Modulation of Growth and of Morphological Characteristics in Glioma Cells by Nerve Growth Factor and Glia Maturation Factor

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ABSTRACT

Anaplastic glioma T9 cells were treated with either nerve growth factor (NGF) or glia maturation factor (GMF) or both. It was found that, when T9 cells were treated with these factors in a chemically defined medium, both NGF and GMF induced characteristic changes of cell morphology and growth pattern. Several differences in the effects of NGF and GMF were noted. NGF retarded growth rate, whereas GMF did not. The cells treated with NGF were characterized by a flattened extended cytoplasm and growth pattern. Several differences in the effects of NGF and GMF were readily reversible while morphological changes induced by NGF persisted in its absence.

INTRODUCTION

NGF is a polypeptide which is essential for the development of sympathetic and sensory neurons in the peripheral nervous system (1, 2) as well as differentiation of neurons in the central nervous system (3–5). Moreover, the presence of NGF receptors in Schwann cells of embryonic sensory ganglia (6) and glioma cells (7) and the production of NGF or NGF-like proteins in a wide variety of tissues and cell types (8–12) suggest that NGF may also be involved in the development and functions of cells other than neurons. GMF is an acidic protein endogenous to the adult brain, and it has been recently purified to an apparent homogeneity (13). It reversibly promotes the morphological and chemical differentiation of glioblasts (14).

These “differentiation factors” have been shown to modulate characteristics of various tumor cells in culture. PC12 pheochromocytoma cells respond to treatment with NGF by ceasing mitosis and extending long, branching neuronal-like processes (15). NGF can enhance neurite outgrowth in some lines of neuroblastoma cells (16, 17). However, NGF neither reduces the growth rate nor enhances survival of neuroblastoma cells (16). GMF stimulates cell division in the logarithmic phase but prevents overgrowth at confluence, restoring contact inhibition in Schwannoma (18) and glioma cells (19).

As an attempt to examine a reverse-transforming capability of “differentiation factors,” we have treated anaplastic glioma cells with NGF and GMF. It will be shown that the tumor cells respond to either factor by developing distinctively different characteristics in growth pattern and cell morphology.

MATERIALS AND METHODS

Preparation of NGF and GMF. β-NGF was isolated and purified from male mouse submaxillary glands by the procedure of Bocchini and Angeletti (20), and the biological activity was assayed by the PC12 method (21). Partially purified GMF was prepared from bovine brains essentially as described by Lim and Miller (22).

Cell Culture. Stock monolayer cultures of rat anaplastic glioma cell line T9 (23, 24) were maintained in DMEM containing 10% fetal bovine serum (Armour Pharmaceutical, Kankakee, IL), 4 mM glutamine, 100 units/ml of penicillin, and 50 µg/ml of streptomycin in a humidified chamber with 7% CO2 at 37°C. A single cell suspension was prepared by trypsinization, and cells (4 × 104/well) were seeded in 0.8 ml of the stock culture medium onto Gibco 12-well cell culture cluster dishes (4 cm2/well). The wells were coated with 50 µg/ml of poly-D-lysine (M, 30,000 to 70,000) for 5 min (25), washed with Hank’s balanced salt solution, and used immediately. One day after seeding (designated as Day 0), the culture medium was replaced with a chemically defined serum-free medium, HL-1 (Ventricx Laboratories, Portland, ME), containing NGF (50 ng/ml) or GMF (15 µg/ml) or both. The control cultures were maintained in the identical medium without NGF or GMF. HL-1 contains DMEM:F-12 base, 4-(2-hydroxyethyl)-1-piperazineneethanesulfonic acid buffer, insulin, transferrin, testosterone, sodium selenite, ethanolamine, saturated and unsaturated fatty acids, and stabilizing proteins, and it was supplemented with 4 mM glutamine. The medium was changed daily.

For cell counting, cells were seeded in duplicates onto Falcon multiwell plates (35-mm diameter). After specific periods of time, the cell cultures were treated with 0.25% trypsin in Dulbecco’s phosphate-buffered saline (magnesium and calcium free) containing 0.1 mM EDTA and appropriately diluted with the same buffer. The cells were then counted with a hemocytometer.

