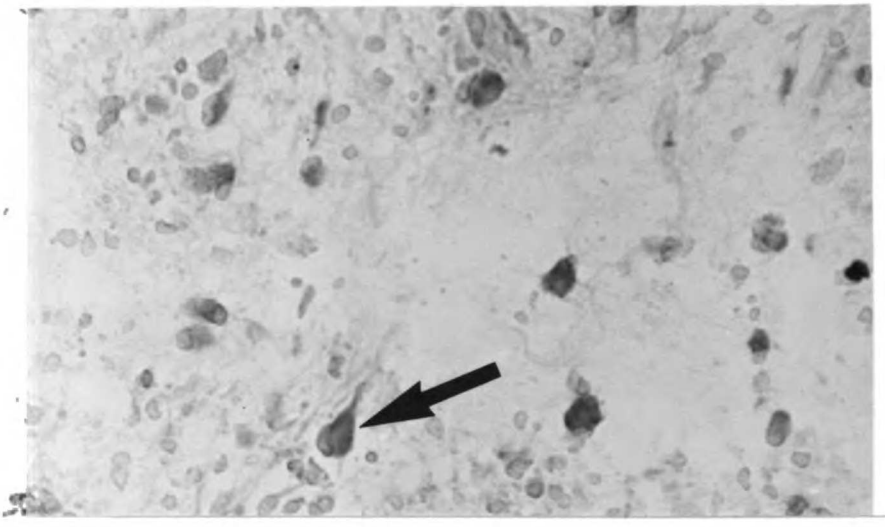


Figure 25: Immunohistochemical localization of TGF-alpha in human glioblastoma multifome. Many cells are positively immunostained for TGF-alpha (arrows). Staining is intracytoplasmic, and vascular elements are unstained. Not all cells are positively immunostained.

Figure 26: Immunohistochemical localization of TGF-alpha in a human moderately anaplastic astrocytoma. A greater percentage of cells are positively identified by the TGF-alpha antibody than are in Figure 24. Staining is intense and is largely intracytoplasmic among malignant cells.



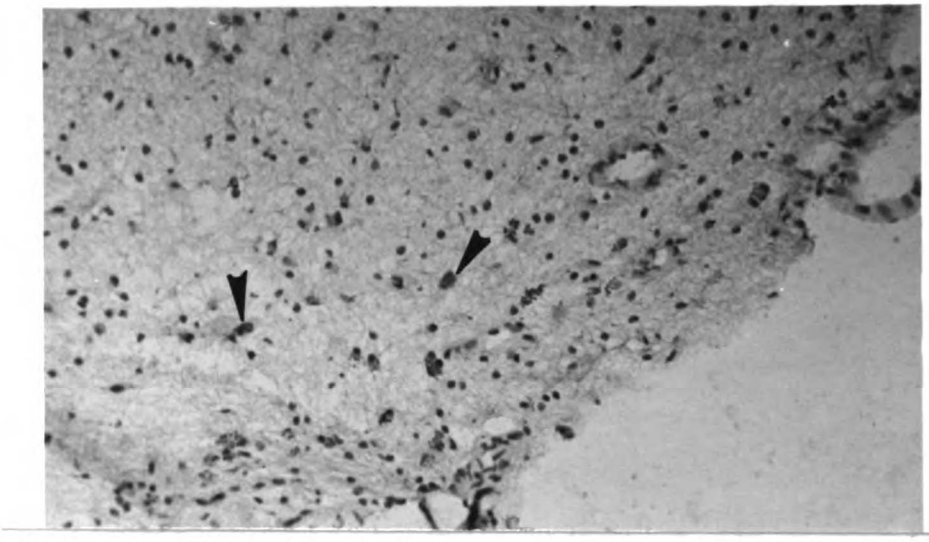


Figure 27: Immunohistochemical demonstrated of faint TGF-alpha immunoreactivity (arrows) amongst primitive neuroectodermal cells in the marginal layer of a human fetal brain specimen, gestational age 18 weeks.

