Title

Detection of autocrine stimulatory signals involving platelet-derived growth factor, vascular endothelial growth factor, and epidermal growth factor and the effects of a recombinant Pseudomonas toxin on human malignant meningiomas

Author

Sehati, Nouzhan,

Publication Date

2000

Peer reviewed|Thesis/dissertation

Detection of Autocrine Stimulatory Signals Involving Platelet-Derived Growth Factor, Vascular Endothelial Growth Factor, and Epidermal Growth Factor
and
The Effects of A Recombinant *Pseudomonas* Toxin on Human Malignant Meningiomas

Thesis

Nouzhan Sehati
MD with Thesis
School of Medicine
University of California, San Francisco

Michael W. McDermott, MD

Mark Israel, MD

Zena Werb, PhD

Table of Contents

Index of Tables and Figures

Abstract

Meningiomas are the second most common primary tumors of the central nervous system. Although most are benign, those afflicted with the malignant variety often face a poor prognosis due to lack of effective treatment modalities. This inquiry examined the presence of autocrine growth stimulatory mechanisms involving platelet-derived growth factor (PDGF), transforming growth factor-α (TGF-α), and vascular endothelial cell growth factor (VEGF); and determined the cytotoxic effects of the recombinant Pseudomonas exotoxin, TP-38, targeted to the epidermal growth factor (EGF) receptor as a novel therapeutic agent in three human malignant meningiomas cell lines. Northern blot analysis was used to detect mRNA for PDGF, PDGF receptor, TGF-α, EGF receptor, and VEGF receptor; and enzyme-linked immunosorbent assay detected secreted VEGF. Results indicated significant secretion of VEGF by the three cell lines without evidence of expression of VEGF receptor (subtype 2). PDGF (subtype BB) and its cognate receptor (subtype β) were found to be coexpressed in all cultures, whereas EGF receptor was expressed in variable quantities without coexpression of TGF-α. TP-38 was found to be cytotoxic in all three cultures with a median lethal dose ranging from 0.2 to 6 ng/ml. This effect was consistent with relative levels of EGF receptor expressed by the cells and was completely inhibited in presence of excess EGF, indicating that TP-38 exerts its effects through the EGF receptor. This study suggests the potential presence of an autocrine growth stimulatory loop for $PDGF_{BB}$ but not for TGF-α or VEGF in the cell lines. Also, the recombinant toxin TP-38 was found to be effective in promoting cytotoxicity in cells bearing EGF receptors and may have use as a novel chemotherapeutic agent in the treatment of malignant meningiomas.

Introduction

Meningiomas are primary tumors of the central nervous system that arise from the arachnoid meninges which sheath the brain and spinal cord (1). Most of these neoplasms, which represent 15-20% of all primary brain tumors, are benign (2), with an incidence that varies from 1 to 6 per 100,000 population (3) and increases with age (4). Meningiomas are most common in the seventh decade of life for women and the sixth decade for men (5), with a male to female ratio of 1:1.25-2.0 (6). These tumors are extremely rare in children; however, when they do occur, they often have an aggressive course (7).

The World Health Organization has classified meningiomas as *typical (benign)*, *atypical*, and *anaplastic (malignant)*. Benign meningiomas are generally slow-growing (8); and although various histologic types exist, none is believed to have any prognostic significance (2). Atypical tumors are distinguished from those that are benign by evidence of cellular sheeting, high cellularity, nuclear pleomorphism, prominent nucleoli, increased number of mitoses, and necrosis (2). Such features portend an increased likelihood for recurrence and aggressiveness to atypical meningiomas (9). In addition to these characteristics, the malignant variety, which comprises nearly 5% of all meningiomas (7), shows invasion into the brain and frequent metastases. Common sites of extracranial dissemination include lungs, bones, abdominal organs, muscles, and lymph nodes (6, 10).

Although there have been many advances in cancer therapy during recent years, treatment of primary malignancies of the central nervous systems, especially meningiomas, remains relatively constant, with surgical resection constituting the main

therapeutic modality. Furthermore, the capacity of malignant meningiomas for local invasion, combined with their occurrence in a spatially constrained and anatomically complex organ, presents a surgical challenge. Therefore, control of tumor growth by alternative means of treatment is often advantageous. However, the only validated form of adjuvant therapy at this time is radiation since many of the chemotherapeutic agents that are commonly effective against soft-tissue tumors have had only a modest impact on survival of patients suffering from malignant meningiomas (2). And even with the best therapy available, the overall rate of recurrence of this malignant neoplasm is 78% at 5 years (11) with a survival rate of only 50% during the same period of time (12). Given these limitations of the current modes of therapy, there has been accumulating interest in investigation of malignant meningiomas on the molecular level – namely study of the role of growth factors – in order to devise new therapeutic approaches in the form of specifically-targeted agents.

Growth factors are polypeptides that are required by normal cells for proper growth and differentiation (13). They are released by cells and generally exert their effects by both autocrine and paracrine mechanisms. The former involves attachment of the molecule to receptors which belong to the cell itself; whereas in the latter, the released polypeptide binds to receptors on adjacent cells (6). The receptors for many growth factors belong to the transmembrane receptor family with intrinsic tyrosine kinase activity. In this group binding of the ligand leads to dimerization and activation of the receptor with subsequent reciprocal phosphorylation by tyrosine kinase portion of the receptors, located on the intracellular domain. A cascade of second messengers is then initiated which ultimately leads to gene expression and cell growth. In the absence of

proper regulatory mechanisms, any processes that lead to constitutive activation of such tyrosine kinase receptors have the potential to promote aberrant cellular proliferation and tumorigenesis (14). Many identified human oncogenes, including those described in conjunction with central nervous system malignancies, encode components of the biochemical pathway mediated by activation of growth factor receptors (13). Three such examples are platelet-derived growth factor (PDGF), vascular endothelial cell growth factor (VEGF), and transforming growth factor-alpha (TGF-α) (8, 14-44).

Initially discovered as a molecule contained in human platelets (25), PDGF is a potent mitogen for a wide variety of cells including glia (16). Biochemical characterization has determined that PDGF is composed of two distinct subunits A and B, which share 60% identity in their amino acid sequence in humans. Functionally active as dimers linked by disulfide bonds (15) these molecules exist as both the heterodimer ($PDGF_{AB}$) and homodimers ($PDGF_{AA}$ and $PDGF_{BB}$) (16). The receptor for this growth factor (PDGF-R) exists in α and β subunits that can combine to form αα, αβ, and ββ isoforms; however, only the ββ form ($PDGF\text{-}R_{\beta}$) has been found in meningiomas (18, 23).

The potential for presence of PDGF autocrine and paracrine stimulatory mechanisms has been postulated for a variety of neoplasms including melanoma (45), esophageal (46), gastric (47), and colorectal adenocarcinoma (48, 49), basal cell cancer (50), choriocarcinoma (51), breast cancer (52), Kaposi's sarcoma (53), ovarian cancer (54), non-small cell lung carcinoma (55), and prostate cancer (56). Initial indications for the importance of this growth factor in the development of brain tumors resulted from observations that intracranial injection of the simian sarcoma virus carrying v-*sis*, the

homologue of $PDGF_{BB}$ gene, led to the induction of brain tumors in monkeys (57). Furthermore, only cell lines that expressed PDGF receptors could be transformed by transfection with v-*sis* (26). In addition, several studies have described coordinate and elevated expression of $PDGF_{BB}$ and PDGF-R_β as a common feature of human meningiomas (18, 29, 58, 59). These findings, along with the observation that PDGF-R_β has affinity only for the $PDGF_{BB}$ chain (16), suggest the involvement of an autocrine mechanism for PDGF in meningioma tumorigenesis (17, 20, 26, 27, 29). However, the number of such studies focusing on malignant meningiomas is very limited, which warrants an investigation into the role of PDGF and its receptor in the pathophysiology of these tumors.

VEGF is another growth factor that has been implicated in angiogenesis (60-62), endothelial cell migration and proliferation (63-65), vascular permeability (24, 66), and facilitation of tumor invasion through stromal degradation (67). Four isoforms of VEGF, resulting from alternate splicing, have been identified which contain 121, 165, 189, and 206 amino acids (68-71), with $VEGF_{165}$ being the predominant isoform (72). The biological functions of this growth factor are mediated through two receptors, Flt-1 (*fms*-like tyrosine kinase-1) and KDR (kinase-insert-domain-containing receptor), of which the latter, also known as VEGF receptor subtype 2 (VEGF-R_2) seems to be biologically more important (73-75). These receptors are primarily expressed on vascular endothelial cells, consistent with the main biological function of VEGF as a highly potent angiogenic growth factor (76-78). The expression of the ligand and growth factor are coordinately upregulated in the developing brain (76, 77, 79), with low to undetectable levels of both in the normal adult central nervous system (70). However, studies have found that the

progression of low-grade astrocytomas to a highly vascularized glioblastoma is associated with increased VEGF messenger ribonucleic acid (mRNA) expression by the tumor cells (80, 81). The endothelial cell growth associated with malignant astrocytomas has also been linked to upregulation of VEGF-R_2 mRNA (70, 81-83). These data are consistent with a paracrine role for VEGF in brain tumor angiogenesis, with this growth factor being released by the cancer cells and stimulating the growth of local vascular endothelial cells. Three recent studies have suggested a similar role for VEGF in meningiomas where levels of this agent were strongly correlated with peritumoral edema, which is commonly seen in the setting of malignant meningiomas (22, 24, 31). One of these studies also found a strong correlation between VEGF in peritumoral edema and vascularity (31), further suggesting the involvement of a paracrine mechanism. However, there have been very few investigations into the role of VEGF as a growth-stimulating agent for the neoplastic cells themselves. Given the polyfunctional nature of this growth factor, its involvement in paracrine angiogenesis, and the observation that VEGF shares 20% homology with PDGF (84), it appears reasonable to examine the potential involvement of this agent in proliferation of malignant meningiomas.

