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Effects of growth factors on a human glioma cell line during invasion into rat brain aggregates in culture*

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Summary. Cultures of fetal rat brain cell aggregates and tumor spheroids from the human glioma cell line GaMG were treated with epidermal growth factor (EGF), fibroblast growth factor (FGF) or isoforms of platelet-derived growth factor (PDGF AA or BB). Radioreceptor binding studies displayed a high binding capacity for EGF and FGF, but not binding of PDGF isoforms in the glioma cells. In serum-free culture, 10 ng/ml of both EGF and FGF caused increased growth and cell shedding in the tumor spheroids, whereas PDGF produced no such effect. Similarly, EGF and FGF stimulated tumor cell migration. EGF increased the proliferation and outgrowth of glial fibrillary acidic protein (GFAP)-positive cells in brain cell aggregates, while PDGF AA and BB both stimulated the outgrowth of oligodendrocyte-like cells which were negative for GFAP and neuron-specific enolase. FGF stimulated $GFAP^+$ as well as $GFAP^-$ cell types. In co-culture experiments using brain aggregates and tumor spheroids, both EGF and FGF treatment caused increased tumor cell invasion. PDGF had no effect on the tumor cells, but instead stimulated the proliferation of oligodendrocyte-like cells in the brain aggregates. The present results indicate that growth factors may facilitate glioma growth as well as invasiveness, and cause reactive changes in the surrounding normal tissue.

Key words: Brain neoplasm – Culture – Invasion – Growth factor

Tumors often express high levels of growth factors and their receptors. It is believed that growth factors may cause increased tumor growth [39] and angiogenesis [38]. In addition, the synthesis of tumor-derived proteolytic enzymes, which have frequently been linked to invasion and metastasis [9, 14, 15, 23] may be stimulated by growth factors. Furthermore, a high density of growth factor receptors has been correlated to a bad prognosis in some neoplastic diseases (see, e.g., [25, 34]).

Glioblastoma multiforme is a neoplasm characterized by rapid tumor growth and a strong capacity of the malignant cells to infiltrate the normal brain parenchyma (for review see [21]). Reactive changes, such as the proliferation of glia and vascular endothelial cells [33] are frequently observed, indicating a sensitivity of the normal tissues to tumor-secreted substances.

In glioblastomas, an increased synthesis of growth factors and their receptors has been reported in biopsy material [16, 35, 37] as well as in cultured cells [22, 28, 40]. The concurrent synthesis of ligand and receptor within one tumor may lead to an autocrine stimulation of tumor growth [27, 36, 42], since growth factors act as mitogens [40, 43] and also increase cell motility [41]. Previously, we have characterized a model system for the study of glioma cell invasion in vitro [4–7, 10, 17]. This system has now been highly standardized and is routinely used in studies of therapy and biology in glioma cell invasion [8, 11, 19]. The validity of this in vitro model is suggested by its biological and histological resemblance to animal models [6] and to human glioma biopsy specimens [7, 10].

In a recent report, we have shown that epidermal growth factor (EGF) stimulated glioma cell growth, motility and invasion in vitro [19]. The present study describes the effect of three different growth factors [EGF, and basic fibroblast and platelet-derived growth factors (bFGF, PDGF)] on glioma cell invasion using a co-culture model consisting of glioma spheroids of the GaMG cell line and rat brain aggregates. To exclude the influence of serum components, the present study was performed in a defined culture medium.

Materials and methods

Culture materials

Cells were grown in a standard tissue culture incubator (37 °C, 100% humidity, 5% CO_2). Two different media were used.

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Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % newborn calf serum, four times the prescribed concentration of non-essential amino acid L-glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml) was from Flow Laboratories (Glasgow, Scotland). The SF-X serum substituted modification of Iscove's medium was from Costar (Bad Hoevedorp, the Netherlands). All tissue culture plastic was from Nunc (Roskilde, Denmark), except in the binding studies, where Falcon (Becton-Dickinson, Mountain View, New Jersey) tissue culture dishes were used. For nonadherent three-dimensional culture, the plastic base was coated with 0.75 % agar noble (Difco, Detroit, Michigan) in medium (DMEM or SF-X).