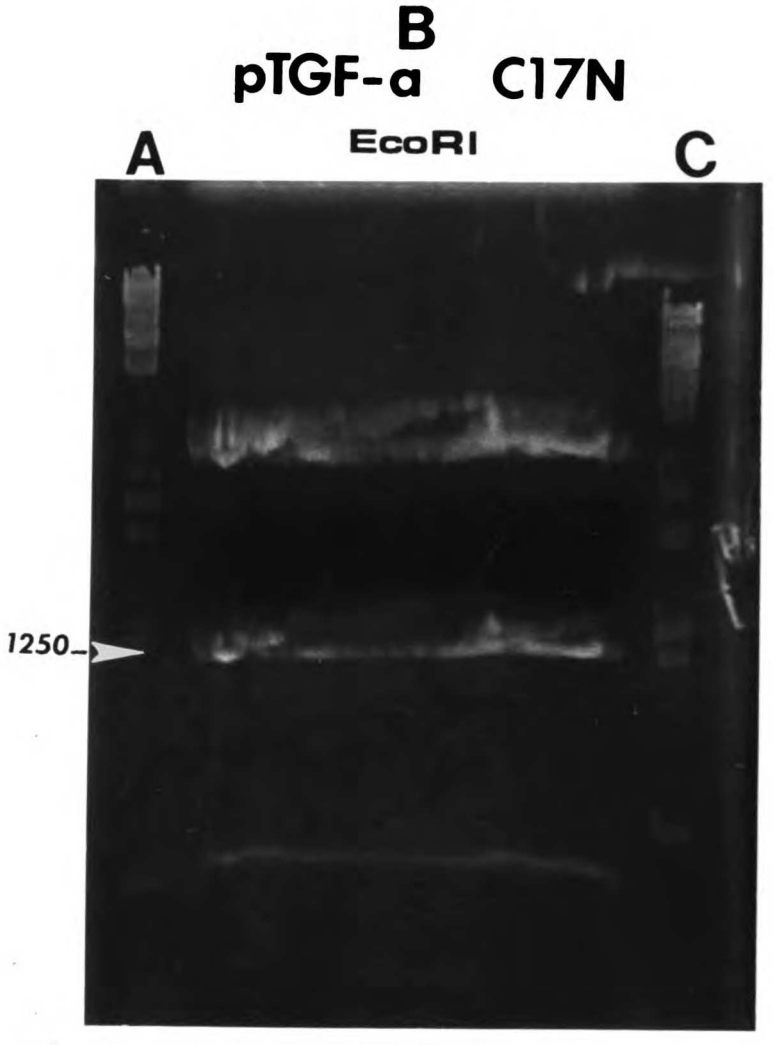


Figure 28: Preparative agarose gel for DNA isolation. Following a restriction endonuclease cut with EcoR1 for 2 hr at 37 C, the TGF-alpha cDNA insert can be identified at approximately 1250 bases (lower arrow). Lambda phage cuts with restriction endonucleases HindIII and Bste I are shown in lanes A and B. Lane C represents uncut plasmid; lane D, the cut plasmid; lane E, the purified insert.