Scanning Electron Microscopy. Cells were plated onto Lux Thermoplastic tissue culture coverslips (Miles Scientific, Naperville, IL) coated with poly-D-lysine as above and incubated in Falcon 24-well plates with 0.5 ml/well (2 cm2) of the culture medium. These plastic coverslips were found to float during the medium change unless the medium was applied dropwise directly onto the surface of the coverslips. Such an application, however, caused the cells, particularly those treated with the factors, to partially or totally dislodge from the surface. A special procedure for the medium change was therefore devised in order to minimize the cell detachment. After 2 days of culture in the experimental medium, 0.2 ml of the medium were replaced by 0.2 ml of the fresh medium containing either 2.5 µg of NGF or 7.5 µg of GMF; on the following day, 0.1 ml of the fresh medium containing the same amount of NGF or GMF was added without withdrawing the existing medium. After 4 days of culture, the cells were fixed by carefully adding 0.5 ml of 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) on top of the medium. The cells were postfixed with 1% osmium tetroxide, dehydrated with ethanol, and coated with gold. The specimens were examined with the JEOL JSM-35C scanning electron microscope at 15 kV.

RESULTS AND DISCUSSION

T9 glioma cells grown in the presence of 10% fetal bovine serum have a polygonal shape with only few processes and a large nuclear:cytoplasmic ratio (Fig. 1). No detectable morphological changes are observable in these cells with NGF or GMF under these culture conditions. When the fetal serum is eliminated and T9 cells are grown in the chemically defined Ventrex HL-1 medium, characteristic changes in the pattern of growth
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and cell morphology are inducible with both NGF and GMF. While slight changes of cell morphology are observable at concentrations as low as 20 ng/ml for NGF and 100 ng/ml for GMF, the degree and the rate of morphological changes become greater as their concentrations increase, approaching the maximal responses at 5 μg/ml of NGF and 15 μg/ml of GMF. A daily replacement of GMF in particular and NGF to some extent further enhances the rate of morphological changes as compared with changing of the medium every other day. In this study, the appropriate seeding density (4 x 10^5 to 10^6/4 cm²), the daily medium change, and optimal concentrations of NGF (5 μg/ml) and GMF (15 μg/ml) have been chosen so as to obtain the maximal effects in the shortest period of time.

Growth curves of T9 cells in the presence or the absence of the factors are presented in Fig. 2. Since T9 cells grow exponentially up to 10^5 cells/well (9.6 cm²) in DMEM containing 10% fetal bovine serum, it is apparent that the rate of cell growth is gradually reduced following changing to the serum-free HL-1 medium. As clearly seen in Fig. 2, NGF retards cell growth, whereas GMF does not. The treatment with both NGF and GMF produces retardation of growth similar to that observed with NGF alone. NGF has been reported to have a mitogenic activity on cultured chromaffin cells (26), and GMF is a known mitogen of cultured glioblasts (13, 14). In the present study, stimulation of T9 cell proliferation was observed at 50 to 500 ng/ml of NGF and at 0.4 to 7.5 μg/ml of GMF.

While T9 cells grown in the chemically defined medium exhibit a low degree of morphological changes (Fig. 3, CONT), the treatment of these cells with either NGF (Fig. 3, NGF) or GMF (Fig. 3, GMF) for 4 days produces distinctive changes in growth pattern and cell morphology. The cells treated with NGF are characterized by a flattened extended cytoplasm with numerous protruding processes. The cell masses are somatically connected by cell bridges. GMF, on the other hand, produces slender cells with long branching processes forming an interconnecting cell net. When both NGF and GMF are administered (Fig. 3, NGF + GMF), T9 cells show the effects of both factors with more pronounced GMF-induced characteristics than NGF-associated features. These characteristics become noticeable as early as 2 days of treatment with either factor, but the maximal responses are attained with 3 to 4 days of treatment. Changes of growth pattern and morphological characteristics in the presence of both NGF and GMF have followed a time course similar to that observed with NGF or GMF alone. When T9 cells are grown on a culture surface not coated with polylysine, the control cells stay attached, but the cells treated with NGF or GMF become totally dislodged from the surface after 1 to 2 days of culture. It has further been noted that, although the coating of culture surface with polylysine substantially stabilizes the attachment of the treated cells, they tend to partially detach from the surface and round up by slight mechanical shock. This tendency has been found to be highest for the cells treated with NGF plus GMF followed by those treated with GMF. The cells treated with NGF are relatively stable, while attachment of the control cells is resistant to moderate mechanical shock. These observations indicate that the changes of cell configuration resulting from exposure to these factors are accompanied by alterations of cell surface properties.