TGF-α has also been implicated in the proliferation of brain tumors. This polypeptide binds with high affinity to the epidermal growth factor receptor (EGF-R) (85), which is the proto-oncogene counterpart of the v-*erb-B1* oncogene, implicating a potential role for this receptor in tumorigenesis. Although meningiomas have been shown to produce both EGF and TGF-α, the latter seems to be predominant (38). EGF-R has also been found at an elevated level in human astrocytoma (86) and glioblastoma cell lines (87) and in meningiomas (33, 43, 85). In addition, stimulation of EGF-R has been

found to enhance *in vitro* growth of glial tumors (88) and meningiomas (89), while inhibition of the receptor halted such effects in gliomas (90). Furthermore, one study detected the presence of TGF-α mRNA in 11 of 15 malignant glioma cell lines and EGF-R mRNA in all 15 cultures (91). These data strongly suggest that TGF-α and EGF-R may be involved in growth of brain tumors through an autocrine mechanism (85), and a study of this growth factor and receptor system in malignant meningiomas is warranted.

Despite growing knowledge of cancer biology, there have been few recent advances affecting clinical outcome in neuro-oncology. Treatment failure in patients with brain neoplasms involves various processes including intrinsic resistance of tumors to radiation and chemotherapy (92, 93) and local recurrence (94). However, with the aid of accumulating data regarding the role of growth factors in the pathophysiology of meningiomas, new chemotherapeutic agents are being devised. One such class of drugs is recombinant toxins, which are cytotoxic agents that are constructed using gene splicing techniques, where growth factors, serving as the targeting moiety, are fused to protein toxins. Several bacterial toxins have been used to produce these chimeric molecules (95), and TP-38 is prototypic of a growing group of recombinant toxins designed for such targeted cancer therapy using the EGF-R.

TP-38 is composed of TGF-α and a genetically engineered form of the *Pseudomonas* exotoxin A, PE-38. The exotoxin itself is a 66kD protein with three distinct domains: Ia/Ib, II, and III. Domain Ia (amino acids 1-252) binds to a receptor which is present on most animal cells; the function of domain Ib (amino acids 365-399) is currently not known. Domain II (amino acids 253-364) mediates translocation of the toxin fragment into the cytosol (96, 97), where domain III (amino acids 400-613),

containing adenosine 5'-diphosphate ribosylating activity, leads to cell death by inactivating Elongation Factor 2, a vital component of the cell's protein synthetic machinery. In TP-38, the native Ia/Ib domain is replaced with TGF-α, which results in a modified version of the toxin. This agent contains amino acids 253-364 and 381-613, and selectively targets cells expressing EGF-R with negligible toxicity to other cells due to its inability to bind to proper cell membrane receptors (98, 99). Many primary brain tumors, including meningiomas, have high EGF-R expression, whereas the concentration of these receptors in normal brain is relatively low (100, 101). Therefore, cytotoxic agents targeted to may EGF-R have a high therapeutic index for treatment of such tumors (98).

In vitro studies have demonstrated the specificity of this agent for the EGF-R by demonstrating the ability of TP-38 to inhibit the binding of radiolabelled EGF to its receptor on non-small cell lung carcinoma cells. The concentration required to inhibit binding by 50% ranged from 100-2000 ng/ml in these cells (102). In another study, the concentration of TP-38 needed to inhibit 50% of protein synthesis in glioblastoma cells expressing EGF-R was 200-fold less, ranging from 0.5-10 ng/ml (95). However, there have been no studies evaluating the effects of TP-38 on malignant meningiomas; therefore, an investigation into its effects on these tumors of the central nervous system is appropriate.

To further elucidate the role of growth factors in the genesis and progression of malignant meningiomas, this inquiry aims to detect the presence of autocrine stimulatory mechanisms involving PDGF-R_β, VEGF-R_2, and EGF-R in three low-passage human malignant meningioma cell lines. Furthermore, in order to evaluate the potential clinical

applications of the recombinant toxin, TP-38, in the treatment of these tumors, this study evaluates the effects of this agent on the proliferation of these cells. It is hoped that this information will aid in the development of a more effective therapeutic modality for these neoplasms, which has thus far been hindered by the limited understanding of their molecular biology.

Materials and Methods

Origin of Tumor Cell Lines

To obtain a diagnosis, a portion of each tumor specimen was fixed in formalin, embedded in paraffin, cut into thin sections, and stained with hematoxylin and eosin immediately following surgical resection. These specimens were then microscopically examined, and the diagnosis was made according to standard classification criteria by a neuropathologist.

Monolayer Cell Cultures

For establishment of the primary cultures, a representative portion of the tumor was minced into pieces smaller than 1 mm^3 within 4 hours of surgical excision. The tissue was then dissociated into single cells using an enzyme cocktail consisting of pronase (0.05% of proteolytic units/mg), collagenase (0.02% of 125 units/mg), and DNAse (0.2% of 7×10^4 dornase units/mg) for 30 minutes at 37 °C. The cells were then transferred into 75-cm^2 flasks, and incubated in a 5% CO_2 humidified atmosphere in 15 ml of enriched medium consisting of Modified Eagle's Medium (MEM) supplemented

with 20% fetal bovine serum (FBS). The medium was changed once weekly until the cells reached confluence at which time they were subcultured by treatment with 0.05% trypsin containing ethylenediamine tetraacetic acid, and grown in the same medium at a density of 1×10^6 cells per 75-cm^2 flask. Each subsequent passage was done by the same procedure. All assays were done at passages 9 or 10.

Immunohistochemical Staining

Immunohistochemistry studies, staining for vimentin, epithelial membrane antigen (EMA), and EGF-R, were performed on the three cell lines. Following fixation of a confluent monolayer in ethanol, glass slides were incubated in the primary antibody for 30 minutes at room temperature (vimentin and EMA) or overnight at 4 °C (EGF-R). A biotinylated secondary antibody recognizing the primary antibody was applied for 10 minutes prior to a ten-minute application of an avidin-biotin-peroxidase complex. Diaminobenzidine in tris-hydroxymethyl-aminomethane buffer was used as substrate for development of the peroxidase reaction. Monolayers were then counterstained with hematoxylin following the immunoperoxidase reaction. Tissues known not to express vimentin, EMA, and EGF-R served as control for the corresponding stain.

Enzyme Linked Immunosorbent Assay (ELISA)

To determine the concentrations of VEGF present in tissue culture medium conditioned by the three cell lines, the quantitative ELISA method was employed using the QuantiGlo™ Human VEGF Imunoassay Kit (R&D Systems, Inc.) according to manufacturer's recommended protocol. Briefly, a 96-well microtiter plate was precoated

with murine monoclonal antibody against VEGF. Following addition of a buffered protein base to each well, samples of supernate from each of the three cell lines (at ~90% confluence following 6 days of incubation) were placed in triplicates into separate wells, and incubated at room temperature for two hours. The wells were then rinsed with a concentrated solution of buffered surfactant (washing buffer), and incubated at room temperature for 3 hours in a solution containing polyclonal antibody against human VEGF conjugated to horseradish peroxidase. Following a rinse with the washing buffer, the wells were subjected to enhanced luminol and hydrogen peroxide at room temperature for 25 minutes, after which time the emitted light was measured in Relative Light Units (RLU) using a luminometer. A standard curve was generated by plotting RLU versus concentration using values for standard VEGF samples. Concentrations of VEGF in the samples of cell lines were then determined by interpolation from the standard curve, and adjusted for the total number of cells. Samples of medium from stock and from a culture containing normal meningeal cells were used as negative control and the medium conditioned by a culture of established glioblastomas was used as positive control and found to be in agreement with previously published data (103).

Northern Blot Analysis

For each of the three cultures, RNA isolation was initiated by incubation of cells in a monophasic reagent containing phenol and guanidium isothiocyanate for 5 minutes at room temperature. Following addition of 0.2 ml of chloroform for each 1 ml of reagent, the solution was further incubated at room temperature for 3 minutes. Samples were then centrifuged at 12,000g for 15 minutes at 4 °C to produce a triphasic solution. The

aqueous phase was isolated, and RNA was precipitated by incubation with 0.5 ml of isopropyl alcohol for each 1 ml of reagent initially used for 10 minutes at room temperature. Following centrifugation at 12,000g for 10 minutes at 4 °C, the supernate was discarded and the remaining RNA pellet was suspended in 1 ml of 75% ethanol for each 1 ml of reagent initially used. The solution was then centrifuged at 7,500g for 5 minutes at 4 °C, the liquid phase removed, and the remaining RNA pellet was air dried at room temperature and dissolved in RNAse-free water. The concentration of RNA was measured by determination of absorbance at 260 nm with a spectrophotometer, and purity ascertained by calculation of ratio of absorbances at 260 nm and 280 nm. Integrity of the isolated RNA was determined by separation of a representative portion of the sample on a 1% denaturing formaldehyde-agarose gel.

