Antiproliferative function of glia maturation factor β

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Recombinant human glia maturation factor β (GMF- β) reversibly inhibits the proliferation of neoplastic cells in culture by arresting the cells in the G_0/G_1 phase. This phenomenon is not target-cell specific, as neural and nonneural cells are equally inhibited. When tested simultaneously, GMF- β suppresses the mitogenic effect of acidic fibroblasts growth factor (aFGF), but the two are synergistic in promoting the morphologic differentiation of cultured astrocytes. GMF- β also counteracts the growth-stimulating effect of pituitary extract and cholera toxin on Schwann cells. The results underscore the regulatory role of GMF- β and its intricate interaction with the mitogenic growth factors.

Introduction

In 1972 this laboratory detected the effect of crude brain extract in promoting the morphologic expression of cultured astrocytes (Lim *et al.*, 1972, 1973; Lim and Mitsunobu, 1974). The active agent responsible for this phenomenon was named glia maturation factor (GMF). Subsequently, a mitogenic activity was also observed in the partially purified GMF (Lim *et al.*, 1976, 1985). Because of the presence of other growth factors in the brain, the question arose as to whether the mitogenicity was indeed an intrinsic function of GMF or a result of contamination. Our purification effort led to the isolation of a 17-kDa acidic protein designated GMF- β (Lim *et al.*, 1989). The protein has now been sequenced (Lim *et al.*, 1990b), cloned, and expressed in *E. coli* (Kaplan *et al.*, 1990). The availability of pure recombinant GMF- β , free of contamination from other mammalian proteins,

permitted us to reexamine the problem of mitogenicity. In this paper, we report that human recombinant GMF- β , which is identical in amino acid sequence with the bovine natural protein, is not mitogenic but is strongly antiproliferative for many tumor cells in culture. The growth inhibitory action of GMF- β is not limited to cells of neural origin.

Results

GMF- β reversibly inhibits tumor cell proliferation

Figure 1 shows the effect of GMF- β on a number of cell types in culture, ranging from normal to highly malignant. Although a small inhibition was observed even with the normal cells, the antiproliferative effect was most obvious with the transformed cells. In general, the more rapid the growth rate, the more pronounced was the inhibition. Furthermore, the effect was not confined to cells of neural origin, as cells derived from other organs were also affected. The growth curves of C6 glioma cells under the influence of GMF- β are depicted in Figure 2, which demonstrates the reversibility of the inhibition. Essentially the same response was observed when reversibility was tested on immortalized Schwann cells (ISC) and immortalized intestinal epithelial cells (IEC-17) (results not shown).

GMF- β arrests cells at the G_0/G_1 phase

To study the mechanism of inhibition by GMF- β , we subjected C6 cells exposed to GMF- β for 3 d to cell-cycle analysis with the use of a flow cytometer. Table 1 shows that cells grown in the presence of GMF- β have a higher percentage of population in the G_0/G_1 phase than controls, a result consistent with the maturation effect of GMF- β .

Natural and recombinant GMF- β exhibit similar antiproliferative function

When natural and recombinant GMF- β were tested at the same time on astrocytes and C6 cells, both factors behaved indistinguishably, showing a strong growth inhibition on C6 cells and a mild effect on astrocytes (Figure 3). An antiserum developed against the natural protein equally neutralized the antimitogenic action of

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¹ Abbreviations used: aFGF, acidic fibroblast growth factor; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GMF, glia maturation factor; IEC-17, intestinal epithelial cells; ISC, immortalized Schwann cells.

Figure 1. Effect of GMF- β on the proliferation of normal and neoplastic cells. Cells were cultured for 3 d in 24-well trays in media (see Materials and methods) containing 5% serum in the absence ($\square$) or presence ($\blacksquare$) of 250 ng/ml of GMF- β . Initial cell number was 2×10^5 per well. Final cell numbers are indicated by the lengths of the bars. Each value is the mean of four wells $\pm$ SD. All cells were of rat origin, except the following, which were derived from mouse: 3T3, G26, and N18. (A) All normal cells were purified, and homogeneous populations were obtained as follows. Astrocytes and fibroblasts were secondary cultures from fetal rat brains and skin, respectively (Lim and Miller, 1984). Schwann cells were secondary cultures from neonatal rat sciatic nerves (Assouline *et al.*, 1983). Normal intestinal epithelial cells were derived from 20-d-old rats and were used within the 25th passage (Quaroni and May, 1980). (B) Immortalized cells were cells that had originated from normal tissues but had acquired an unlimited life span and transformed properties after repeated passages. ISC cells were derived from Schwann cells as described before (Porter *et al.*, 1987; Langford *et al.*, 1988). 3T3 cells were obtained from American Type Culture Collection (ATC). IEC-17 cells were derived from intestinal epithelial cells (see above) and were used after 101 passages (De Rose and Claycamp, 1989). (C) Tumor-derived cells were established tumor lines obtained from the following sources: L929 (ATC), HTC (D.R. LaBrecque), C6 (ATC), G26 (S.E. Pfeiffer), N18 (M. Nirenberg), 354A (W. Shain).

both natural and recombinant GMF- β (Figure 3B).

