Regulation of DNA Synthesis and Growth of Cells Derived from Primary Human Meningiomas

Andrea S. Weisman, Jean-Guy Villemure, and Paul A. Kelly

ABSTRACT

We have studied the effects of insulin, epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor, and steroid hormones (estradiol, progesterone, and cortisol) on human meningioma cell proliferation and DNA synthesis in a serum-free culture system. The growth factors, particularly EGF and FGF, increased DNA synthesis in a dose-dependent manner as measured by \( ^{3}H \) thymidine incorporation, and they stimulated submaximal cell proliferation. No individual factor or combination of factors was able to successfully reproduce the effects of 10% fetal calf serum (FCS) on cell growth, although a combination of platelet-derived growth factor (5 units/ml) and EGF (10 ng/ml) synergistically stimulated DNA synthesis to near maximal levels. In addition, serum dependency was observed in studies involving the mitogenic effects of insulin, EGF, or FGF. Both EGF and FGF (10 ng/ml) maximally stimulated cell growth in the presence of 5% FCS.

The effects of steroid hormones on cell proliferation, individually or in combination with growth factors or charcoal-treated FCS, were also evaluated. Estradiol (100 nm) significantly increased cell number over control values only in the presence of charcoal-treated FCS; no effects of progesterone or cortisol on cell proliferation were observed.

In conclusion, both EGF and FGF stimulated cell proliferation and DNA synthesis in human meningioma cultures in a serum-free system, whereas steroid hormones were without effect. It appears that additional serum components are required for both estradiol-stimulated growth and for maximal proliferation of human meningioma cells under serum-free conditions.

INTRODUCTION

Meningiomas are mesenchymal tumors which comprise 18% of all primary intracranial tumors and 25% of all intraspinal tumors. Two-thirds of all meningiomas are reported in women, and tumor growth is accelerated during the luteal phase of the menstrual cycle and during pregnancy. There is also an increased incidence of meningiomas in women with breast carcinomas (1, 2). Recent studies have revealed the presence of specific, high affinity binding sites for progesterone, and to a lesser extent, binding sites for estradiol in the cytosol fraction of human meningiomas (2–6). These binding sites are similar in number and affinity to steroid hormone receptors that have been previously characterized in human breast tumors (5, 6).

Meningiomas are primarily benign tumors which can be removed successfully by surgery, but a small percentage are inoperable, invasive, or malignant. Benign metastasizing meningiomas have also been reported in which the secondary tumors retain their benign histopathological features (7). These tumors cannot be eliminated by surgical excision alone. In addition, 20% of all meningiomas recur following surgery, and 75% recur after incomplete excision (2). The development of a specific hormonal therapy would be useful as an alternative or adjuvant therapy in inoperable or recurrent cases. Since few models exist to study cerebral tumors, an in vitro system is necessary to identify and characterize the growth factors and hormones which are essential for tumor growth.

In the past, little research has been performed on human meningioma cells in culture. Duffy et al. (8) studied the effects of micromolar concentrations of polyamines on meningioma cell growth in culture and concluded that putrescine stimulated growth, whereas spermidine and spermine were cytotoxic. Recent studies by Jay et al. (9) suggested that physiological concentrations of estradiol, progesterone, and tamoxifen enhanced growth in cultures derived from four different tumors. The interpretation of these results is complicated by the fact that both studies were conducted in medium containing high concentrations of fetal calf serum. Serum provides a complex mixture of nutrients, hormones, and attachment factors which are necessary for cellular proliferation (10, 11). Unfortunately, its presence often limits the definition and characterization of factors required for growth and differentiation. The present study was undertaken to determine the effects of various growth factors and steroid hormones on DNA synthesis and cell division of meningioma cells in a serum-free medium.

MATERIALS AND METHODS

Clinical Material. Tumor specimens were obtained from three patients. The first patient (Tumor 1, passage 5: T1P5) was a 72-yr-old male who underwent surgery for removal of a left frontoparietal sphenoidal meningioma. The second patient (Tumor 2, passage 2: T2P2) was a 73-yr-old female who underwent removal of a recurrent sphenoidal wing meningioma that was of the meningoenothelial type. The third patient (Tumor 3, passage 2: T3P2) was a 46-yr-old male who developed seizures and underwent removal of a sphenoidal and angiofollicular meningioma.