Growth factors

Tissue culture grade EGF (Sigma, St. Louis, Missouri) and bovine recombinant basic FGF (Amersham Int., Amersham, GB) were dissolved in Hank's balanced salt solution. Human recombinant PDGF AA and BB, generous gifts from Dr. C.-H. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden), were dissolved in 0.1 M acetic acid. All stock solutions of growth factors (1 μg/ml) were sterile filtered, stored at −70 °C and thawed immediately prior to use. In all experiments, there was one control group receiving no growth factor, and treatment groups receiving either EGF, FGF, PDGF AA or BB.

Cell cultures

The GaMG cell line is established at the Gade Institute from a patient with a glioblastoma multiforme [1, 18]. The cells proliferate and invade in the absence of serum [17] and readily from spheroids in agar overlay culture. For each series of experiments, cells at passage 30–34 were thawed from frozen stocks, and seeded into monolayer culture in 80-cm^2 culture flasks in 15 ml of DMEM. The medium was changed every 5th day, until confluence. Spheroids were generated from trypsinized monolayers by seeding 3×10^6–5×10^6 cells in 15 ml of DMEM into 80-cm^2 culture flasks base-coated with 10 ml of medium agar. After 4–8 days, spheroids with a diameter of 200–400 μm were formed. Brain cell aggregates, consisting of differentiated normal neurons and glia cells in a prominent neuropil, were generated by culturing reaggregated cells from fetal rat brain over a 20-day period by standard procedures described previously [4, 5]. Aggregates and spheroids with a diameter of 300 ± 50 μm were selected for experiments.

Radioreceptor assay

EGF/PDGF receptors. GaMG cells were seeded in 35-mm^2 tissue-culture dishes (Falcon) at 0.2×10^6 cells per dish and incubated overnight. The cells were washed once in binding buffer [phosphate-buffered saline (PBS) with 1 mg/ml human serum albumin, 0.01 mg/ml of $CaCl_2$ and 0.01 mg/ml of $MgSO_4$]. Each dish then received 1 ml of binding buffer with 1 ng of ^{125}I-labelled EGF, PDGF AA, or PDGF BB (all kind gifts from Dr. C.-H. Heldin), and varying amounts of unlabelled growth factor. Incubation was carried out at room temperature for 2 h. The cultures were then washed four times with binding buffer (using 1 % newborn calf serum instead of human serum albumin). Cell-associated radioactivity was extracted with 0.5 ml/dish of lysis buffer (1 % Triton-X-100, 20 mM Hepes pH 7.4, 1 % glycerol). Lysates were counted in a Packard gamma counter. Receptor binding parameters for displacement experiments were calculated using the LIGAND computer program [24].

FGF receptors. Seeding was carried out as above. Cells were washed once in DMEM with 30 mM Hepes and 0.15 % gelatine. Samples were added in 1 ml of the above medium, each dish received 1 ng of ^{125}I-labelled acidic FGF (aFGF) and varying concentrations of non-labelled aFGF. Incubation was carried out for 2 h at +4 °C and the cultures were washed three times with DMEM. Cell-associated radioactivity was extracted and counted as described above.

Control cell lines. In the binding experiments cell lines that have previously been shown to express receptors for the different growth factors were included as control. The cell lines were the normal human foreskin fibroblast line AG 1518 (Human Mutant Cell Repository, Camden, N. J.) and the human malignant glioma cell line U-343MGa Cl2 [20].

Cell migration assay

Single tumor spheroids and brain cell aggregates were placed separately in 24-well dishes supplied with 1.0 ml of DMEM. This experiment was not performed in SF-X, because cell migration is reduced in this medium (unpublished observations). To generate a dose-response curve, the growth factors were added to the medium in concentrations ranging from 0.01 to 100 ng/ml (tenfold concentration increments, $n = 4$, each group). Cell outgrowth was observed during a period of 4 days (tumor cells) and 6 days (brain cells). The cultures were then fixed for 5 min in 4 % formaldehyde, and stained with hematoxylin-eosin. The size of the colonies was measured by morphometry with a light microscope connected via a video camera to a Mitsubishi AT personal computer equipped with the Videoplan morphometry software (Kontron, Eching, Germany).