GMF- β interacts with acidic fibroblast growth factor (aFGF)

The combined effect of GMF- β and aFGF was tested on normal and neoplastic glial cells. Figure 4A shows that aFGF alone stimulated the proliferation of normal astrocytes, whereas GMF- β alone produced a slight inhibition of

Figure 2. Reversibility of inhibition by GMF- β . C6 cells were seeded at 2×10^5 cells per well and grown in F12/DMEM medium containing 5% FCS, as in Figure 1, for 12 d. The cells were exposed to 250 ng/ml of GMF- β for various lengths of time as follows: curve I, never exposed to GMF- β ; curve II, exposed to GMF- β for the first 3 d; curve III, exposed to GMF- β for the first 6 d; curve IV, always exposed to GMF- β . Culture medium was renewed at 3, 6, and 9 d. Growth curves were determined with a Coulter counter after trypsinization. All values are means $\pm$ SD of four wells. (Similar curves were obtained using immortalized Schwann cells [ISC] and immortalized intestinal epithelial cells [IEC-17].)

growth. When used together, the mitogenic effect of aFGF was partially suppressed by GMF- β . The appearance of the cells is presented in Figure 5. It is interesting to note that, whereas aFGF or GMF alone caused only minimal morphologic change, the combination of the two factors resulted in a pronounced morphologic expression of the cells, as is evident in the elongation of the cell bodies and the outgrowth of processes.

Likewise, the mitogenic action of aFGF on C6 glioma cells was also suppressed by GMF- β

Table 1. Cell cycle status of C6 cells grown in the presence of GMF- β

Condition	G ₀ /G ₁ (%)	G ₂ + M (%)	S (%)
Control	65	6	29
GMF- β	80	7	13

C6 glioma cells were grown to near confluence on 60-mm tissue culture dishes in F12/DMEM in the presence of 5% FCS. GMF- β was then added and the cells were incubated for 3 additional days. The cells were harvested by trypsinization and stained at 4°C for 15 min with a solution consisting of 0.05 g of propidium iodide, 1 g of sodium citrate, and 1 ml of Triton X-100 per liter of double-distilled water. The stained cells were analyzed with a Coulter EPICS 753 flow cytometer using laser illumination at 488 nm by argon ion (Coherent Laser). A total of 1×10^5 cells were sorted for each analysis.

Figure 3. Comparison between natural and recombinant GMF- β on cell proliferation. The cells were seeded at 2×10^5 cells per well and grown in F12/DMEM medium containing 5% FCS as in Figure 1. The cells were grown for 3 d in the presence of 250 ng/ml of either natural or recombinant GMF- β , or in their absence (control). Where indicated, rabbit antiserum lot No. 88-02, developed against natural bovine GMF- β , was added simultaneously with the growth factor to a final dilution of 1:50. (A) Astrocytes; (B) C6 glioma cells. Ab, antibody added. All values are means $\pm$ SD of four wells.

(Figure 4B). The morphologic effect of GMF- β on C6 cells, consisting of cell elongation, has been published (Lim *et al.*, 1989). No additional changes were produced by aFGF (results not shown).

GMF- β suppresses mitogen-induced proliferation of Schwann cells

To find out whether GMF- β inhibits the growth-promoting effect of other mitogenic factors on other cell types, we tested normal Schwann cells under stimulation by pituitary extract and cholera toxin. Normal Schwann cells grow slowly even in the presence of serum unless stimulated by pituitary extract, which contains a Schwann cell mitogen (Raff *et al.*, 1978a), or by cholera toxin, which raises intracellular cyclic

adenosine monophosphate level (Raff *et al.*, 1978b). Figure 6 shows that in the concurrent presence of GMF- β , the stimulatory effect of pituitary extract and cholera toxin was markedly inhibited.