Isolation and Culture of Human Meningioma Cells. Human meningiomas were obtained using surgical procedures and placed in DMEM (Flow Laboratories, McLean, VA) buffered with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4. The tissue was weighed, minced into 1-mm² pieces, and placed in DMEM (wt/vol, 1:15) containing 0.75% collagenase (Sigma Chemical Co., St. Louis, MO) for 30 min at 37°C. A pellet was obtained, fresh medium was added, and cells were mechanically dispersed. The cell solution was filtered through a stainless steel screen (300 μm) to remove small bone and tissue fragments. Cells were seeded in Primaria 25-cm² flasks (Falcon, Oxnard, CA) containing 10 ml of DMEM supplemented with 10% FCS, penicillin (50 units/ml), streptomycin (50 μg/ml) (Flow Laboratories, McLean, VA), and Fungizone (2.5 μg/ml) (Squibb, Montreal, Canada). Cells were grown to confluence, passed 1 to 4 times, and frozen at -70°C for use in future studies.

Light Microscopy. Meningioma cells were grown to near confluence in DMEM supplemented with 10% FCS in tissue culture chamber/slides (Miles Laboratories, Naperville, IL). Cells were fixed and stained in situ with hematoxylin eosin. Light micrographs were obtained using a Zeiss photomicroscope and Kodak Panatomic-X film.

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The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HBSS, Hank's balanced salt solution; FGF, fibroblast growth factor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor.
Transmission Electron Microscopy. Meningioma cells were grown to near confluence in Primaria culture dishes (60 x 15 mm) and were fixed in situ for 90 min at 25°C using 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. Cultures were rinsed 3 times and scraped into microfuge tubes. Cell pellets were obtained using high speed centrifugation, and pellets were embedded in Epon 812 using standard techniques. Thin sections (60 to 80 nm) were stained with uranyl acetate and lead citrate prior to examination in a Siemens 1A electron microscope (Siemens Corp., Iselin, NJ).

\[^{3}H\]Thymidine Incorporation Studies. \[^{3}H\]Thymidine incorporation studies were performed using a modified method of Jacobs et al. (12). In brief, cells were plated in Primaria 24-well dishes and grown in DMEM containing 10% FCS until cultures were confluent (6 to 8 days). The medium was removed, and the cultures were rinsed in HBSS (Flow Laboratories, McLean, VA). DMEM supplemented with 10% FCS, or serum-free DMEM supplemented with transferrin (25 \(\mu\)g/ml), 0.1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO), the trace elements (in \(\mu\)mol) MnCl\(_2\) (5), (NH\(_4\))\(_2\)MoO\(_4\) (0.5), NiSO\(_4\) (0.25), Na\(_2\)SeO\(_3\) (15), NaSiO\(_3\) (250), SnCl\(_2\) (0.25), CdSO\(_4\) (5), CuSO\(_4\) (10), ZnSO\(_4\) (50), lipoic acid (0.2 \(\mu\)g/ml), linoleic acid (3 \(\mu\)g/ml), growth factors, and steroid hormones, was added to each well, and cells were incubated for 21 h. Concentrated solutions of estradiol, progesterone, and cortisol (Sigma, St. Louis, MO) were prepared in absolute ethanol and diluted to appropriate concentrations in HBSS or medium. Bovine pancreatic insulin (Sigma Chemical Co., St. Louis, MO), FGF, EGF, and PDGF (Collaborative Research, Boston, MA) were diluted in HBSS immediately prior to addition to cultures. One \(\mu\)Ci of \[^{3}H\]thymidine (60 to 90 Ci/mmol; ICN, Irvine, CA) was added to each well, and this was followed by 1 to 3 additional h of incubation. Cultures were rinsed twice with HBSS following the \[^{3}H\]thymidine pulse and treated with a trypsin:EDTA solution (0.05:0.02%). Cell aliquots were freeze-thawed, treated with 10% trichloroacetic acid, and centrifuged to obtain the acid-precipitable pellet. The pellets were solubilized in 0.1 N NaOH and counted in 8 ml of Beckman MP scintillation on a LKB 1211 liquid scintillation counter. All results were expressed as mean ± SE of triplicate determinations and represented at least three experiments except where indicated otherwise. Statistical analyses were performed using Duncan-Kramer’s multiple range test (13) after logarithmic transformation.

Cell Growth Studies. Cells were replicately plated at a density of 1 x 10^4 cells/ml in Primaria 24-well dishes and grown for 5 days, except where indicated otherwise, in DMEM supplemented with various concentrations of FCS or serum-free DMEM, in the presence or absence of the aforementioned hormones and growth factors. Cells were trypsinized and counted using a hemacytometer and an American Optical light microscope. Cell viability was determined using trypan blue exclusion.