To perform the Northern blot, 20 μg of RNA from each cell line along with 5 μl of formaldehyde loading buffer were loaded on a 1% formaldehyde-agarose gel following denaturation with 2.5 μl of 10X 3-[N-morpholino]propanesulfonic acid (MOPS) running buffer, 4.5 μl of 12.3M formaldehyde, and 12.5 μl of formamide at 55 °C for 15 minutes. Electrophoresis was then performed for 3 hours at 100 V and the RNA was ultraviolet cross-linked to a nylon membrane. The blots were then prehybridized at 42 °C for 4 hours in a mixture of degraded salmon sperm DNA (5 mg/ml), 50% formamide, 5X standard sodium citrate (0.15M NaCl, 0.015M sodium citrate), 10X Denhardt's solution, 50 nM $NaPO_4$, 1% sodium dodecylsulfate (SDS), and 10 μg/ml free acid. The blots were hybridized overnight at 42 °C in a solution of 50% formamide, 5X standard sodium citrate, 1X Denhardt's solution, 20 mM $NaPO_4$, 0.5% SDS, 5% dextran sulfate, 20 μg/ml free acid, degraded salmon sperm DNA (5 mg/ml),

and 10^6 cpm/ml of ^{32}Phosphorus-labelled complementary DNA probe. Blots were then washed twice serially in 2X standard sodium citrate containing 0.1% SDS at 42 °C for 5 minutes, then for 30 minutes at the same temperature, followed by 30 minutes at 55 °C. After washing, blots were exposed to x-ray film for 16 hours to 1 week and the autoradiograms were quantified by densitometry. The band densities were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to account for RNA loading differences among the cell lines.

The DNA fragments used for hybridization in the Northern Blot included a 2.6 kb BamH1 $PDGF_{BB}$ chain cDNA, 2.65kb Acc1 and BamH1 PDGF-R_β cDNA, 925 bp EcoRI TGF-α cDNA, 2.4 kb Cla1 EGF-R cDNA, 3.1 kb SmaI VEGF-R_2 cDNA, and 280 bp HindIII and PstI GAPDH cDNA. Using random primer translation, cDNA fragments were labelled with Phosphorus-32 to achieve a specific activity of 25×10^6 cpm/μg DNA.

Chemosensitivity Assay using TP-38

Cells from each of the three cell lines were transferred into 25-cm^2 flasks at a density of 1×10^5 cells per flask, containing a total fluid volume of 5 ml. Flasks were subsequently divided into four distinct groups, the components of which are summarized in Table 1. Following seven days of incubation at 37 °C in a 5% CO_2 humidified atmosphere, the medium was discarded and flasks were treated with 0.05% trypsin in order to mobilize the cells and facilitate determination of number of live cells per flask by using a hemocytometer. Trypan blue was utilized to determine cell viability. In a previous study the *Pseudomonas* toxin/TGF-α construct was found to retain full activity after seven days of incubation at 37 °C, without any evidence of degradation (95).

Table 1. Chemosensitivity assay design

Group	Components
Control-A	MEM + 20% FBS
Experimental-A	MEM + 20% FBS + TP-38 (0.005 ng/ml) MEM + 20% FBS + TP-38 (0.05 ng/ml) MEM + 20% FBS + TP-38 (0.5 ng/ml) MEM + 20% FBS + TP-38 (5.0 ng/ml) MEM + 20% FBS + TP-38 (50 ng/ml)
Control-B*	MEM + 20% FBS + EGF (1 ng/ml) MEM + 20% FBS + EGF (10 ng/ml) MEM + 20% FBS + EGF (100 ng/ml) MEM + 20% FBS + EGF (1,000 ng/ml) MEM + 20% FBS + EGF (10,000 ng/ml)
Experimental-B*	MEM + 20% FBS + EGF (1 ng/ml) + TP-38 (0.005 ng/ml) MEM + 20% FBS + EGF (10 ng/ml) + TP-38 (0.05 ng/ml) MEM + 20% FBS + EGF (100 ng/ml) + TP-38 (0.5 ng/ml) MEM + 20% FBS + EGF (1,000 ng/ml) + TP-38 (5.0 ng/ml) MEM + 20% FBS + EGF (10,000 ng/ml) + TP-38 (50 ng/ml)

* The concentration of TP-38 required to inhibit the binding of EGF to its receptor (102) was 200 fold more than that needed to inhibit protein synthesis (95). Therefore, in *Control-B* and *Experimental-B* EGF was used at 200 times the TP-38 concentration in order to inhibit binding of the latter to the EGF-R as a competitive inhibitor.

Growth Kinetics in Presence of TP-38

For each of the three cell lines, 25-cm^2 flasks, each containing 1×10^5 cells in 5 ml of MEM + 20% FBS, were incubated at 37 °C in a 5% CO_2 humidified atmosphere. TP-38 was then added to the flasks at four different concentrations of 0, 0.05, 0.5, or 5.0 ng/ml, and each flask was assigned to a distinct group based on the concentration of TP-38 in the flask. On subsequent days, triplicates from each of these groups were treated with 0.05% trypsin immediately following disposal of the medium and the number of live cells per flask was determined by using a hemocytometer. Cell counts were determined every three days and continued for a total period of 12 days. Trypan blue was used to ascertain cell viability.

Statistical Analysis

Differences in mean cell counts for the chemosensitivity analysis were evaluated with the one-tailed Student's t-test for paired data. For all tests, results exceeding the level of $p = 0.05$ were considered statistically significant.

Results

Origin of Tumor Cell Lines

The patient profile and identification of the three tumor cell lines used in this inquiry are listed in Table 2.

Table 2. Patient and tumor profiles for the cell lines

Cell Line	Patient Age/Gender	Tumor Location	Tumor Diagnosis
SF-1625	45/Female	Left Sphenoid Wing	Malignant Meningioma
SF-1826	55/Male	Left Parietal Lobe	Malignant Meningioma
SF-3178	53/Female	Right Temporal Lobe	Malignant Meningioma

Histologic Characteristics

Figures 1A – 1I illustrate the results of histologic staining for cell lines SF-1625, SF-1826, and SF-3178. Immunohistochemical analyses support the concept that meningiomas can express both mesenchymal and epithelial phenotypes. All three cell lines demonstrated staining for vimentin which is an intermediate filament found in cells of mesenchymal origin especially meningiomas (104-106). The epithelial nature of these cells is evidenced by immunoreactivity for epithelial membrane antigen which has been shown to be variable in expression in these tumors (104, 106). In addition, Figure 2

demonstrates positive immunostaining for EGF-R in all three cell lines indicating the presence of this receptor on the surface of these cells.

Enzyme Linked Immunosorbent Assay for VEGF

An ELISA was used to detect the levels of VEGF in the supernate for the three malignant meningioma cell lines, and revealed that each secreted a measurable quantity of VEGF, as shown in Table 3. The relative concentrations of VEGF secreted by each of these cultures under identical conditions (1×10^6 cells in MEM + 20% FBS for 6 days) ranged from 818 pg/ml to 4590 pg/ml. The two control groups, which consisted of the medium alone and a culture of normal meninges exhibited undetectable levels of VEGF.

Table 3. Concentrations of VEGF by ELISA in the conditioned medium of normal meninges and the three human malignant meningiomas cell lines for two determinations

Sample	VEGF Concentration (per 1×10^6 cells)
MEM + 20% FBS	Background (<30 pg/ml)
Normal Meninges	Background (<30 pg/ml)
SF-1625	818 pg/ml
SF-1826	4590 pg/ml
SF-3178	948 pg/ml

Northern Blot Analysis for $PDGF_{BB}$, $PDGF\text{-}R_\beta$, TGF-α, EGF-R, and $VEGF\text{-}R_2$

Figure 3 depicts the results of the Northern blot analysis detecting the presence of mRNA for $PDGF_{BB}$, $PDGF\text{-}R_\beta$, TGF-α, EGF-R, and $VEGF\text{-}R_2$. All three cell lines expressed significant quantities of both $PDGF_{BB}$ and $PDGF\text{-}R_\beta$ mRNA. Levels of EGF-R were variable among the three cell lines with SF-3178 expressing the largest quantity, SF-1625 exhibiting moderate expression, and SF-1826 showing the least amount of EGF-

R mRNA. No TGF-α or VEGF-R_2 mRNA was detected in any of the cell lines.

Effects of TP-38 on the Cell Lines

Figures 4A – 4C show the effects of various concentrations of TP-38 on SF-1625, SF-1826, and SF-3178. This agent was found to cause cell death in all three cell lines, with LD_{50} values (concentration of toxin necessary to kill half of the cells) of 6 ng/ml for SF-1625, 0.5 ng/ml for SF-1826, and 0.2 ng/ml for SF-3178. Cytotoxity was fully inhibited in presence of excess EGF. The difference between Experimental-B and both control groups was statistically significant ($p < 0.005$) at LD_{50} for all three cell lines.

Figures 5A – 5C illustrate the growth kinetics of SF-1625, SF-1826, and SF-3178 in presence of TP-38. All three cell lines showed the largest toxicity at maximum concentration of 5 ng/ml; and consistent with levels of EGF-R mRNA, SF-3178 demonstrated the greatest response and SF-1826 showed the least amount of cytotoxicity. Growth rate of the cells at concentrations of 0.05 and 0.5 ng/ml was variable among the three cultures.

Discussion

Autocrine Stimulatory Mechanisms

Many neoplasms, including meningiomas, contain relatively large numbers of growth factor receptors. Evidence is accumulating to support the hypothesis that these receptors and their ligands may regulate the growth of tumor cells through an autocrine mechanism. This inquiry investigated the presence of such mechanisms involving PDGF, TGF-α, and VEGF in three human malignant meningioma cell lines. The results

indicated the expression of PDGF, PDGF-R_β, VEGF, and EGF-R in all three cell lines, whereas TGF-α and VEGF-R_2 were not detected.

The coexpression of PDGF-R_β and its cognate ligand, $PDGF_{BB}$, suggests that this growth factor and its receptor may be involved in autocrine growth stimulation in the malignant meningiomas. Various other studies had also demonstrated the coincidence of $PDGF_{BB}$ and PDGF-R_β in benign meningiomas (8, 28) and in all cases neutralizing antibodies against PDGF were successful in retarding cell proliferation. These data lend further support to the hypothesis that an autocrine stimulatory mechanism may indeed be present in cells bearing this growth factor receptor and its ligand, and that this system may be involved in neoplastic transformation and/or growth of meningiomas. However, to further investigate this possibility it is necessary to demonstrate that the PDGF-R mRNA is indeed translated into a functional receptor in these cell lines and that activation of that receptor by its ligand promotes cell proliferation. Furthermore, it should be shown that inactivation of the secreted PDGF, by neutralizing antibodies, leads to hindrance of this proliferation.