Immunoperoxidase staining

Six-day-old explants of brain aggregates on tissue culture plastic were washed in PBS, and fixed in 4 % paraformaldehyde. The cell membranes were then permeabilized for 30 min with 5 % dimethylsulfoxide, rinsed with PBS, incubated for 15 min with 20 % swine serum (all immunochemicals from Dakopatts, Glostrup, Denmark) in PBS and incubated overnight at 4 °C with an affinity-purified polyclonal antibody to GFAP diluted 1:200 with 20 % swine serum in PBS. Similarly, parallel cultures were incubated with an antibody to neuron-specific enolase (NSE). Immunoperoxidase reaction was obtained by incubating the specimens for 10 min in 150 ml 3′3-diaminobenzidine tetrahydrochloride in PBS supplemented with 75 μl 35 % H_2O_2. As a negative control, serum from non-immunized rabbits was used instead of the primary antibodies.

Three-dimensional growth and cell shedding

Single spheroids and brain aggregates ($n = 6$, each group) were rinsed three times for 5 min in SF-X, and cultured on agar base in 24-well multiwell dishes containing 1.5 ml of medium and 10 ng/ml of EGF, FGF or PDGF. The culture diameters were measured regularly for 20 days (tumor cells) or 10 days (brain cells) in a phase-contrast microscope with a calibrated reticle in the eyepiece, culture volumes were thereafter calculated as described previously [3]. Growth factor treatment caused increased cell shedding and the appearance of new tumor spheroids. Therefore, cells being shed from the spheroids were counted regularly in each well by phase-contrast microscopy. At day 20, cells and spheroids were transferred to 10-ml plastic tubes which were placed vertically. The spheroids rapidly sank to the bottom, allowing them to be separated from the single cells, which were then centrifuged, resuspended in 1 ml of medium, and counted with a hemocytometer. Viability of the single cells was confirmed by plating them on tissue culture plastic with DMEM. In the EGF and FGF treatment groups shedding cells reaggregated into new spheroids. The total spheroid mass in each experimental group was, therefore, calculated by measuring all the spheroids, and their volumes were summarized.

Glioma-brain invasion assay

Pairs of aggregates and tumor spheroids ($n = 5$–7) were rinsed as above, and transferred to 96-well dishes base-coated with 100 μl of medium-agar, and filled with 300 μl of DMEM or SF-X and growth factors at a concentration of 10 ng/ml. After 4 or 7 days, the specimens were fixed for light microscopy in 2 % glutaraldehyde in 0.1 M succrose-adjusted cacodylate buffer (pH 7.4, 300 ± 50 mOsm).

Co-culture morphology and morphometry

The three-dimensional cultures were postfixed in OsO_4, dehydrated and embedded in Epon 812 by standard techniques described previously [4, 13]. Polymerization was carried out overnight at 40 °C, and then at 60 °C for 24 h. Semithin epon sections (1.5 μm thickness) from the middle part of the co-cultures were cut on a Reichert 2040 microtome, mounted on glass slides and stained with toluidine blue. To quantify invasion, the sections were examined by a recently described morphometry procedure [19]. Briefly, the area represented by invading tumor cells located inside the circumference of the brain aggregate is determined. This area is compared to the total area inside the brain aggregate circumference (invaded plus uninvaded portions of the brain aggregate) and the percentage of brain which has been invaded is then calculated.

Results

Radioreceptor studies

The GaMG cells were analyzed for expression of growth factor receptors using ^{125}I-labelled ligands. In Fig. 1A it is shown that GaMG cells bound ^{125}I-labelled EGF to a lesser extent than U-343MGa cells (Scatchard analysis of the GaMG cells revealed a receptor density of 200 000 sites/cell with the K_D in the expected nanomolar range). No specific binding could be obtained with either PDGF AA or PDGF BB while the control cell line AG1518 readily bound ^{125}I-labelled PDGF of both isoforms (Fig. 1B–C). In Fig. 1D the binding of ^{125}I-labelled aFGF to the GaMG cells is shown. Scatchard analysis of FGF receptors was not obtained due to the high nonspecific binding of ^{125}I-labelled aFGF.

Cell migration

Tumor spheroids. The GaMG cells were sensitive to EGF as well as FGF in the migration assay, showing a dose-dependent response with maximum stimulation in the range of 10–100 ng/ml. PDGF AA and BB had no effect on cell migration (Fig. 2). In the migration experiments, the growth factor-stimulated cells were spread on the tissue-culture plastic plates without being in direct contact with other cells. This indicated that the increased area represented by the cells was caused by increased cell motility, and not only by increased proliferation.