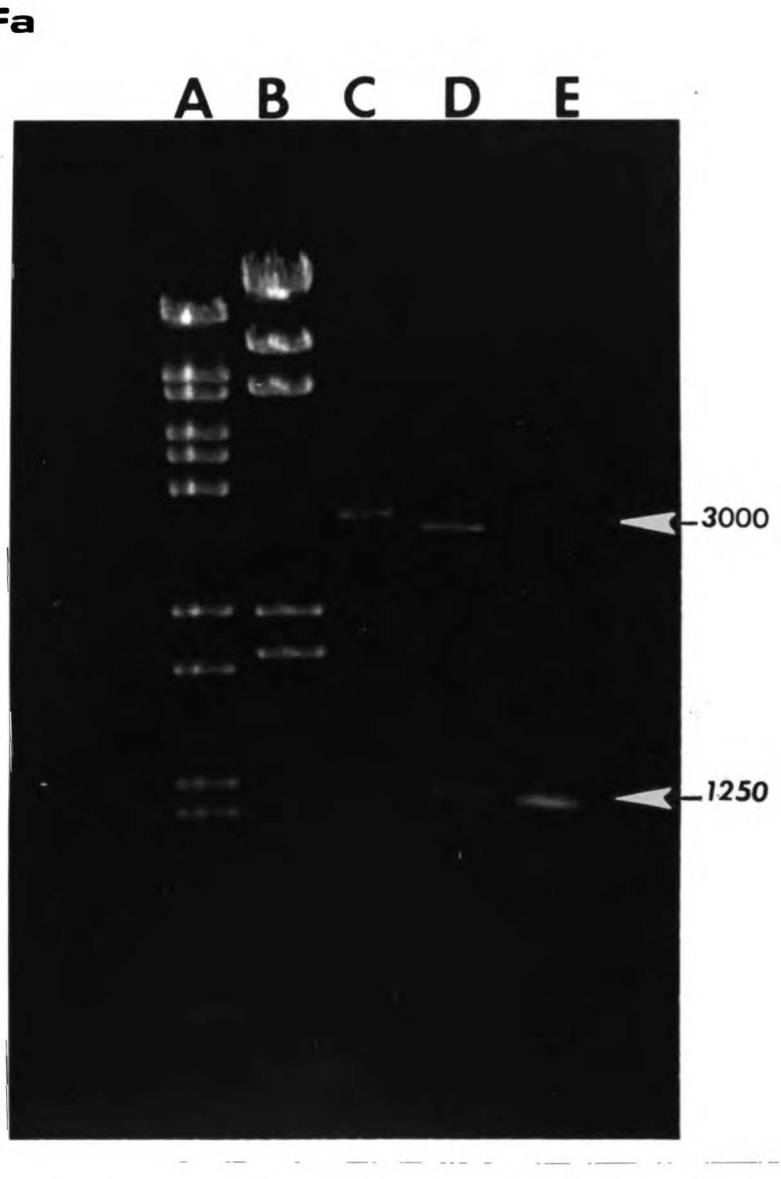


Figure 29: Purification of the TGF-alpha cDNA insert. Following chloroform-phenol extractions, only the cDNA insert was recovered from the agarose DNA gel (arrows).

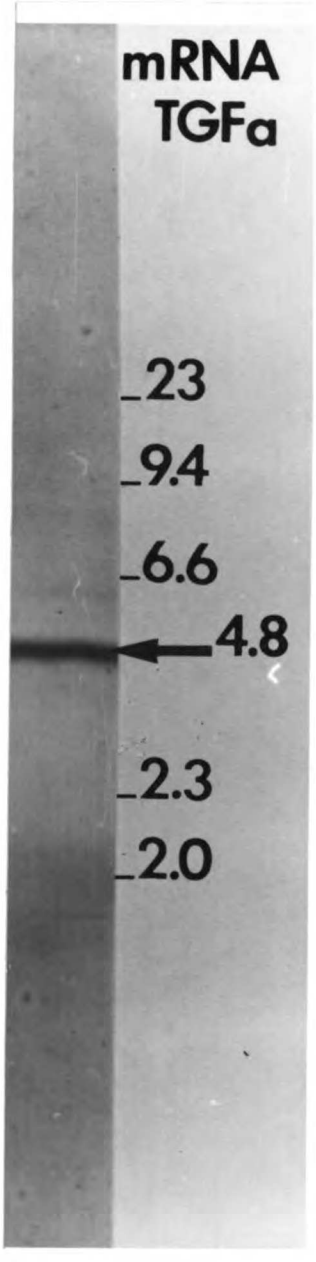


Figure 30: TGF-alpha mRNA level as determined by Northern hybridization in RNA preparation from U 343 MG-A tumor cells. A 4.8 kilobase message for TGF-alpha is clearly identified. RNA standards are as shown. No other tumor specimen or glioma-derived cell line was found to contain the message for TGF-alpha.