Discussion

Our earlier studies with crude GMF revealed a dual effect of mitogenicity, on the one hand, and the enhancement of phenotypic expression, on the other. For example, normal astrocytes and Schwannoma and glioma cells responded to crude GMF by cell proliferation and outgrowth of processes (Lim *et al.*, 1976; Bosch *et al.*, 1984). Schwannoma and glioma cells responded to the same preparation by an initial surge in cell division when the cells were sparse, but exhibited contact inhibition when the culture became confluent (Lim *et al.*, 1981, 1986). Although it is possible that a single growth factor could mediate both effects, especially in a time-depen-

Figure 4. Antagonism between GMF- β and aFGF on cell proliferation. (A) Astrocytes grown in F12/DMEM containing 5% FCS in the presence of either aFGF (100 ng/ml), GMF- β (250 ng/ml), or both. Cells were obtained and grown for a 3-d period as described in Figure 1. (B) C6 glioma cells grown in serum-free defined medium in the presence of either aFGF (100 ng/ml), GMF- β (250 ng/ml), or both. C6 cells were grown for a 3-d period as in Figure 1, except for the use of serum-free medium. (The rapid growth of C6 cells in the presence of serum masked the stimulatory effect of aFGF.) The defined medium consisted of F12/DMEM (1:1; v/v) containing 10 nM hydrocortisone, 30 nM sodium selenite, 50 μ g/ml transferrin, 10 ng/ml biotin, 5 μ g/ml insulin, and 1.5 μ g/ml fibronectin. All values are means $\pm$ SD of four wells.

Figure 5. Synergism between GMF- β and aFGF on astrocyte morphology. The experiment was that presented in Figure 3A. Phase-contrast photomicrographs were taken at the termination of the experiment (after 3 d of stimulation by growth factors). (A) No growth factor added; (B) aFGF added (100 ng/ml); (C) GMF- β added (250 ng/ml); (D) aFGF and GMF- β added. Note elongation of cells and process outgrowth in the concurrent presence of both factors. (Bar = 100 μ m.)

dent manner, we could not rule out the interaction of two or more growth factors present in the same GMF preparation. With the availability of pure GMF- β , and especially the recombinant protein expressed in bacteria, we can now test its biological function without intervention by other eukaryotic proteins. The results presented in this paper clearly demonstrate the universal suppressive effect of GMF- β on the proliferation of tumor cells in culture. Although the data were obtained with recombinant GMF- β , we have confirmed the anti-tumor effect with the natural protein isolated from bovine brain (Lim *et al.*, 1989, 1990a). That the antiproliferative action of GMF- β is a regulatory function and not a cytotoxic phenomenon is supported by the reversibility of the inhibition.

In this paper, we also demonstrate a mild growth inhibition on normal astrocytes by both

natural and recombinant GMF- β . Although we previously detected a mitogenic action of purified natural GMF- β on astrocytes (Lim *et al.*, 1989), we were subsequently unable to confirm the observation with numerous experiments.

Because it is now obvious that the dual effect previously observed with crude brain extract was due to a combination of GMF- β and some other growth factors from the brain, we examined the interaction of GMF- β and a brain-derived mitogenic factor, the aFGF. What is of interest in the outcome of this study is that the two factors are antagonistic in cell division, but are synergistic in morphologic expression on astrocytes. Thus, by combining two well-defined growth factors isolated from the brain, we have in some measure reconstructed the phenomenon observed with crude brain extract. Despite the lack of sequence homology, GMF- β and aFGF have similar molecular weights and isoelectric points. During the purification of GMF, the two seemed to be associated until they were separated by heparin binding. We suspect that the physical affinity between the two factors, although not rigorously defined, may be of biological significance, as the interplay between the two may dictate the optimal extent of cell proliferation and degree of differentiation, a "microhomeostasis" necessary for the maintenance of a healthy tissue.

Growth factors can be broadly defined as endogenous molecules, usually proteins, that can

Figure 6. Inhibitory effect of GMF- β on Schwann cell mitogens. Normal Schwann cells were obtained as described in Figure 1 and seeded in 24-well trays at 2×10^5 cells per well in F12/DMEM containing 5% FCS. The cells were grown for 3 d under no stimulation (No), or stimulation by pituitary extract (PE), cholera toxin (CT), or a combination of the two (PE + CT). □, cells cultured in the absence of GMF- β ; ■, in the presence of 250 ng/ml of GMF- β . Pituitary extract was used at 500 μ g/ml; cholera toxin (Sigma Chemical, St. Louis, MO) at 1 μ g/ml. All values are means $\pm$ SD of four wells.

regulate the survival, proliferation, or phenotypic expression (or any combination of the three) of target cells. Many of the reported growth factors are mitogenic for cultured cells (although they may exhibit differentiation effect under proper conditions). A few are well known for their antiproliferative function, such as nerve growth factor (NGF) (Greene and Tischler, 1976), transforming growth factor β (TGF- β) (Sporn and Roberts, 1989), and tumor necrosis factor (TNF) (Goeddel *et al.*, 1986). GMF- β belongs in the second category. In this respect, it is functionally more akin to NGF and TGF- β than to TNF because it is a component of the normal tissue rather than a cytotoxic agent produced under pathologic conditions.