Brain aggregates. The fetal brain cells showed less migration than the tumor cells. As compared to control, EGF, FGF, PDGF AA and BB all increased cell spreading from the aggregates over a 6-day period. EGF induced the outgrowth of cells with a morphology resembling astrocytes [20]. PDGF AA and BB predom-

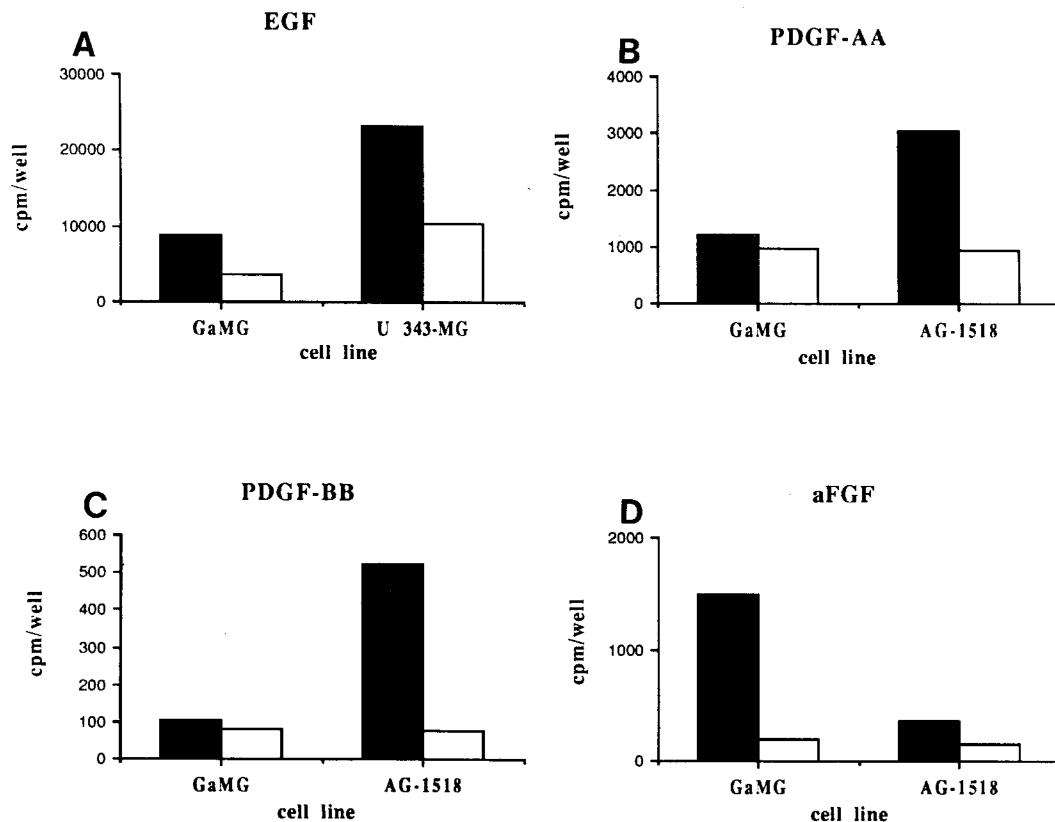
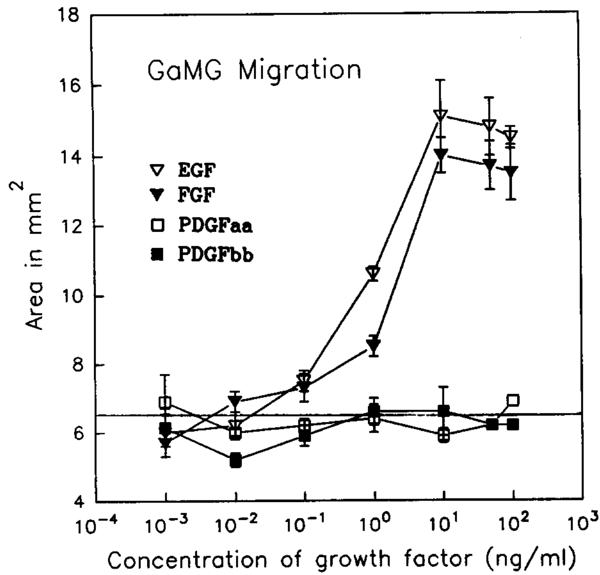


Fig. 1A–D. Analysis of growth factor receptors in GaMG glioma cells. ^{125}I-labelled growth factor was added to the cells in the absence (■) or presence (□) of unlabelled ligand. *EGF, PDGF, FGF:* Epidermal, platelet-derived and fibroblast growth factors, respectively



inantly stimulated cells with morphology resembling cells of the O-2A/oligodendrocyte lineage [20]. FGF stimulated the outgrowth of both astrocyte-like and O-2A/oligodendrocyte-like cells (Fig. 3).