TABLE I:

Effects of Glioma Conditioned Medium on 3H-Tdr Incorporation of NRK Indicator Cells:

Cell Line CM	Mitogenic Response NRK Cells*	Dose Mitogenic†
SF-126	+	10 ug/ml
SF-188	+	0.5 ug/ml
SF-210	+	5 ug/ml
SF-268	+	4 ug/ml
SF-295	+	9 ug/ml
SF-539	+	10 ug/ml
U 343 MG-A	+	7.5 ug/ml
U 251 MG	+	1 ug/ml

* Stimulation of tritiated thymidine incorporation within NRK cells to a degree greater than 200% of serum free control is considered positive mitogenicity (+).

† Dose at which the glioma conditioned medium initially caused a stimulation of NRK cells

TABLE II

PHYSICOCHEMICAL CHARACTERISTICS OF A TRANSFORMING GROWTH FACTOR ISOLATED FROM HUMAN GLIOBLASTOMA MULTIFORME -- U 343 MG-A

CONDITIONS	NUMBER OF NRK COLONIES
Purified Factor (HPLC 100 ng/ml)	82 ± 4
+ trypsin (100 ug/ml)	0
+ pH 2.0	89 ± 7
+ pH 12.0	4 ± 1
+ 60 C (30 min)	75 ± 4
+ DTT (0.05 M)	0
+ EGF (10 ng/ml)	81 ± 7
+ TGFß (10 ng/ml)	196 ± 5
+ anti-TGF alpha (100 ug/ml)	5 ± 1
+ heparin-Sepharose	78 ± 4
+ anti-PDGF	80 ± 4

TABLE III

PHYSICOCHEMICAL CHARACTERISTICS OF A TRANSFORMING GROWTH FACTOR ISOLATED FROM HUMAN GLIOBLASTOMA MULTIFORME -- SF-210

CONDITIONS	NUMBER OF NRK COLONIES
Purified Factor HPLC (100 ng/ml)	146 ± 7
+ trypsin (100 ug/ml)	0
+ pH 2.0	138 ± 9
+ pH 12.0	9 ± 1
+ 60 C (30 min)	121 ± 12
+ DTT (0.05 M)	13 ± 2
+ EGF (10 ng/ml)	149 ± 13
+ TGFß (10 ng/ml)	133 ± 5
+ anti-TGF alpha (100 ug/ml)	125 ± 9
+ heparin-Sepharose	110 ± 4
+ anti-PDGF antibodies	129 ± 12

TABLE IV

PARTIAL PURIFICATION OF TRANSFORMING GROWTH FACTOR ACTIVITY FROM HUMAN GLIOBLASTOMA MULTIFORME CELL LINE U-343 MG-A

Process	Total Protein(mg)	Activity (U*)	Specific Activity (U/mg)	Purification	Recovery
Conditioned Medium (8 L)	75	6.25×10^3	83	1	100 %
Acid Extract	10	5.0×10^3	500	6	80 %
BioGel P60	4	4.0×10^3	1000	12	64 %
uBondPak c18 HPLC	0.15	2.5×10^3	16660	200	40 %

* 1 Unit defined as the amount of transforming growth factor required to produce half maximal response seen with TGF-alpha in the standard soft agar assay with NRK cells (= 40 colonies)

TABLE V

PARTIAL PURIFICATION OF TRANSFORMING GROWTH FACTOR LIKE ACTIVITY FROM HUMAN GLIOBLASTOMA MULTIFORME SF - 210:

Process	Total Protein (mg)	Activity (U*)	Specific Activity (U/mg)	Purification	Recovery
Conditioned Medium (4L)	40	4×10^3	100	1	100%
Acid Extract	5	3.3×10^3	660	6.6	83%
Bio Gel P60	2	3.0×10^3	1500	15	78%
uBondPak c18 HPLC	0.12	1.92×10^3	16000	160	48%