Although the genetic aberrations that establish meningiomas and lead to their proliferation have not been well characterized, it has been demonstrated that $PDGF_{BB}$ mRNA and its protein product are absent in normal pachymeninges, whereas PDGF-R was found to be expressed in these cells (29). Furthermore, intracranial injection of the simian sarcoma virus carrying v-*sis*, the homologue of $PDGF_{BB}$ gene, led to the induction of brain tumors in monkeys (57); and only cells expressing PDGF-R could be transformed by transfection with v-*sis* (26). It has also been demonstrated that approximately half of all meningiomas have deletions in chromosome 22 (107-109)

suggesting that loss of function of a tumor suppressor gene at this locus, such as NF2, may be involved in the etiology of meningiomas (110). This conjecture, although requiring confirmation, may provide clues into the molecular events that take place in the transformation of normal meninges into benign or malignant meningiomas.

PDGF appears to be related not only to tumor cell proliferation, but also to metastasis. In a study of breast cancer, the expression frequency of PDGF mRNA of tumors with lymph node metastases was as high as 92.3% which was two fold higher than that of tumors without metastases (111). This is in accordance with the finding that PDGF possesses protease activity (112, 113), which is required in the process of metastasis. The observation that the three cell lines in this inquiry are derived from meningiomas with a malignant diagnosis and that all express PDGF is also consistent with the theory that this growth factor may play a role in metastasis, which is a feature of malignant tumors.

Various investigators have also demonstrated that PDGF may exert its effects in meningiomas in conjunction with other growth factors. For instance, activation of PDGF-R and EGF-R stimulates cell proliferation in human meningioma cultures, with a combination of both exerting a synergistic effect on DNA synthesis (89). This inquiry demonstrated that EGF-R, but not TGF-α, is expressed in the three malignant meningioma cell lines. Several other studies of benign meningiomas have also demonstrated the overexpression of EGF-R (114) in the absence of TGF-α (85); thus, suggesting that an autocrine stimulatory mechanism in benign or malignant meningiomas is unlikely to exist for this growth factor. However, this finding does not rule out the possibility of an autocrine mechanism involving the EGF-R, since this receptor has

several other ligands, such as EGF, which may be secreted by these tumors.

A role for EGF-R in tumorigenesis is also suggested by several studies that have demonstrated the simultaneous occurrence of EGF-R gene amplification and a chromosome 10 deletion in high grade gliomas (115). In addition, the latter was found in all tumors with EGF-R gene amplification (116). Thus, although the precise relationship between these two alterations remains unclear (32), their coexistence implies a significant role for them in the glioma growth stimulatory pathway. That is, the potential loss of tumor suppressor activity through deletion of chromosome 10 may lead to aberrant cell growth and tumorigenesis in setting of EGF-R amplification. Given the documented presence of EGF-R in both benign and malignant meningiomas, investigation of the above proposed model in these tumors may elucidate pathways that lead to initiation or growth of these tumors.

Activation of EGF-R or PDGF-R_β has also been shown to induce the expression of VEGF in brain tumors (38). All three cell lines in the current investigation were demonstrated to express EGF-R and PDGF-R_β, and to secrete significant quantities of VEGF. Although the mere coexpression of these growth factors and receptors in the same cell does not necessarily demonstrate a relationship between tumor cell proliferation and angiogenesis, it raises the possibility that such an association may indeed exist. This is a reasonable concept since it implies that the same growth factors that may lead to cell proliferation would ensure a sufficient blood supply to the growing tumor by inducing vascular proliferation through secretion of VEGF. Given the observation that normal brain endothelial cells do not express VEGF receptors (117, 118), inhibition of VEGF may, therefore, be an approach to the treatment of malignant

meningiomas with potentially low side effects. However, in order to design effective agents, the molecular biology of VEGF action must be better understood.

Recently, the transcriptional upregulation of VEGF was shown to be mediated by activation of Ras which is an intracellular signal transduction protein (119). Stimulation of EGF-R and PDGF-R also leads to Ras induction (31); therefore, it can be postulated that activation of these two receptors causes upregulation of Ras, leading to both a mitogenic signal to the nucleus and VEGF expression. Despite having a rational basis, these proposed molecular events need to be validated in experimental paradigms.

The current study did not show the presence of VEGF-R on any of the cell lines; thus, making the presence of a direct autocrine growth stimulatory signal in these cells unlikely. However, by enhancing the blood supply of the growing tumor, increased vascularity brings more growth factors and nutrients to the neoplastic cells in a self-propagating cycle; thus, indirectly leading to the increase in tumor size.

Cytotoxicity of TP-38 In Vitro and Clinical Implications

TP-38 is a chimeric toxin composed of TGF-α (a ligand for the EGF-R) serving as the targeting moiety and replacing the nonspecific cell surface-binding domain of the *Pseudomonas* Exotoxin A. This construct was found to be active on the three human malignant meningioma cell lines – all of which were shown to bear the EGF-R on their surface. The potency of the cell killing is evident in the LD_{50} ranging from as low as 0.2 ng/ml to 6 ng/ml. The variability of activity observed among the three different cell lines may be in part due to the dissimilarity in the number of EGF receptors expressed – as demonstrated by mRNA analysis – or due to the differential efficiency of each cell line to

internalize and process the toxin.

The specificity of the TP-38 for the EGF-R was confirmed by incubation in presence of excess EGF which demonstrated essentially no activity by the toxin, most likely due to hindrance of TP-38 access to the receptor by competitive inhibition. In addition, this agent has been shown in a different study to be inactive on a human leukemia cell line known to not express the EGF receptor (95).

The presence of EGF-R in various brain tumors, including malignant meningiomas, has also been described by several other investigations. Furthermore, normal brain specimens have been demonstrated to lack this growth factor receptor (120) – a reasonable finding in light of the observation that significant cell division does not take place in the normal adult central nervous system. This allows for a therapeutic advantage whereby a potential toxic agent that specifically targets EGF-R can exert cytotoxic effects on these neoplastic cells without significant toxicity to the normal tissue. TP-38 is one such agent with additional properties that make it an attractive adjuvant chemotherapeutic agent for the treatment of brain tumors. First, it is extremely potent; requiring the entrance of only a few molecules into the cytosol to kill the cell (95, 121). Furthermore, the cytotoxic effects are not cell-cycle specific, allowing high efficacy even in slow-growing tumors. Also, the small size of TGF-α molecule as the targeting moiety reduces the antigenicity and increases penetration across blood vessels (95). Finally, activity has been demonstrated even in hypoxic conditions which have traditionally limited the effectiveness of irradiation and chemotherapy (95, 122)..

Despite these advantages, however, the use of this recombinant toxin *in vivo* may be limited. One reason is that EGF receptors are present in many major organs including

liver, kidney, and the bone marrow (95) and administration of TP-38 may lead to systemic toxicity before any therapeutic effects have been achieved. For these reasons and because the properties of cells may differ when grown *in vitro*, it is essential to assess the characteristics of this agent in an *in vivo* model. An intracranial xenograft will be invaluable in elucidating the systemic and local toxicity, efficacy, and potency of TP-38 in a system that more closely demonstrates the effects from potential altered accessibility of the toxin due to systemic administration. In addition, this model allows intrathecal or intracerebral delivery of the toxin, which may allow the administration of higher doses of the drug without significant systemic effects. Moreover, the immunoprotective environment of the central nervous system may allow for repeated delivery of the drug without development of neutralizing antibodies (95). Given the promising potential for TP-38 to be an effective chemotherapeutic agent for the treatment of brain tumors, especially malignant meningiomas, further investigation into this agent is warranted.

Acknowledgements

The author would like to thank the individuals whose assistance contributed to this project, namely Dr. James Rubenstein (ELISA), Dr. John Fike (provision of TP-38), and Ms. Miranda Harmon-Smith and Ms. Carol Julin (Northern blot).

References

1. Tsujino K, Yamate J, Tsukamoto Y, et al. 1997. Establishment and characterization of cell lines derived from a transplantable rat malignant