Immunocytochemistry

The EGF-treated fetal brain cultures were dominated by GFAP-positive cells (Fig. 3C), whereas the oligodendrocyte-like cells produced by PDGF treatment were

Fig. 2. Dose-response-mediated effects of growth factors on cell migration of tumor cells. Mean values ± standard error of the mean. *Baseline:* control level

Fig. 3A–D. Brain aggregate explanted on plastic. **A** EGF, 10 ng/ml, showing cells with large cell bodies. **B** PDGF, 10 ng/ml, showing a network of cells with small cell bodies and branched extensions. **C** EGF treatment showing GFAP-positive astrocytes, immunoperoxidase stain. **D** Quantitation of explant areas. *Baseline:* Control level. **A, C** ×300, **B** ×450

GFAP/NSE negative (not shown). GFAP-positive as well as -negative cells were observed in the controls and in FGF-treated cultures.

Growth and cell shedding

Tumor spheroids. The GaMG spheroids grew in the SF-X medium with an average volume-doubling time of 6.5 ± 0.5 (SEM) days from the start to the end of the experiment. EGF and FGF reduced the doubling time to 1.6 ± 0.3 and 2.0 ± 0.4 days respectively, whereas spheroids treated with the isoforms of PDGF displayed a doubling time of 6.4 ± 0.6 days (average values of three experiments; Fig. 4A). Summarizing the volumes of the initial spheroids and satellite spheroids caused by reaggregation of shed cells, the net volumes of all spheroids from the six wells at day 20 were 0.29 mm^3 (control, PDGF isoforms), 1.3 mm^3 (EGF) and 0.9 mm^3 (FGF). Cell shedding was strongly increased by EGF and FGF treatment (Fig. 4B). In DMEM growth factor effects on tumor cells were similar to those seen in SF-X (not shown).

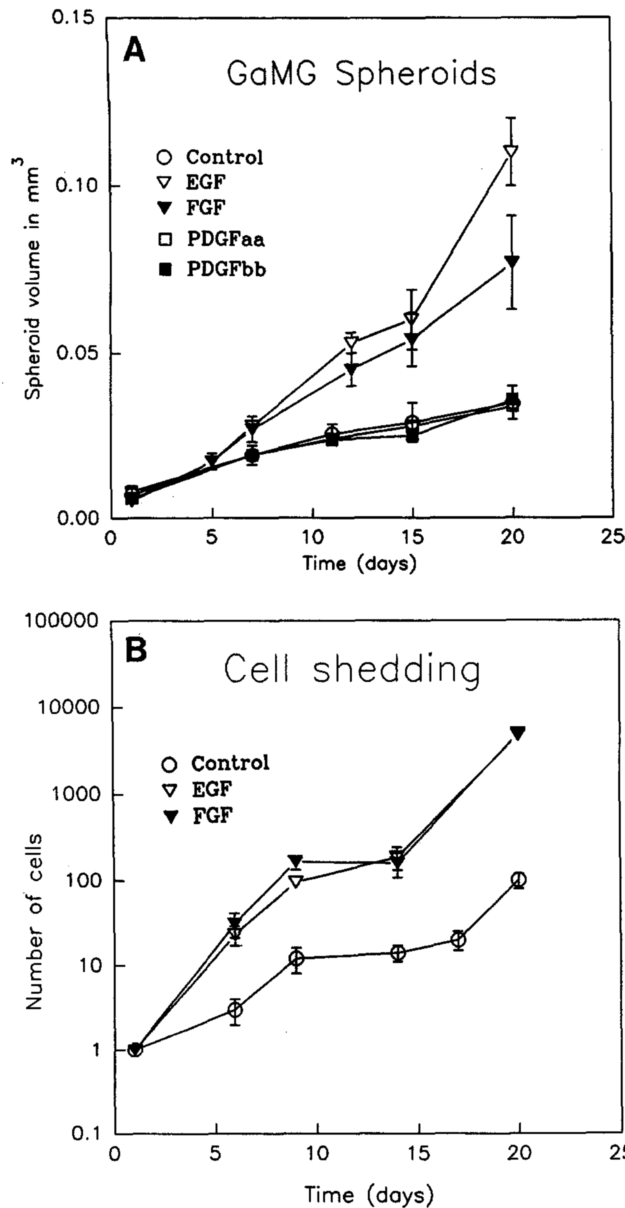


Fig. 4A, B. Effect of growth factors on (**A**) the volume increase of, and (**B**) cell shedding from, GaMG glioma spheroids treated with growth factors in serum-free medium. PDGF treatment produced cell shedding similar to that of control (not shown). *Bars* = Standard error of the mean