* 1 unit defined as the amount of transforming growth factor required to produce half maximal response seen with TGF-alpha in the standard soft agar assay with NRK cells (= 40 colonies)

TABLE VI

IMMUNOHISTOCHEMICAL LOCALIZATION OF TRANSFORMING GROWTH FACTOR-ALPHA IN HUMAN BRAIN TUMORS:

SPECIMEN	NUMBER EXAMINED	IMMUNOREACTIVITY
Glioblastoma Multiforme	5	+ (2/5)
Highly Anaplastic Astrocytoma	5	+ (1/5)
Moderately Anaplastic Astro	5	+ (5/5)
Meningioma	1	-
Schwannoma	1	-
Hemangioblastoma	1	-
Normal Brain (Adult)	2	-
Normal Brain (fetal)	3	+ (3/3)

+ = postive immunoreactivity
- = no immunoreactivity

Numbers in parenthesis represent the number of specimens positively identified by the anti-TGF-alpha monoclonal antibody

CHAPTER 10

SUMMARY

The glioblastoma multiforme is the most common and malignant of the primary human brain tumors. Little, however, is known regarding its pathogenesis. One of the most conspicuous histopathological features of the glioblastoma multiforme is the marked proliferation of mesenchymal (endothelial and leptomeningeal) cells within and around the tumor. This striking degree of mesenchymal proliferation must be considered an unusual yet fairly specific manifestation of the pathological interaction that often occurs between glial and mesenchymal elements in the CNS. Occasionally the stromal response to a glioblastoma multiforme may be so intense that the mesenchymal component assumes the morphology of a fibrosarcoma. In these instances, a mixed tumor consisting of two distinct neoplasms -- glioblastoma multiforme and sarcoma -- is found and has been termed "gliosarcoma".

Determining the pathogenesis of gliosarcoma formation has been the subject of this thesis. Although clinicopathological data abound in support of the hypothesis that glioma cells induce the malignant transformation of previously normal mesenchymal cells, to date there have been no convincing experimental approaches to test this hypothesis. The induction of neoplasia in a normal cell population which is different from that of the original tumor suggests that malignant glioma cells may secrete a TGF that acts in a paracrine manner. In this thesis, data is presented for the first time which supports the paracrine hypothesis of human gliosarcoma formation.

We devised an in vitro model system to test the hypothesis that malignant glioma cells secrete TGFs which cause the phenotypic transformation of previously normal mesenchymal cells. In our system, normal human leptomeningeal cells served as the targets for the glioma-derived growth factors present in glioma conditioned medium.

The human leptomeningeal cells in culture could be faithfully propagated, and were reproducibly identified and characterized by the presence of desmosomes, tonofilaments, and marked plasma membrane interdigitation. In contrast, attempts to cultivate pure populations of adult human glial cells were unsuccessful from samples of normal brain. Enriched

populations of human glial cells might have provided a means by which the autocrine hypothesis of glioma formation could have been tested.

The source of TGFs for these studies was the conditioned medium harvested from a series of well-characterized human malignant glioma cell lines. Of the three glioma cell lines that were found to secrete TGF-like factors, U-343 MG-A and U-251 MG carry the glial specific marker GFAP over serial passages suggesting that they are probably of glial origin. The other glioma cell line -- SF-210 -- expressed neither GFAP nor interstitial collagens. The glioma cell conditioned medium was concentrated, acid-extracted, and subjected to protein fractionation and purification by sequential molecular sieve chromatography and reverse-phase HPLC. TGF-activity was determined at each step of the purification process by determining leptomeningeal or NRK colony formation in the the soft agar assay. The physicochemical properties of the active fractions from the glioma lines were then analyzed.