meningioma: morphological heterogeneity and production of nerve growth factor. *Acta Neuropathologica*. **93**: 461-70.
2. De Monte F. 1995. Current management of meningiomas. *Oncology*. **9**: 83-91.
3. Schoenberg BS, Christine BW, and Whisnant JP. 1976. The descriptive epidemiology of primary intracranial neoplasms: the Connecticut experience. *American Journal of Epidemiology*. **104**: 499-510.
4. Kurland LT, Schoenberg BS, Annegers JF, et al. 1982. The incidence of primary intracranial neoplasms in Rochester, Minnesota, 1935-1977. *Annals of New York Academy of Science*. **381**: 6-16.
5. Rohringer M, Sutherland GR, Louw DF, et al. 1989. Incidence and clinicopathological features of meningioma. *Journal of Neurosurgery*. **71**: 665-72.
6. McCutcheon IE. 1996. The biology of meningiomas. *Journal of Neuro-Oncology*. **29**: 207-16.
7. Langford LA. 1996. Pathology of meningiomas. *Journal of Neuro-Oncology*. **29**: 217-21.
8. Shamah SM, Alberta JA, Giannobile WV, et al. 1997. Detection of activated platelet-derived growth factor receptors in human meningioma. *Cancer Research*. **57**: 4141-7.
9. de la Monte SM, Flickinger J, and Linggood RM. 1986. Histopathologic features predicting recurrence of meningiomas following subtotal resection. *American Journal of Surgical Pathology*. **10**: 836-43.
10. Younis GA, Sawaya R, DeMonte F, et al. 1995. Aggressive meningeal tumors: review of a series. *Journal of Neurosurgery*. **82**: 17-27.
11. Jaaskelainen J, Haltia M, and Servo A. 1986. Atypical and anaplastic meningiomas: radiology, surgery, radiotherapy, and outcome. *Surgical Neurology*. **25**: 233-42.
12. McDermott MW and Wilson CB. 1996. Meningiomas. In Neurological Surgery. Youmans JR (Ed). Philadelphia: WB Saunders. P 2782-825.
13. Ashley DM, Batra SK, and Bigner DD. 1997. Monoclonal antibodies to growth factors and growth factor receptors: their diagnostic and therapeutic potential in brain tumors. *Journal of Neuro-Oncology*. **35**: 259-73.
14. Kirsch M, Wilson JC, and Black P. 1997. Platelet-derived growth factor in human brain tumors. *Journal of Neuro-Oncology*. **35**: 289-301.
15. Antoniades HN, Scher CD, and Stiles CD. 1979. Purification of human platelet-derived growth factor. *Proceedings of the National Academy of Sciences of the United States of America*. **76**: 1809-13.
16. Antoniades HN. 1991. PDGF: a multifunctional growth factor. *Baillieres Clinical Endocrinology and Metabolism*. **5**: 595-613.
17. Bejcek BE, Li DY, and Deuel TF. 1989. Transformation by v-sis occurs by an internal autoactivation mechanism. *Science*. **245**: 1496-9.
18. Black PM, Carroll R, Glowacka D, et al. 1994. Platelet-derived growth factor expression and stimulation in human meningiomas. *Journal of Neurosurgery*. **81**: 388-93.
19. Christov C, Lechapt-Zalcman E, Adle-Biassette H, et al. 1999. Vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) and its

receptor flt-1 in microcystic meningiomas. *Acta Neuropathologica*. **98**: 414-20.
20. Deuel TF, Pierce GF, Yeh HJ, et al. 1987. Platelet-derived growth factor/sis in normal and neoplastic cell growth. *Journal of Cellular Physiology*. **Supplement 5**: 95-9.
21. Dietzmann K, von Bossanyi P, Warich-Kirches M, et al. 1997. Immunohistochemical detection of vascular growth factors in angiomatous and atypical meningiomas, as well as hemangiopericytomas. *Pathology, Research and Practice*. **193**: 503-10.
22. Goldman CK, Bharara S, Palmer CA, et al. 1997. Brain edema in meningiomas is associated with increased vascular endothelial growth factor expression. *Neurosurgery*. **40**: 1269-77.
23. Harsh GR, Keating MT, Escobedo JA, et al. 1990. Platelet derived growth factor (PDGF) autocrine components in human tumor cell lines. *Journal of Neuro-Oncology*. **8**: 1-12.
24. Kalkanis SN, Carroll RS, Zhang J, et al. 1996. Correlation of vascular endothelial growth factor messenger RNA expression with peritumoral vasogenic cerebral edema in meningiomas. *Journal of Neurosurgery*. **85**: 1095-101.
25. Kaplan DR, Chao FC, Stiles CD, et al. 1979. Platelet alpha granules contain a growth factor for fibroblasts. *Blood*. **53**: 1043-52.
26. Keating MT and Williams LT. 1988. Autocrine stimulation of intracellular PDGF receptors in v-sis-transformed cells. *Science*. **239**: 914-6.
27. Mauro A, Bulfone A, Turco E, et al. 1991. Coexpression of platelet-derived growth factor (PDGF) B chain and PDGF B-type receptor in human gliomas. *Childs Nervous System*. **7**: 432-6.
28. Mauro A, Di Sapio A, Mocellini C, et al. 1995. Control of meningioma cell growth by platelet-derived growth factor (PDGF). *Journal of the Neurological Sciences*. **131**: 135-43.
29. Maxwell M, Galanopoulos T, Hedley-Whyte ET, et al. 1990. Human meningiomas co-express platelet-derived growth factor (PDGF) and PDGF-receptor genes and their protein products. *International Journal of Cancer*. **46**: 16-21.
30. Maxwell M, Galanopoulos T, and Antoniades H. 1996. Coexpression of EGF receptor and TGFα mRNA and protein occurs in primary meningiomas. *International Journal of Oncology*. **9**: 917-22.
31. Provias J, Claffey K, delAguila L, et al. 1997. Meningiomas: role of vascular endothelial growth factor/vascular permeability factor in angiogenesis and peritumoral edema. *Neurosurgery*. **40**: 1016-26.
32. Tang P, Steck PA, and Yung WK. 1997. The autocrine loop of TGF-alpha/EGFR and brain tumors. *Journal of Neuro-Oncology*. **35**: 303-14.
33. Todo T, Adams EF, and Fahlbusch R. 1993. Inhibitory effect of trapidil on human meningioma cell proliferation via interruption of autocrine growth stimulation. *Journal of Neurosurgery*. **78**: 463-9.
34. Todo T, Adams EF, Fahlbusch R, et al. 1996. Autocrine growth stimulation of human meningioma cells by platelet- derived growth factor. *Journal of Neurosurgery*. **84**: 852-8.
35. Tsai JC, Goldman CK, and Gillespie GY. 1995. Vascular endothelial growth

factor in human glioma cell lines: induced secretion by EGF, PDGF-BB, and bFGF. *Journal of Neurosurgery*. **82**: 864-73.

36. Tsai JC, Hsiao YY, Teng LJ, et al. 1999. Regulation of vascular endothelial growth factor secretion in human meningioma cells. *Journal of the Formosan Medical Association*. **98**: 111-7.
37. Yoshioka H, Hama S, Taniguchi E, et al. 1999. Peritumoral brain edema associated with meningioma: influence of vascular endothelial growth factor expression and vascular blood supply. *Cancer*. **85**: 936-44.
38. Carroll RS, Black PM, Zhang J, et al. 1997. Expression and activation of epidermal growth factor receptors in meningiomas. *Journal of Neurosurgery*. **87**: 315-23.
39. Dorward NL, Hawkins RA, and Whittle IR. 1993. Epidermal growth factor receptor activity and clinical outcome in glioblastoma and meningioma. *British Journal of Neurosurgery*. **7**: 197-9.
40. Horsfall DJ, Goldsmith KG, Ricciardelli C, et al. 1989. Steroid hormone and epidermal growth factor receptors in meningiomas. *Australian and New Zealand Journal of Surgery*. **59**: 881-8.
41. Johnson MD, Horiba M, Winnier AR, et al. 1994. The epidermal growth factor receptor is associated with phospholipase C-gamma 1 in meningiomas. *Human Pathology*. **25**: 146-53.
42. Shiurba RA, Eng LF, Vogel H, et al. 1988. Epidermal growth factor receptor in meningiomas is expressed predominantly on endothelial cells. *Cancer*. **62**: 2139-44.
43. Torp SH, Helseth E, Dalen A, et al. 1992. Expression of epidermal growth factor receptor in human meningiomas and meningeal tissue. *Apmis*. **100**: 797-802.
44. Linggood RM, Hsu DW, Efird JT, et al. 1995. TGF alpha expression in meningioma--tumor progression and therapeutic response. *Journal of Neuro-Oncology*. **26**: 45-51.
45. Forsberg K, Valyi-Nagy I, Heldin CH, et al. 1993. Platelet-derived growth factor (PDGF) in oncogenesis: development of a vascular connective tissue stroma in xenotransplanted human melanoma producing PDGF-BB. *Proceedings of the National Academy of Sciences of the United States of America*. **90**: 393-7.
46. Yoshida K, Kuniyasu H, Yasui W, et al. 1993. Expression of growth factors and their receptors in human esophageal carcinomas: regulation of expression by epidermal growth factor and transforming growth factor alpha. *Journal of Cancer Research and Clinical Oncology*. **119**: 401-7.
47. Chung CK and Antoniades HN. 1992. Expression of c-sis/platelet-derived growth factor B, insulin-like growth factor I, and transforming growth factor alpha messenger RNAs and their respective receptor messenger RNAs in primary human gastric carcinomas: in vivo studies with in situ hybridization and immunocytochemistry. *Cancer Research*. **52**: 3453-9.
48. Lindmark G, Sundberg C, Glimelius B, et al. 1993. Stromal expression of platelet-derived growth factor beta-receptor and platelet-derived growth factor B-chain in colorectal cancer. *Laboratory Investigation*. **69**: 682-9.
49. Sundberg C, Ljungström M, Lindmark G, et al. 1993. Microvascular pericytes express platelet-derived growth factor-beta receptors in human healing wounds

and colorectal adenocarcinoma. *American Journal of Pathology*. **143**: 1377-88.