Brain aggregates. The brain aggregates responded to growth factor treatment with the strongest effect seen in serum-free culture. Volume changes (v/v_0) during a 10-day period were, for control and PDGF BB: 0.75 (1.0), EGF: 1.3 (3.6), FGF: 1.0 (1.0), PDGF AA: 1.3 (2.6), (parentheses indicate values for SF-X). Using light microscopy it was observed that, in the aggregates, EGF caused an increase in astrocyte-like cells and PDGF increased the number of small cells with a dark nucleus. These oligodendrocyte-like cells were found predominantly on the surface of the aggregates. The effect of growth factors on aggregate morphology was most profound in serum-free culture.

Invasion

The GaMG cells invaded the brain aggregates in a progressive manner as has previously been described [17]. Both EGF and FGF caused increased invasiveness as compared to controls, whereas no effect was seen with PDGF (Fig. 5). An increase of cell lysis and disorganization of the brain cells was observed with EGF or FGF treatment. This was most evident in 7-day-old co-cultures maintained in SF-X medium (Fig. 6). Due to the disruption and disorganization of brain tissue in 7-day-old EGF- and FGF treated SF-X co-cultures, it was impossible to determine the percentage of invasion by morphometry in those specimens. In SF-X co-

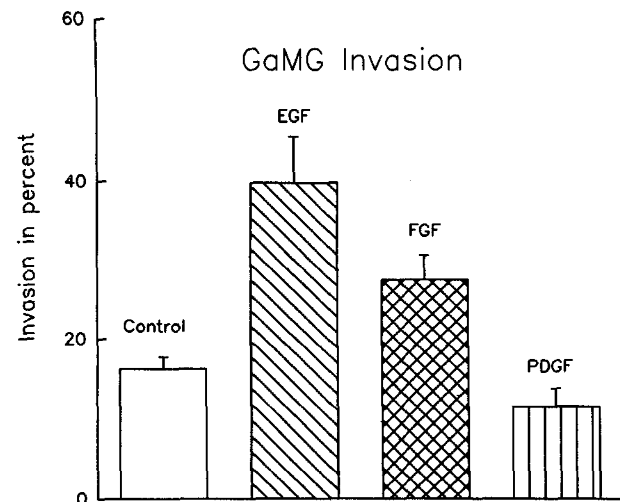


Fig. 5. Graphic presentation of invasiveness of GaMG spheroids into fetal brain aggregates co-cultured for 4 days. *Bars* = Standard error of the mean