RESULTS

Light and Electron Microscopy. The three meningioma cultures used in this study represent the most common histological features of this intracranial tumor. The tumors, all benign and classified according to their histological patterns on routinely stained tissue, were synovial (T\(_2\)P\(_3\)), endotheliomatous (T\(_3\)P\(_2\)), and angioblastic/syncytial (T\(_3\)P\(_3\)) in nature. Outgrowth of meningioma cells isolated from these tumors occurred within 24 to 48 h after culture initiation, and cells formed a monolayer within 1 wk. Numerous whorls were observed in primary cultures, but upon subculture, the number and size of whorls decreased. The morphological appearance of the cultured meningioma cells was similar to that described in previous studies (Fig. 1; Ref. 13). The cells are polyhedral in shape, have centrally located nuclei with one to three nucleoli, and manifest formations suggestive of whorls. Cytoplasmic filaments, nuclear invaginations, microvilli-like processes, and numerous small vesicles were observed in later passages using electron microscopy (Fig. 2). These features are characteristic of meningioma cells seen in culture (14) and in tumor sections (15).

\[^{3}H\]Thymidine Incorporation Studies. The effects of insulin, EGF, FGF, and PDGF on \[^{3}H\]thymidine incorporation into DNA by serum-free cultures were studied. All four growth factors stimulated DNA synthesis in a dose-dependent manner in the three meningioma cultures tested (Fig. 3, A to D). However, the maximal stimulation of \[^{3}H\]thymidine incorporation by the individual growth factors was only 60% of the amount of \[^{3}H\]thymidine incorporated in the presence of 10% FCS (control), in these experiments.

Insulin increased DNA synthesis to 50% of control values in two of three tumor-derived cultures. PDGF stimulated the least amount of \[^{3}H\]thymidine incorporation when compared to the other growth factors tested. The highest concentration of PDGF (5 units/ml) stimulated DNA synthesis to only 25% of control values. EGF increased DNA synthesis in a similar

Fig. 1. Light micrographs of human meningioma cells (syncytial tumor, T\(_3\)P\(_3\)). In A, this paraffin section of the original tumor displays polygonal cells in a whorl-like pattern. H & E. x 80. In B, human meningioma cells cultured for 1 wk show preservation of whorling formation. Large arrows indicate spontaneously appearing foci, and arrowheads indicate large, multinucleated cells. H & E. x 32.
manner in all tumor cells with a slight variation in potency. The maximal amount of incorporation (induced by 10 to 50 ng/ml) was equal to 60% of control values. The FGF-induced responses were more variable, although FGF was equipotent in all the cultures tested and maximally stimulated DNA synthesis to 50% of control values. Interestingly, both EGF and FGF appeared to be slightly inhibitory at the highest concentrations tested (1 μg/ml or 160 nM, and 250 ng/ml or 19 nM, respectively).

Numerous studies have shown the synergistic effects of growth factors on DNA synthesis and cell proliferation in other cell systems (16, 17). Since EGF, FGF, and PDGF stimulate DNA synthesis individually in our culture system, the ability of two factors to act synergistically with one another is best assessed at submaximal concentrations. Fig. 4 shows that EGF (10 ng/ml) synergizes with PDGF to induce maximal stimulation of DNA synthesis in this culture (T3P3). However, an inhibitory effect on DNA synthesis was observed at the highest dose of PDGF (5 units/ml) used in this study. The effects of FGF (10 ng/ml) and PDGF were essentially additive, but synergism was observed at higher concentrations of PDGF (1.25, 2.5 units/ml).

Cell Proliferation and Serum Dependency Studies. Cells obtained from T3P3 were used in all subsequent studies involving growth factor or hormone-induced cell proliferation. When cells were plated on Primaria in the presence of 10% FCS and then exposed to serum-free medium, the addition of insulin (10 ng/ml), EGF (10 ng/ml), FGF (10 ng/ml), and PDGF (2.5 units/ml) only stimulated cell proliferation to 22.5, 46, 36, and 33% of control values, respectively (Fig. 5). Various combinations of growth factors did not act synergistically to enhance growth, although some additive effects (P < 0.01) on cell proliferation were observed. All cultures, regardless of culture conditions, showed a 24-h lag period prior to resuming proliferation (results not shown). Following this lag period, cultures exposed to medium containing 10% FCS proliferated with an average population doubling time of 27 h during the logarithmic growth phase. In contrast, the cultures maintained in serum-free
REGULATION OF HUMAN MENINGIOMA CELL GROWTH IN VITRO

Fig. 3. Effects of growth factors on [3H]thymidine incorporation into DNA. DNA synthesis was estimated by incorporation of [3H]thymidine (64 Ci/mmol, 1 μCi/ml, 1 h) into acid-insoluble product following a 21-h incubation period with growth factors. Control is defined as incorporation in the presence of 10% FCS and was 10,000 cpm/1.8 x 10^6 cells/h. Points, mean of triplicate wells representative of at least three experiments per treatment; bars, SE.