50. Pontén F, Ren Z, Nistér M, et al. 1994. Epithelial-stromal interactions in basal cell cancer: the PDGF system. *Journal of Investigative Dermatology*. **102**: 304-9.
51. Holmgren L, Flam F, Larsson E, et al. 1993. Successive activation of the platelet-derived growth factor beta receptor and platelet-derived growth factor B genes correlates with the genesis of human choriocarcinoma. *Cancer Research*. **53**: 2927-31.
52. Seymour L, Dajee D, and Bezwoda WR. 1993. Tissue platelet derived-growth factor (PDGF) predicts for shortened survival and treatment failure in advanced breast cancer. *Breast Cancer Research and Treatment*. **26**: 247-52.
53. Stürzl M, Roth WK, Brockmeyer NH, et al. 1992. Expression of platelet-derived growth factor and its receptor in AIDS-related Kaposi sarcoma in vivo suggests paracrine and autocrine mechanisms of tumor maintenance. *Proceedings of the National Academy of Sciences of the United States of America*. **89**: 7046-50.
54. Versnel MA, Haarbrink M, Langerak AW, et al. 1994. Human ovarian tumors of epithelial origin express PDGF in vitro and in vivo. *Cancer Genetics and Cytogenetics*. **73**: 60-4.
55. Antoniades HN, Galanopoulos T, Neville-Golden J, et al. 1992. Malignant epithelial cells in primary human lung carcinomas coexpress in vivo platelet-derived growth factor (PDGF) and PDGF receptor mRNAs and their protein products. *Proceedings of the National Academy of Sciences of the United States of America*. **89**: 3942-6.
56. Fudge K, Wang CY, and Stearns ME. 1994. Immunohistochemistry analysis of platelet-derived growth factor A and B chains and platelet-derived growth factor alpha and beta receptor expression in benign prostatic hyperplasias and Gleason-graded human prostate adenocarcinomas. *Modern Pathology*. **7**: 549-54.
57. Deinhardt F. 1980. Biology of primate retroviruses. In Viral Oncology. Klein G (Ed). New York: Raven Press. P 357-98.
58. Figarella-Branger D, Pellissier JF, Bouillot P, et al. 1994. Expression of neural cell-adhesion molecule isoforms and epithelial cadherin adhesion molecules in 47 human meningiomas: correlation with clinical and morphological data. *Modern Pathology*. **7**: 752-61.
59. Kuratsu JI, Seto H, Kochi M, et al. 1994. Expression of PDGF, PDGF-receptor, EGF-receptor and sex hormone receptors on meningioma. *Acta Neurochirurgica*. **131**: 289-93.
60. Samoto K, Ikezaki K, Ono M, et al. 1995. Expression of vascular endothelial growth factor and its possible relation with neovascularization in human brain tumors. *Cancer Research*. **55**: 1189-93.
61. Carmeliet P, Ferreira V, Breier G, et al. 1996. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature*. **380**: 435-9.
62. Ferrara N, Carver-Moore K, Chen H, et al. 1996. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature*. **380**: 439-42.
63. Senger DR, Van de Water L, Brown LF, et al. 1993. Vascular permeability

factor (VPF, VEGF) in tumor biology. *Cancer Metastasis Reviews.* **12**: 303-24.

64. Gospodarowicz D and Lau K. 1989. Pituitary follicular cells secrete both vascular endothelial growth factor and follistatin. *Biochemical and Biophysical Research Communications.* **165**: 292-8.
65. Ferrara N and Henzel WJ. 1989. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochemical and Biophysical Research Communications.* **161**: 851-8.
66. Senger DR, Galli SJ, Dvorak AM, et al. 1983. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science.* **219**: 983-5.
67. Ferrara N. 1996. Vascular endothelial growth factor. *European Journal of Cancer.* **32A**: 2413-22.
68. Schott RJ and Morrow LA. 1993. Growth factors and angiogenesis. *Cardiovascular Research.* **27**: 1155-61.
69. Neufeld G, Tessler S, Gitay-Goren H, et al. 1994. Vascular endothelial growth factor and its receptors. *Progress in Growth Factor Research.* **5**: 89-97.
70. Ferrara N, Houck K, Jakeman L, et al. 1992. Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endocrine Reviews.* **13**: 18-32.
71. Connolly DT. 1991. Vascular permeability factor: a unique regulator of blood vessel function. *Journal of Cellular Biochemistry.* **47**: 219-23.
72. Houck KA, Ferrara N, Winer J, et al. 1991. The vascular endothelial growth factor family: Identification of a fourth molecular species and characterization of alternate splicing of RNA. *Molecular Endocrinology.* **5**: 1806-14.
73. Terman BI, Dougher-Vermazen M, Carrion ME, et al. 1992. Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. *Biochemical and Biophysical Research Communications.* **187**: 1579-86.
74. Barleon B, Hauser S, Schöllmann C, et al. 1994. Differential expression of the two VEGF receptors flt and KDR in placenta and vascular endothelial cells. *Journal of Cellular Biochemistry.* **54**: 56-66.
75. de Vries C, Escobedo JA, Ueno H, et al. 1992. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science.* **255**: 989-91.
76. Millauer B, Wizigmann-Voos S, Schnürch H, et al. 1993. High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell.* **72**: 835-46.
77. Breier G, Albrecht U, Sterrer S, et al. 1992. Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. *Development.* **114**: 521-32.
78. Brown LF, Berse B, Jackman RW, et al. 1993. Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in adenocarcinomas of the gastrointestinal tract. *Cancer Research.* **53**: 4727-35.
79. Berse B, Brown LF, Van de Water L, et al. 1992. Vascular permeability factor (vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages, and tumors. *Molecular Biology of the Cell.* **3**: 211-20.
80. Shweiki D, Itin A, Soffer D, et al. 1992. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature.* **359**:

843-5.

81. Plate KH, Breier G, Weich HA, et al. 1992. Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. *Nature*. **359**: 845-8.
82. Dvorak HF, Sioussat TM, Brown LF, et al. 1991. Distribution of vascular permeability factor (vascular endothelial growth factor) in tumors: concentration in tumor blood vessels. *Journal of Experimental Medicine*. **174**: 1275-8.
83. Millauer B, Shawver LK, Plate KH, et al. 1994. Glioblastoma growth inhibited in vivo by a dominant-negative Flk-1 mutant. *Nature*. **367**: 576-9.
84. Tischer E, Mitchell R, Hartman T, et al. 1991. The human gene for vascular endothelial growth factor. *Journal of Biological Chemistry*. **266**: 11947-54.
85. Sanfilippo JS, Rao CV, Guarnaschelli JJ, et al. 1993. Detection of epidermal growth factor and transforming growth factor alpha protein in meningiomas and other tumors of the central nervous system in human beings. *Surgery, Gynecology and Obstetrics*. **177**: 488-96.
86. Filmus J, Pollak MN, Cairncross JG, et al. 1985. Amplified, overexpressed and rearranged epidermal growth factor receptor gene in a human astrocytoma cell line. *Biochemical and Biophysical Research Communications*. **131**: 207-15.
87. Westphal M, Harsh GRt, Rosenblum ML, et al. 1985. Epidermal growth factor receptors in the human glioblastoma cell line SF268 differ from those in epidermoid carcinoma cell line A431. *Biochemical and Biophysical Research Communications*. **132**: 284-9.
88. Frappaz D, Singletary SE, Spitzer G, et al. 1988. Enhancement of growth of primary metastatic fresh human tumors of the nervous system by epidermal growth factor in serum-free short term culture. *Neurosurgery*. **23**: 355-9.
89. Weisman AS, Villemure JG, and Kelly PA. 1986. Regulation of DNA synthesis and growth of cells derived from primary human meningiomas. *Cancer Research*. **46**: 2545-50.
90. Werner MH, Humphrey PA, Bigner DD, et al. 1988. Growth effects of epidermal growth factor (EGF) and a monoclonal antibody against the EGF receptor on four glioma cell lines. *Acta Neuropathologica*. 77: 196-201.
91. Nistér M, Libermann TA, Betsholtz C, et al. 1988. Expression of messenger RNAs for platelet-derived growth factor and transforming growth factor-alpha and their receptors in human malignant glioma cell lines. *Cancer Research*. **48**: 3910-8.
92. Phillips PC. 1991. Antineoplastic drug resistance in brain tumors. *Neurologic Clinics*. **9**: 383-404.
93. Ozols RF, Masuda H, and Hamilton TC. 1988. Mechanisms of cross-resistance between radiation and antineoplastic drugs. *National Cancer Institute Monograph*. **6**: 159-65.
94. Silverman CL and Simpson JR. 1982. Cerebellar medulloblastoma: the importance of posterior fossa dose to survival and patterns of failure. *International Journal of Radiation Oncology, Biology, Physics*. **8**: 1869-76.
95. Kunwar S, Pai LH, and Pastan I. 1993. Cytotoxicity and antitumor effects of growth factor-toxin fusion proteins on human glioblastoma multiforme cells. *Journal of Neurosurgery*. **79**: 569-76.

96. Pastan I, Chaudhary V, and FitzGerald DJ. 1992. Recombinant toxins as novel therapeutic agents. *Annual Review of Biochemistry*. **61**: 331-54.
97. Pastan I and FitzGerald D. 1991. Recombinant toxins for cancer treatment. *Science*. **254**: 1173-7.
98. Phillips PC, Levow C, Catterall M, et al. 1994. Transforming growth factor-alpha-Pseudomonas exotoxin fusion protein (TGF-alpha-PE38) treatment of subcutaneous and intracranial human glioma and medulloblastoma xenografts in athymic mice. *Cancer Research*. **54**: 1008-15.
99. Hwang J, Fitzgerald DJ, Adhya S, et al. 1987. Functional domains of Pseudomonas exotoxin identified by deletion analysis of the gene expressed in E. coli. *Cell*. **48**: 129-36.
100. Hall WA and Fodstad O. 1992. Immunotoxins and central nervous system neoplasia. *Journal of Neurosurgery*. **76**: 1-12.
101. Libermann TA, Razon N, Bartal AD, et al. 1984. Expression of epidermal growth factor receptors in human brain tumors. *Cancer Research*. **44**: 753-60.
102. Draoui M, Siegall CB, FitzGerald D, et al. 1994. TGF alpha-PE40 inhibits non-small cell lung cancer growth. *Life Sciences*. **54**: 445-53.
103. Kim KJ, Li B, Winer J, et al. 1993. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature*. **362**: 841-4.
104. Winek RR, Scheithauer BW, and Wick MR. 1989. Meningioma, meningeal hemangiopericytoma (angioblastic meningioma), peripheral hemangiopericytoma, and acoustic schwannoma. A comparative immunohistochemical study. *American Journal of Surgical Pathology*. **13**: 251-61.
105. Russell DS and Rubinstein LJ. 1989. Pathology of tumours of the nervous system (5th Edition). London: Edward Arnold.
106. Artlich A and Schmidt D. 1990. Immunohistochemical profile of meningiomas and their histological subtypes. *Human Pathology*. **21**: 843-9.
107. Cogen PH, Daneshvar L, Bowcock AM, et al. 1991. Loss of heterozygosity for chromosome 22 DNA sequences in human meningioma. *Cancer Genetics and Cytogenetics*. **53**: 271-7.
108. Dumanski JP, Carlbom E, Collins VP, et al. 1987. Deletion mapping of a locus on human chromosome 22 involved in the oncogenesis of meningioma. *Proceedings of the National Academy of Sciences of the United States of America*. **84**: 9275-9.
109. Seizinger BR, de la Monte S, Atkins L, et al. 1987. Molecular genetic approach to human meningioma: loss of genes on chromosome 22. *Proceedings of the National Academy of Sciences of the United States of America*. **84**: 5419-23.
110. Lutchman M and Rouleau GA. 1995. The neurofibromatosis type 2 gene product, schwannomin, suppresses growth of NIH 3T3 cells. *Cancer Research*. **55**: 2270-4.
111. Anan K, Morisaki T, Katano M, et al. 1996. Vascular endothelial growth factor and platelet-derived growth factor are potential angiogenic and metastatic factors in human breast cancer. *Surgery*. **119**: 333-9.
112. Chua CC, Geiman DE, Keller GH, et al. 1985. Induction of collagenase secretion in human fibroblast cultures by growth promoting factors. *Journal of*