The conditioned medium from glioma cell line U-343 MG-A contains an acid- and heat-stable TGF complex with molecular weight species at 6, 12, and 15 kD. The activity of the U-343 MG-A TGF is eliminated by 0.05 M DTT, and by incubation with monospecific anti-TGF-alpha monoclonal antibodies. The NRK colony stimulating activity of this TGF is potentiated by the addition of TGF-beta. The partially purified U-343 MG-A TGF competes with radiolabeled [^{125}I]-EGF for the EGF-receptor on A431 epidermoid carcinoma cells. A total RNA preparation from U 343 MG-A cells contains a 4.8 kb mRNA for TGF-alpha. These data suggest that U-343 MG-A malignant glioma cells secrete a soluble polypeptide(s) with TGF-alpha-like activity.

In contrast, the purified SF-210 TGF has neither TGF-alpha- nor TGF-beta-like activity. By HPLC, this TGF is a single protein with a molecular weight of 20 kD whose activity but not migration is altered by reducing agents. The phenotypic transformation of normal mesenchymal cells in the soft agar system is not changed when the SF-210 TGF is incubated with anti-TGF-alpha or anti-PDGF antibodies, or is passaged over a heparin-Sepharose column. The SF-210 TGF does not compete with radiolabeled EGF for the EGF-receptor. In addition, the SF-210 TGF is capable of inducing human fetal leptomeningeal cells to grow anchorage independently. Thus, it appears that the soluble peptide isolated from the malignant glioma line SF-210 is a novel growth factor with TGF-gamma-like activity. The salient features of the

glioma-derived TGFs isolated in this study and other known and purified growth factors are compared in Table I.

These data imply that human malignant gliomas secrete TGFs which can cause the phenotypic transformation of previously normal cells. The growth of human leptomeningeal cells in soft agar when exposed to the novel SF-210 TGF provides in vitro data to support the hypothesis that, by secreting TGFs, malignant glioma cells may induce peritumoral leptomeningeal cells to undergo phenotypic transformation (Figure 1). Such a phenomenon may help to explain the formation of complex mixed neoplasms in the CNS such as the gliosarcoma.

Ultimately, there are several areas in which growth factor research could conceivably have significant therapeutic applications in human systems of neoplasia. These include: 1) The synthesis of specific growth factor anatagonists to control the proliferation of cancer cells, 2) The design of fraudulent, incompetent growth factors which nonetheless could compete with a specific growth factor receptor, and 3) The use of certain growth factors as immunostimulants to bolster the host immune-response to neoplasia. While these potential benefits yet to be realized from growth factor research are beyond the scope and the data presented within the confines of this thesis, it is hoped that effective therapy against human brain tumors will be realized with an increased understanding of the pathogenesis of these tumors.

TABLE I

COMPARISON OF GLIOMA-DERIVED TGFs WITH KNOWN GROWTH FACTORS

	GROWTH FACTOR						
	SF-210	U343 MG	TGF-a	TGF-ß	EGF	FGF	PDGF
Mature factor size (kDa)	20	6,12,15	6	25	6	17	32
Acid/Heat Stable	+	+	+	+	-	-	-
DTT	-	-	-	-	-	-	-
Receptor	?	EGF-R	EGF-R	TGFß-R	EGF-R	?	PDGF-R
NRK Colonies	+	+	+	+	-	-	-
SF-673 Colonies	+	-	-	-	-	-	-
Heparin Affinity	-	-	-	-	-	+	-
TGF-a MAb	-	+	+	-	-	-	-