Fig. 4. Comparison of the ability of EGF or FGF to synergistically stimulate DNA synthesis in the presence of increasing concentrations of PDGF. Conditions and measurement of [3H]thymidine incorporation were as described in Fig. 3. Control is defined as the amount of incorporation induced by 10% FCS which was 10,000 cpm/1.8 x 10^6 cells/h in this experiment. Both EGF and FGF were present in 10 ng/ml. Points, mean of triplicate wells from three experiments: bars, SE.

DMEM in the absence of added growth factors did not undergo a population doubling. After 5 days in culture, the final density in serum-free DMEM was equal to the plating density (1 x 10^5 cells/ml). The addition of either EGF or FGF (10 ng/ml) resulted in an average population doubling time of 48 h, almost twice that of control cultures.

In the presence of 2% FCS, insulin, EGF, and FGF increased cell number over control values, but only FGF stimulated mitogenesis in a significant manner (P < 0.05) (Fig. 6). The cell doubling time was 48 h in DMEM containing only 2% FCS. Addition of either EGF or FGF (10 ng/ml) resulted in an average population doubling time of 48 h, almost twice that of control cultures.

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Fig. 5. Effects of insulin (I), EGF (E), FGF (F), and PDGF (P) and their combinations on the proliferation of meningioma cells maintained in serum-free DMEM (SFM). Meningioma cells were seeded at 1 x 10^4 cells/ml/well on Primaria as described in "Materials and Methods." Cells were exposed to serum-free DMEM in the presence or absence of insulin (10 μg/ml). EGF (10 ng/ml), FGF (10 ng/ml), and PDGF (2.5 units/ml), combinations of these factors, or 10% FCS. Cells were harvested 5 days later for determination of cell number. Columns, mean of triplicate wells from two experiments; bars, SE.

Fig. 6. Effects of insulin (1, 10 μg/ml), EGF (10, 100 ng/ml), and FGF (10, 100 ng/ml) in the presence of 2, 5, and 10% FCS on meningioma cell proliferation. Cells were replated at 1 x 10^4 cells/ml/well and exposed to DMEM containing various concentrations of growth factors and FCS. Cells were harvested 4 days later for determination of cell number. Columns, mean of triplicate cultures from two experiments; bars, SE. Symbols indicate that cell number in the presence of growth factors is significantly higher than in their absence at the P < 0.05 (*) and P < 0.01 (***) levels.

ng/ml) and FGF (10 ng/ml) stimulated a greater than 2-fold increase in cell number over control values, and doubling time was shortened to 21 h under these conditions. Interestingly, cell number was reduced at high concentrations of EGF (100 ng/ml, 16 μM) in 5% or 10% FCS, and high concentrations of FGF (100 ng/ml, 7.5 μM) in 10% FCS.

Steroid Hormone Studies: Cell Proliferation and Serum Dependency. The addition of estradiol, progesterone, or cortisol (100 nM) to cultures grown in serum-free medium had no effect on cell proliferation over a 5-day period. Steroid hormones added in the presence of insulin or in the combination of insulin, EGF, and FGF were also without effect (Fig. 7). When cultures were exposed to steroid hormones in a medium containing 10% charcoal-treated serum, only estradiol significantly stimulated cell proliferation over control values (P < 0.01). No other combination of steroids and growth factors, or charcoal-treated FCS increased cell number when compared to control cultures.

DISCUSSION

Meningiomas are primarily benign mesenchymal tumors which can be surgically removed in most cases. However, some tumors are inoperable or invasive, and they cannot be cured by surgical procedures. Studies involving the in vitro regulation of meningioma cell growth and differentiation may help to eluci-
cell proliferation and other anabolic events in vitro and in vivo.

In the present study, we have examined the effects of various growth factors (insulin, EGF, FGF, and PDGF) and steroid hormones on DNA synthesis and proliferation of human meningioma cells in a serum-free culture system. Primary cultures were not used because they could not provide an adequate number of cells for evaluation; therefore, we used early passage cells (passages 2 to 5) in our studies.

Several types of mesenchymal cells require micromolar concentrations of insulin for normal proliferation (18). Supraphysiologic concentrations are necessary because insulin binds to receptors for insulin-like growth factors (somatomedins) with low affinity (19). Somatomedins have been shown to mediate cell proliferation and other anabolic events in vitro and in vivo (20). PDGF, isolated from human platelets, is a heat-stable, cationic polypeptide that ranges in molecular weight from 28,000 to 35,000 (21). PDGF stimulates proliferation of a variety of mesenchymal cells in vitro (22).