Biological Chemistry. **260**: 5213-6.

113. Bauer EA, Cooper TW, Huang JS, et al. 1985. Stimulation of in vitro human skin collagenase expression by platelet-derived growth factor. *Proceedings of the National Academy of Sciences of the United States of America*. **82**: 4132-6.
114. Wang JL, Nistér M, Hermansson M, et al. 1990. Expression of PDGF beta-receptors in human meningioma cells. *International Journal of Cancer*. **46**: 772-8.
115. Rasheed BK, McLendon RE, Herndon JE, et al. 1994. Alterations of the TP53 gene in human gliomas. *Cancer Research*. **54**: 1324-30.
116. von Deimling A, Louis DN, von Ammon K, et al. 1992. Association of epidermal growth factor receptor gene amplification with loss of chromosome 10 in human glioblastoma multiforme. *Journal of Neurosurgery*. **77**: 295-301.
117. Weindel K, Moringlane JR, Marme D, et al. 1994. Detection and quantification of vascular endothelial growth factor/vascular permeability factor in brain tumor tissue and cyst fluid: the key to angiogenesis? *Neurosurgery*. **35**: 439-48.
118. Hatva E, Kaipainen A, Mentula P, et al. 1995. Expression of endothelial cell-specific receptor tyrosine kinases and growth factors in human brain tumors. *American Journal of Pathology*. **146**: 368-78.
119. Rak J, Mitsuhashi Y, Bayko L, et al. 1995. Mutant ras oncogenes upregulate VEGF/VPF expression: implications for induction and inhibition of tumor angiogenesis. *Cancer Research*. **55**: 4575-80.
120. Whittle IR, Hawkins RA, Killen E, et al. 1988. Epidermal growth factor binding in intracranial neoplasms: preliminary biochemical and clinicopathological findings. *British Journal of Neurosurgery*. **2**: 211-5.
121. Yamaizumi M, Mekada E, Uchida T, et al. 1978. One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. *Cell*. **15**: 245-50.
122. McNally NJ. 1982. Enhancement of chemotherapy agents. *International Journal of Radiation Oncology, Biology, Physics*. **8**: 593-8.

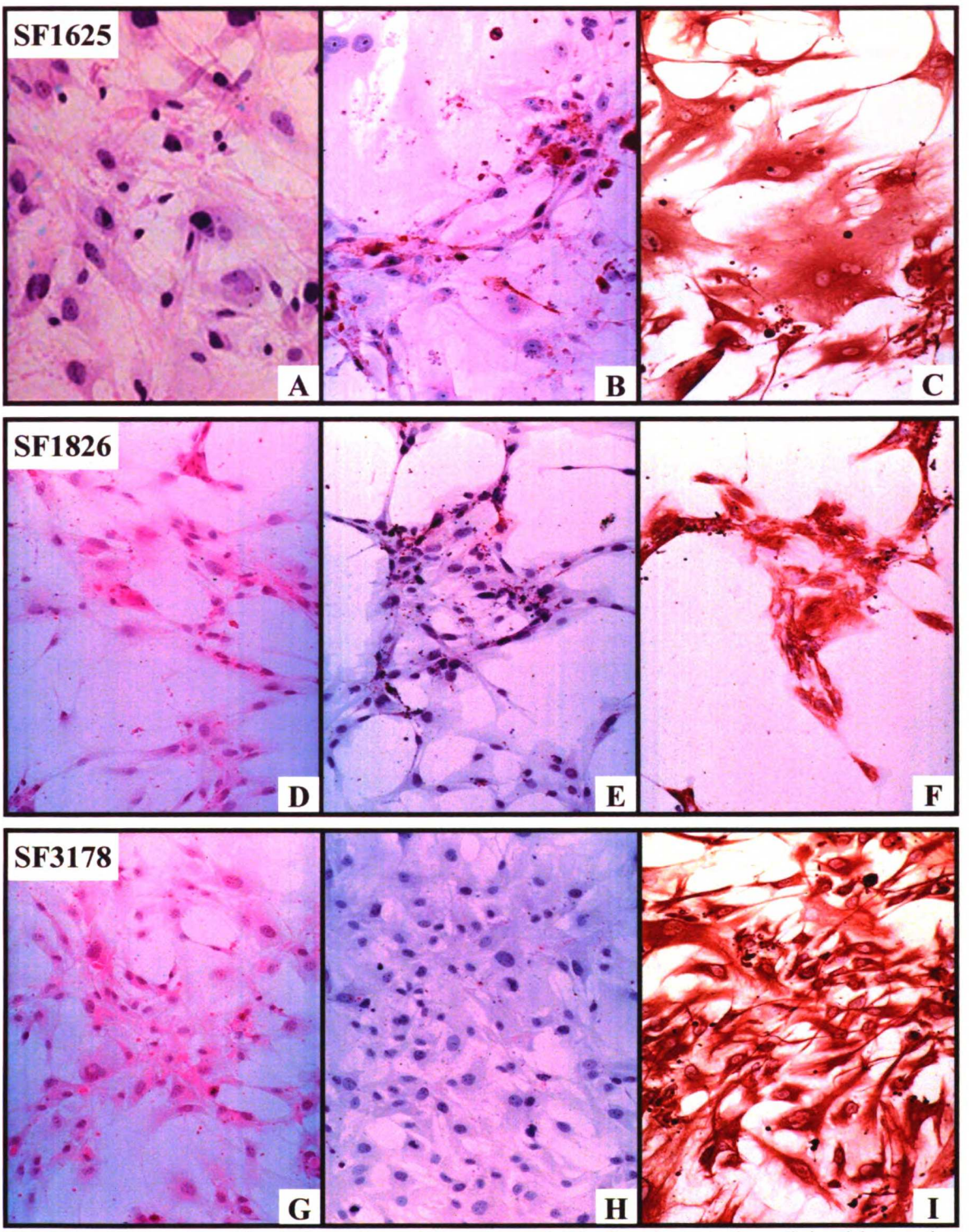

Figure 1. Hematoxylin and eosin staining (A, D, G) showing oval nuclei with regular nuclear membranes, uniform fine granular chromatin, and eosinophilic cytoplasm. Immunohistochemical staining for epithelial membrane antigen reveals mild (H) to moderate (B & E) expression of this marker; while presence of the intermediate filament, vimentin, is demonstrated in all three cell lines (C, F, I).

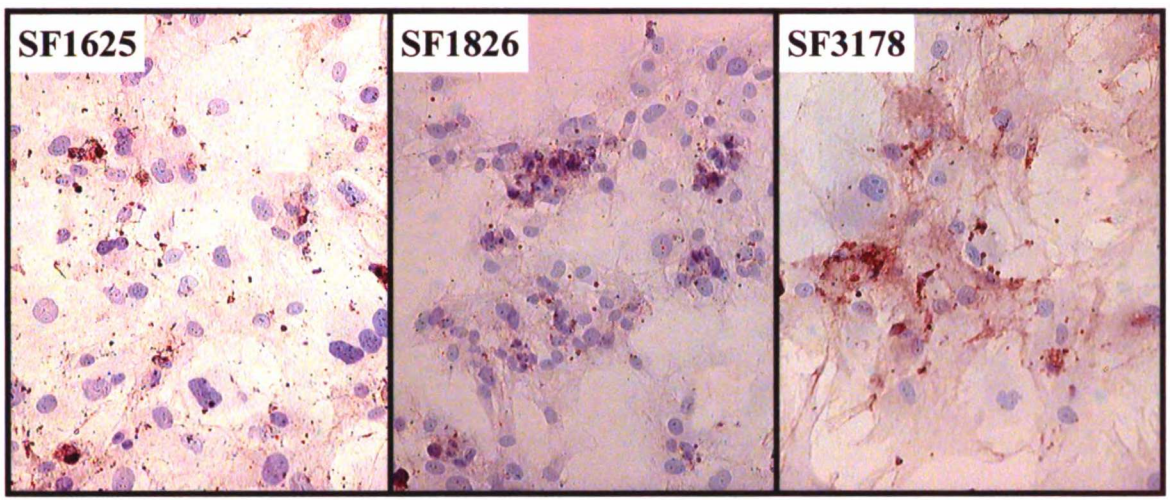

Figure 2. Immunohistochemistry for EGF-R showing positive staining in all cell lines.