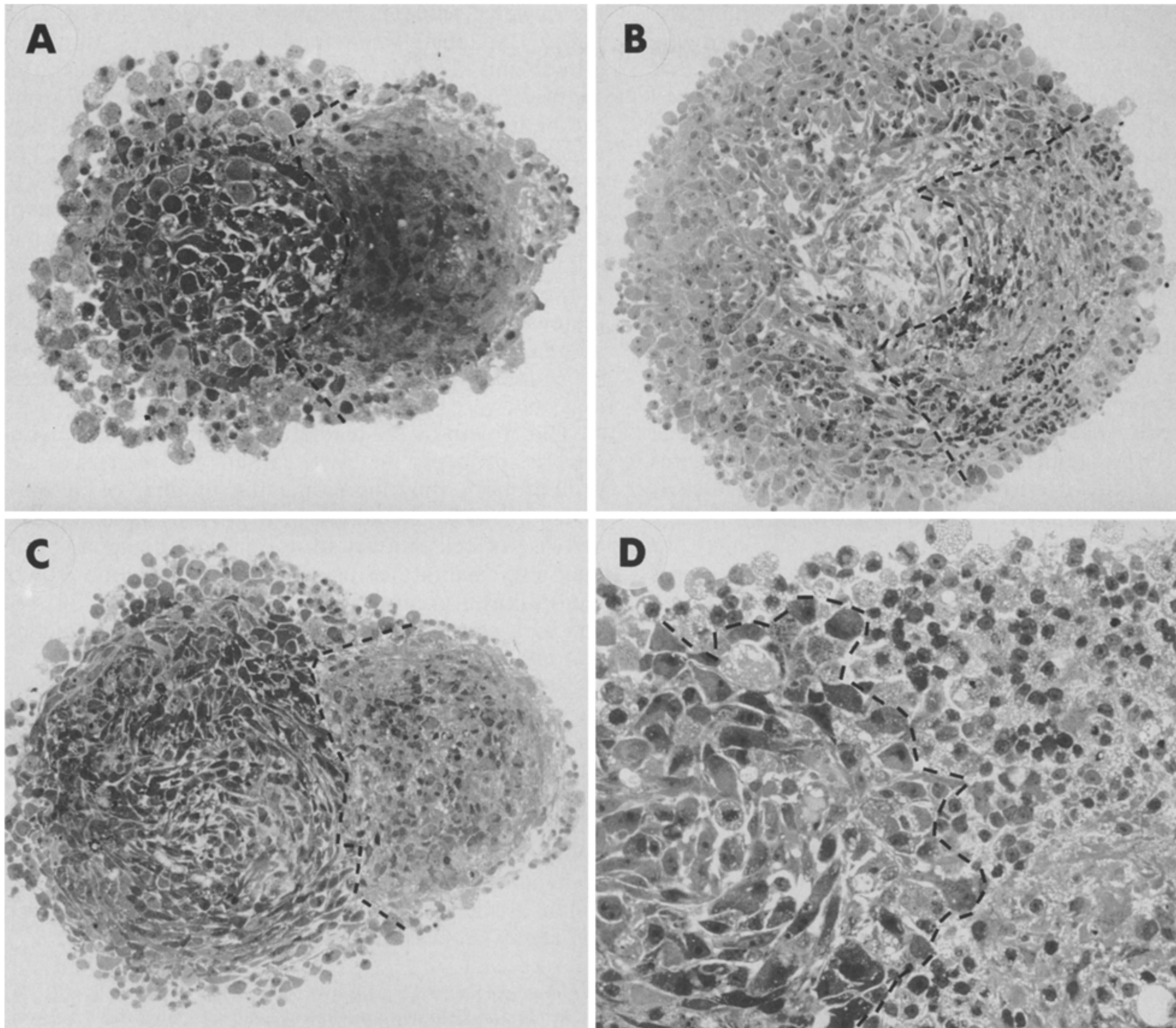


Fig. 6A–D. Morphology of 7-day-old co-cultures, serum-free medium. The brain aggregates are to the *right*. **A** Control, **B** EGF, **C** FGF, **D** PDGF AA (all: 10 ng/ml). Compared to control, there is a near-total destruction of brain tissue in **B** and increased loosening of the neuropil in **C.** In **D,** there is an appearance of oligodendrocyte-like cells on the surface of the brain aggregate. *Dashed lines* indicate the border between normal and malignant tissue. **A** ×275, **B, C** ×200, **D** ×400

cultures that had been treated with PDGF, there was a strong increase of oligodendrocyte-like cells which appeared on the aggregate surface, and which migrated onto the tumor spheroid (Fig. 6D).

Discussion

In this study, it has been shown that the growth factors EGF, FGF and PDGF may stimulate the growth of three-dimensional cultures of brain cells and tumor cells. With the exception of PDGF, the responses produced by the malignant cells exceeded those of the normal cells. Depending on the culture conditions (serum-free vs. serum-supplemented medium), the brain cell responsiveness to growth factors varied. In serum-free medium, brain aggregate volume was markedly increased when growth factors were added, whereas in serum-supplemented culture growth factor effects were weaker. Serum contains differentiating substances, such as Transforming growth factor-β, which may increase the stability of the aggregate culture. However, when explanted on plastic, migrating brain cells showed a marked growth factor response which was less observable in whole aggregates on agar. When the cells move from the brain aggregate along the plastic surface, they are deprived of cell-to-cell contacts; as a consequence contact inhibition is reduced and, therefore, growth factor-mediated proliferation as well as motility may be increased. However, the use of a serum-free medium in this study was necessary to demonstrate that growth factors act without any unknown serum cofactors.