EGF, a small peptide (M, 6,200) isolated from mouse submaxillary glands, and FGF, a peptide (M, 13,400) isolated from the bovine brain and pituitary gland, have been shown to be mitogenic for a variety of cells including fibroblasts, corneal endothelium, vascular smooth muscle, and granulosa cells (10, 23).

In our culture system, insulin, EGF, FGF, and PDGF stimulated DNA synthesis in a dose-dependent manner to a variable extent (data not shown). In addition, cells grown in six different sera incorporated [3H]thymidine in a similar manner when exposed to insulin (1 nM to 1 mM) for 21 h (data not shown). It is also doubtful that the limited effects of EGF and FGF were due to proteolytic degradation of these factors in serum-free medium. Cell number was unaltered when growth factors were added on either Day 1, or Days 1 and 3 (data not shown).

We observed that PDGF and EGF, or PDGF and FGF synergistically stimulated DNA synthesis in our meningioma cultures. These results are in close agreement with other studies (16, 17) in which growth factors such as EGF and FGF induced optimal DNA synthesis in 3T3 cells in the presence of other factors (insulin, corticosteroids, PDGF). Interestingly, the effects of EGF and FGF combined were neither additive nor synergistic in our culture system. It is possible that EGF and FGF have the same mechanism of action in meningioma cells, whereas PDGF, acting on a different receptor, can modulate the effects of these growth factors. PDGF and phorbol acetate have been shown to decrease the affinity of EGF receptors in Swiss 3T3 cells (24, 25), while phorbol acetate synergizes with EGF to stimulate DNA synthesis in the same cell system (26).

In our system, the inhibition of DNA synthesis by high levels of PDGF in combination with EGF may have been the result of a decreased affinity of the EGF receptor. PDGF and EGF receptors may have multiple interactions which result in seemingly paradoxical observations.

Although synergism was observed in our DNA synthesis studies, the factors we tested were unable to stimulate cell proliferation in a synergistic manner. This dissociation of effects may be explained by the fact that a particular hormonal treatment may differentially affect a number of diverse cellular processes (cell death, G0 to G1 recruitment). In many culture systems, cell number is often less dramatically influenced than the actual rate of DNA synthesis (27).

Serum dependency was also observed in studies involving the mitogenic effects of insulin, EGF, and FGF. Similar results were obtained in studies by Gospodarowicz and Bialecki (28) in which serum potentiated the mitogenic effects of EGF and FGF on cultured granulosa cells. However, these authors observed a linear increase in mitogenesis with increasing amounts of serum, whereas our studies indicate an inhibitory effect of high levels of serum (10%) on growth factor-induced mitogenesis.

In our studies involving the effects of estradiol, progesterone, and cortisol on meningioma cell proliferation, we observed a 50% increase over control values for cells grown in the presence of 100 nM estradiol and 10% charcoal-treated FCS. No other combination of steroids and growth factors or charcoal-treated FCS increased cell number over controls.

In additional studies (data not shown), a range of concentrations (1 to 100 nM) of steroid hormones did not affect either DNA synthesis or cell proliferation in the presence or absence of added growth factors (insulin, EGF, FGF). These results differ slightly from those obtained by Jay et al. (9) in which physiological concentrations of estradiol, progesterone, or tamoxifen (1 to 100 nM) in the presence of 10% charcoal-treated FCS enhanced growth in meningioma cultures derived from four tumors. We observed no stimulation of growth by progesterone. In addition, the results from their study were difficult to interpret, because three of four tumors had extremely low levels of estrogen receptors (<3 fmol/mg protein). These authors suggested that the sex steroids were possibly stimulating cell proliferation by binding to a non-steroid receptor protein. Another possibility is that steroid hormones interact with serum.
components and indirectly increase the rate of cellular proliferation. The presence of charcoal-treated serum components appears to be vital for antiestrogen activity in MCF-7 cultures (27). In another study, the addition of a platelet extract was essential for granulosa cell proliferation in a serum-free culture system (29). Interestingly, PDGF was unable to substitute for this extract and actually inhibited cell proliferation in these studies. These authors suggested that the platelet extract contained other factors which either facilitate the activity of PDGF or are mitogenic in themselves.

In summary, we have characterized some of the growth factors and hormones required for human meningioma cell proliferation in vitro. It appears that human meningioma cells require additional serum components (possibly attachment factors) to actively proliferate in a serum-free system. Further studies must be performed to fully characterize the factors necessary for meningioma cell proliferation and differentiation. A better understanding of these factors may eventually result in improved medical treatment for tumors which are inoperable.

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