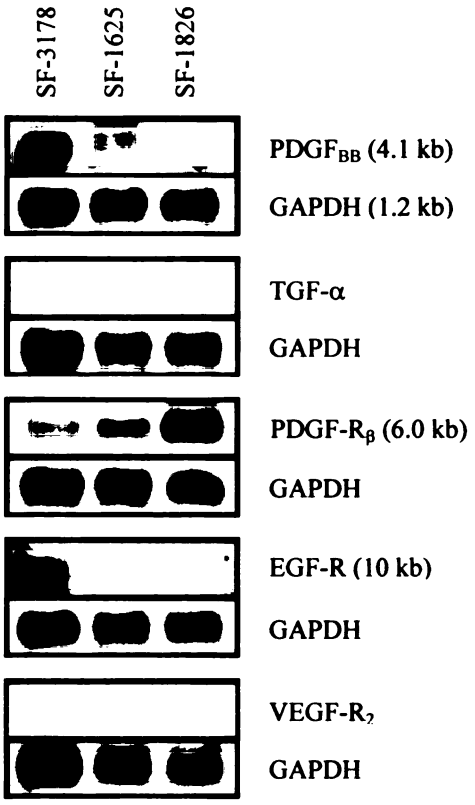

Figure 3. Northern Blot analysis detecting the presence of PDGF$_{BB}$, PDGF-R$_β$, TGF-α, EGF-R, and VEGF-R$_2$ mRNA. GAPDH was used to control for variations in loading of mRNA.

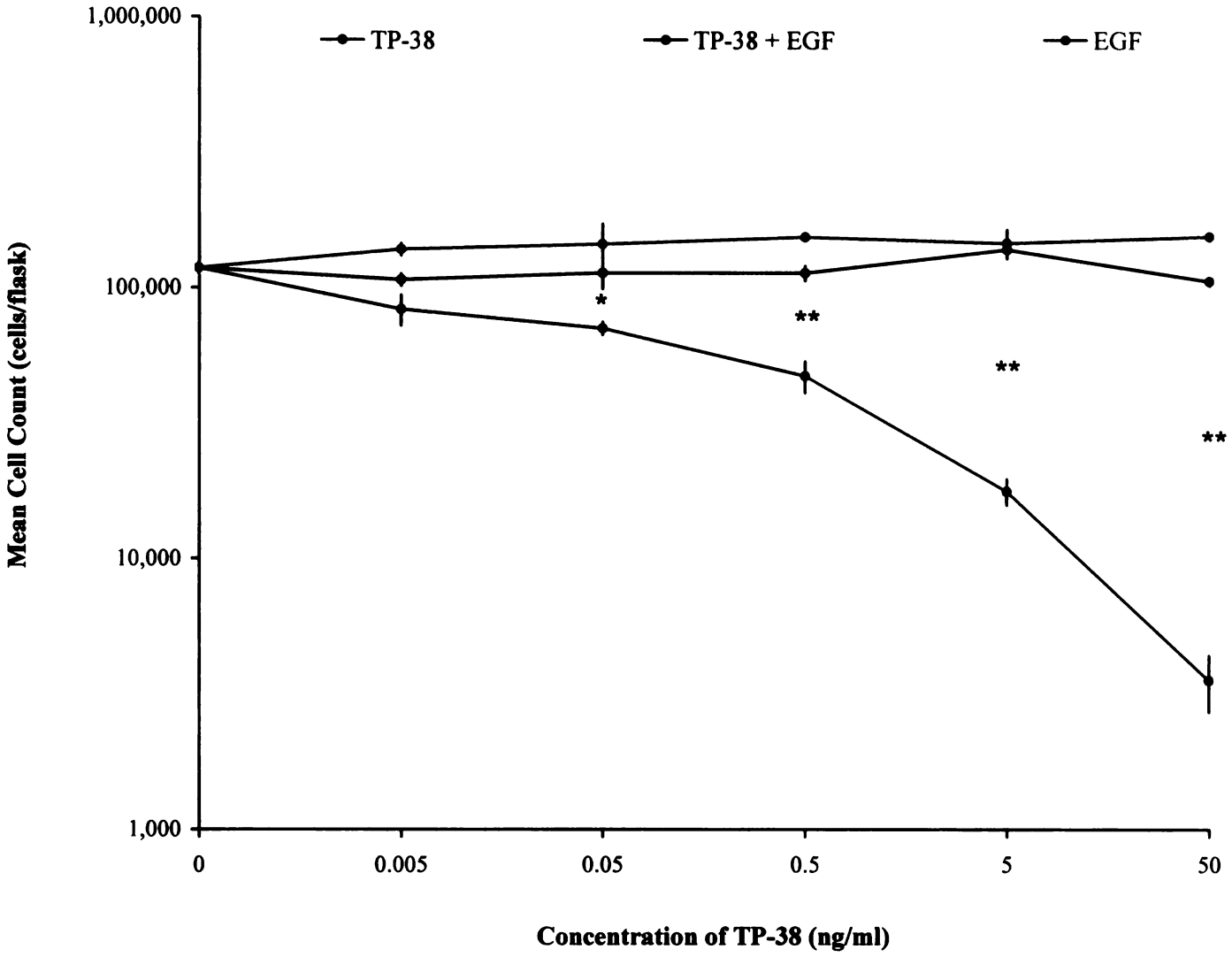

Figure 4C. Effects of TP-38 on cell line SF-3178 in the presence and absence of excess EGF following 7 days of incubation. Concentration of EGF used in the assay is 200 times that of TP-38. The mean value ± standard deviation of 3 determinations is indicated (* $p < 0.025$ and ** $p < 0.005$ for both *EGF* and *TP-38 + EGF*).

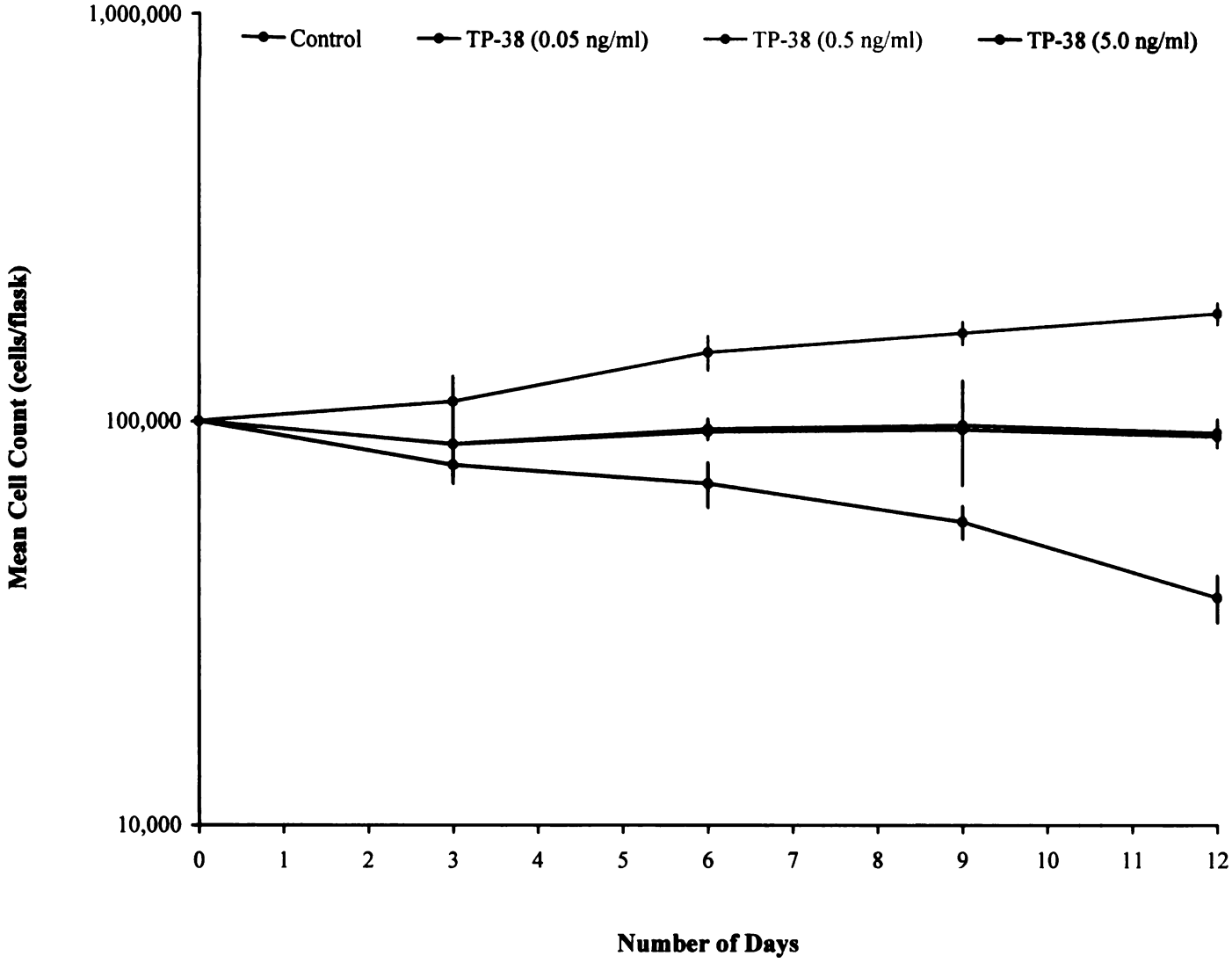

Figure 5B. Effects of three different concentrations of TP-38 on growth of SF-1826. The mean value ± standard deviation of 3 determinations is indicated.