Noble, et al. [19] and Raff et al. [32] have shown that PDGF stimulates the proliferation of O-2A progenitor cells, which then gradually differentiate into postmitotic oligodendrocytes. Furthermore, it has been shown that

EGF is an astrocyte mitogen [12], thus explaining the increased proliferation of astrocytes in outgrowing aggregates. Our results on FGF support the view that this growth factor may stimulate both astrocytes and oligodendrocytes [12]. Therefore, since growth factor effects on rat brain cells have been studied in detail by other groups, a further characterization of these cells has not been done in the present study. A novel observation, however, appears to be that three-dimensionally cultured brain cells exhibit similar responses as those in monolayers.

There is a high degree of cellular heterogeneity in malignant gliomas in vivo. These tumors may have neoplastic oligodendrocytes [33], which may be stimulated by glioma-derived PDGF [28]. Similarly, reactive astrocytosis, may be a consequence of the synthesis of EGF-receptor agonists in the neoplasm [35]. Finally, it is likely that the extensive neovascularization, which is seen in gliomas, may be caused by growth factors like, for example, FGF [22, 38].

Invasion is a co-process between the invasive cells and the host organ. It must be assumed that in the co-cultures, the target tissue contributes somewhat to the invasion process. It is also possible that some of the increased invasion caused, e.g. by EGF, is mediated by growth factor responses expressed by the normal cells. There are, however, two observations which indicate that the main effect is on the tumor cells.

First, PDGF, which had no effect on the glioma cells, did not affect invasion. Second, the addition of FGF or EGF to tumor spheroids caused a potent stimulation of growth and migration which exceeded that on brain cells by severalfold. In the present experimental system, growth factor effects may be demonstrated on normal and malignant cells simultaneously under highly standardized conditions. Histologically, the invasion pattern observed in co-cultures of human glioma cells and fetal rat brain cell aggregates resemble that found in vivo [10, 17]. Furthermore, reactive changes, similar to those seen in a glioma were induced by growth factors. However, although the rat brain aggregates contain structures associated with the differentiated CNS, such as astrocytic processes, myelinated axons and synapses, a limited number of undifferentiated cells are also found [4, 5]. The recruitment of growth factor-responding cells from this population is not known. Furthermore, it is believed that reactive processes, such as astrocytosis, can occur by a transient change in growth factor receptor status and not by an increase in ligand concentration [26]. Therefore, we cannot from the present study conclude that brain tumors in vivo exhibit the same response as that seen in vitro solely by the action of growth factors.

Although growth factors stimulated normal brain cells, the predominant response seen in co-cultures treated with EGF or FGF was an acceleration of malignant cell growth and invasiveness. This is consistent with recent data showing EGF-stimulated growth of glioma monolayers in serum-free medium [31]. EGF caused the strongest effect, inducing tumor cell lysis of brain tissue together with an increased proliferation of tumor cells, while FGF caused a similar, but weaker effect. Previously, we have shown that EGF stimulates growth and invasion in several glioma cell lines. In this study we have used a serum-free medium to demonstrate the biological effects of growth factors on a cell line, which was also stimulated by FGF, but not PDGF. The sensitivity of the GaMG cells to the different growth factors may be explained by the density of growth factor receptors on the cell membrane. The GaMG cell line had a relatively high level of EGF and FGF receptors. However, this line did not bind PDGF isoforms. Complete lack of PDGF binding is rarely observed in human glioma cell lines [28]. Therefore, from the present study we cannot conclude upon the role of PDGF in glioma invasion.

The growth factor-mediated stimulation of invasiveness is probably in part caused by increased cell proliferation, thus increasing the number of invasive malignant cells. In addition, a growth-factor induced increase of cell motility in normal and malignant cells may also facilitate invasiveness [13]. Possibly, growth factors may also cause glioma cell proteolysis [2, 30, 44], and as a consequence stimulate brain cell destruction and malignant cell invasion.

In conclusion, in cultures of rat brain cells and human glioma cells, a stimulatory effect of EGF was seen both on astrocytes and on glioma cells. PDGF caused stimulation predominantly of oligodendrocyte-like cells, whereas FGF stimulated both types of glia as well as glioma cells. It is shown that increased invasiveness was obtained by growth factor action both on normal and malignant cells. As a net result this accelerated the tumor dissociation of the normal tissue, and increased the aggressiveness of the tumor cells.

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