It should be noted that not all tumor cells are inhibited by GMF- β . We previously reported that PC12 cells, which do not possess GMF receptors, do not respond to the factor (Lim *et al.*, 1990a). This observation may have two implications with respect to cancer research. First, the lack of GMF receptors may be the phenotypic feature of some malignancies. Second, in considering the remote possibility of utilizing GMF- β as a therapeutic agent, the assay of GMF receptors may be of prognosticating value on the outcome of the treatment.

One of the exciting developments in growth factor research has been their association with the oncogenes. In this context, the overproduction of the mitogenic growth factors and/or their receptors could be an essential feature of malignancy. However, attention has recently been directed toward the opposite control mechanism, that of the tumor suppressor genes (Sager, 1989), the deficiency of which can be as conducive to neoplastic transformation as the overactivity of the protooncogenes, as exemplified by the study on the retinoblastoma gene (Knudson, 1985). In light of this, GMF- β may well be the product of an as-yet-undiscovered tumor suppressor gene, the malfunctioning of which could provide a permissive environment for the uncontrolled proliferation of the tumor cells. Future investigations may substantiate this prediction.

Materials and methods

Preparation and activation of human recombinant GMF- β

Recombinant human GMF- β was used exclusively in this work except where indicated otherwise. The recombinant protein was obtained by expressing a human cDNA insert (in pET-3b plasmid vector) in *E. coli* strain BL21 (DE3) pLys S (Kaplan *et al.*, 1990). After disrupting the bacteria by freeze-thawing and sonication, we purified recombinant GMF- β to homogeneity from the soluble fraction by gel per-

meation chromatography (BioGel P-30) as described (Kaplan *et al.*, 1990). Pure recombinant GMF- β exhibited a single peak on reverse-phase HPLC and a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The recombinant GMF- β , of human origin, was identical with natural GMF- β from bovine brain by the following criteria: molecular weight, immunoreactivity, tryptic map, amino acid composition, and sequence (Kaplan *et al.*, 1990; Lim *et al.*, 1990b). The recombinant protein was activated by reduction-oxidation (Morehead *et al.*, 1984; Tsuji *et al.*, 1987) as described below. Recombinant GMF- β , at 0.5 mg/ml in 0.1 M Na phosphate buffer, pH 7.4, was mixed with an equal volume of 6 M guanidine-HCl, pH 8.0, and subsequently with 10 mM reduced and 1 mM oxidized glutathione, both freshly weighed. After incubation at room temperature for at least 6 h, the mixture was diluted with 0.1 M Na phosphate buffer, pH 7.4, to a protein concentration of 0.1 mg/ml and dialyzed overnight at 4°C in the same buffer. The redoxed material was stored frozen for up to several months. Before use, the stock solution was subjected to a procedure of heat-cooling to optimize protein conformation (Lim *et al.*, 1989). The sample was put in a plastic tube, which was in turn placed in a beaker of water at room temperature. The beaker was gradually heated to 80°C on a hot plate set at medium control. After stabilizing the temperature at 80°C for another minute (by removing the beaker from the plate), we removed the tube from the beaker and let it stand alone to cool to room temperature. The fully activated GMF- β was kept at 4°C and used within 1 wk. Activated recombinant GMF- β demonstrated a dose-response curve indistinguishable from that of natural GMF- β when assayed for growth inhibition on N18 neuroblastoma and C6 glioma lines. The dose for half maximal activity for both cell lines was ~20 ng/ml medium (Kaplan *et al.*, 1990).

Source of natural GMF- β

Natural GMF- β was purified from bovine brain as described before (Lim *et al.*, 1989).

Source of antibody against GMF- β

Rabbit antiserum, lot No. 88-02, was raised against pure bovine natural GMF- β (Lim *et al.*, 1989). The antiserum was decanted before use.

Preparation of aFGF

aFGF was isolated from bovine brain by the heparin-binding procedure as described by Gospodarowicz (1987).

Preparation of pituitary extract

Bovine pituitary extract containing a mitogenic factor for Schwann cells was prepared according to Brockes *et al.* (1980).

Cell culture

All cells were grown in F12/Dulbecco's modified Eagle's medium (DMEM) (1:1; v/v) supplemented with 5% fetal calf serum (FCS) except for the following: N18 cells were grown in RPMI 1640 medium containing 5% heat-inactivated FCS. HTC cells were grown in DMEM containing 5% FCS, 0.45% (w/v) glucose, and 1% (w/v) L-glutamine. Intestinal epithelial cells (normal and IEC-17) were grown as HTC cells except for the additional presence of insulin (10 μ g/ml). The stimulation of C6 cells by aFGF was carried out in serum-free medium (see Figure 4). All cells were seeded on plastic trays or dishes and allowed to attach for 4 h before the addition of growth factors. The cells were cultured at 37°C in air

containing 5% CO₂ and saturated humidity. Cell number was determined with a Coulter counter after trypsinization.

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