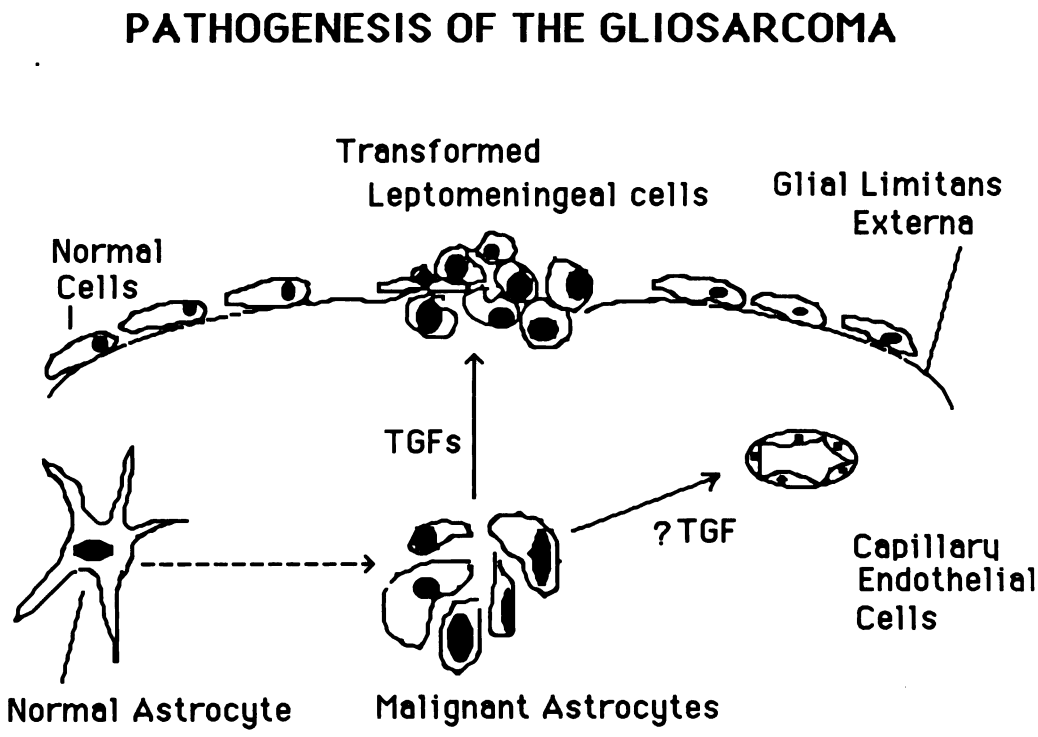


Figure 1: Data presented in this thesis have supported the paracrine hypothesis of gliosarcoma formation. TGFs have been isolated and partially purified from three malignant glioma cell lines. The TGF isolated from SF-210 appears to be a novel TGF with the capabilities of inducing previously normal SF-673M leptomeningeal cells to grow in soft agar. Future directions of investigation should include an attempt to study the effects of these TGFs on culture human brain endothelial cells; and efforts should be focused on testing the autocrine hypothesis of glioma formation.

APPENDIX

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April 10th, 1987

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Dr. John Moossy,
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School of Medicine,
Pittsburgh, PA 15261

Dear Dr. Moossy,

As part of my PhD thesis, I would like to include work that I have published in the Journal of Neuropathology and Experimental Neurology entitled: An ultrastructural and immunocytochemical analysis of leptomeningeal and meningioma cultures. JT Rutka, J Giblin, DV Dougherty, JR McCulloch, SJ DeArmond, ML Rosenblum. This paper appeared in Volume 45, No. 3, May, 1986, pp 285-303.

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Jim Rutka

James T. Rutka, M.D.,
Brain Tumor Research Center, HSW 783,
University of California San Francisco,
San Francisco, CA 94143

May 14, 1987
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May 5, 1987

Dr. James T. Rutka,
Brain Tumor Research Centre HSW 783
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BRAIN TUMOR RESEARCH CENTER
University of California
San Francisco, California 94143

April 5th, 1987

Dr. Emanuel Rubin,
Department of Pathology and Laboratory Medicine,
Hahnemann University,
School of Medicine,
Broad and Vine Streets,
Philadelphia, Penn 19102

Dear Dr. Rubin,

As part of my PhD thesis, I would like to include the work that I have published in Laboratory Investigation entitled: Characterization of normal human brain cultures: Evidence for the outgrowth of leptomeningeal cells. JT Rutka, H Kleppe-Hoifodt, DA Emma, JR Giblin, DV dougherty, JR McCulloch, SD DeArmond, ML Rosenblum. The paper was published in Volume 55, No. 1, p 71-85, 1986.

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