As depicted in Fig. 4, scanning electron microscopy provides further details of the changes in cell structure induced by NGF and GMF. The untreated T9 cells (CONT-a and -b) have few short processes. Overlapping cell bodies are clearly visible. The cells treated with GMF show three dimensionally well-developed cell bodies with long processes which form an interconnecting cell mesh. These processes bear hair-like thin structures. The cells treated with NGF show broad cytoplasmic processes, and the neighboring cells are connected somatically (NGF-a and -b). These processes (NGF-c and -d) often develop flattened cytoplasmic projections (lamellipodia) with numerous long filamentous processes (filopodia) and secondary branches, forming the “crown” at the terminal (NGF-a and -c).

In order to examine reversibility of the morphological changes induced by NGF and GMF, T9 cells were treated with either NGF or GMF for varying periods of time and then grown in the medium without these factors. As can be seen in Fig. 5, the cells treated with NGF for 2 days (NGF 2d) undergo further morphological changes during the succeeding 2 days in the absence of NGF (NGF 2d + 2d) and exhibit the pattern of cell growth and cell morphology similar to those grown in the continuous presence of NGF (NGF 2d). Similarly, the cells grown with NGF for 2 days followed by 4 days without NGF (NGF 2d + 4d), those with NGF for 4 days and then for 2 days without NGF (NGF 4d + 2d), and those kept in the presence of NGF for 6 days (NGF 6d) all show essentially identical morphological characteristics and growth pattern. It thus appears that the effects of NGF persist in its absence. As seen in

![Fig. 1](cancerres.aacrjournals.org) T9 glioma cells grown in DMEM supplemented with 10% fetal bovine serum. Phase-contrast. Bar, 100 μm.

![Fig. 2](cancerres.aacrjournals.org) Effects of NGF and GMF on cell growth. T9 cells were seeded in DMEM containing 10% fetal bovine serum. One day after seeding (Day 0), the culture medium was changed to HL-1 (CONT) or HL-1 containing NGF, GMF, or both. Each well has a surface area of 9.6 cm².
Fig. 3. Effects of NGF and GMF on growth pattern and cell morphology. One day after seeding, T9 cells were cultured for 4 days in HL-1 (CONT) or HL-1 containing NGF, GMF, or both. Phase-contrast. Bar, 100 μm.

Flattening of cells and shortening of processes are observable immediately following the withdrawal of GMF, indicating that the effects of GMF are readily reversible. Differentiation of glioblasts by GMF has also been reported to be reversible (14).

We have shown in this study that NGF and GMF induce morphological changes in glioma cells. The present finding, together with the previous observations that NGF induces differentiation of pheochromocytoma (15) and neuroblastoma cells (16, 17) and that GMF restores contact inhibition in Schwannoma (18) and glioma cells (19), indicates that these factors are capable of reversing some of the transformed properties of susceptible tumor cells. It has been reported that administration of NGF reduces the numbers of neurogenic
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Fig. 4. Scanning electron microscopic examination of T9 glioma cells treated with NGF and GMF. One day after seeding, T9 cells were grown for 4 days in HL-1 (CONT) or HL-1 containing NGF or GMF. NGF induces a prominent cell surface structure characterized by a flattened cytoplasmic projection, lamellipodia (La) with long slender filamentous processes, filopodia (Fi). The cells treated with GMF develop thick cytoplasmic bodies with long processes bearing hair-like structures. Bar, 10 μm.
Fig. 5. Reversibility of morphological changes induced by NGF. Top row, T9 cells treated with NGF for 2 days (NGF 2d) and then grown without NGF for 2 (NGF 2d + 2d) or 4 days (NGF 2d + 4d); middle row, 4 days with NGF (NGF 4d) and then without NGF for 2 (NGF 4d + 2d) or 4 days (NGF 4d + 4d); bottom row, 6 days with NGF (NGF 6d) and then without NGF for 2 (NGF 6d + 2d) or 6 days (NGF 6d + 6d). The morphological changes induced by NGF persist in the absence of this factor. Phase-contrast. Bar, 100 μm.
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Fig. 6. Reversibility of morphological changes induced by GMF. Top row, T9 cells treated with GMF for 2 days (GMF 2d) and then grown without GMF for 2 days (GMF 2d + 2d) or 4 days (GMF 2d + 4d); middle row, 4 days with GMF (GMF 4d) and then without GMF for 2 (GMF 4d + 2d) or 4 days (GMF 4d + 4d); bottom row, 6 days with GMF (GMF 6d) and then without GMF for 2 (GMF 6d + 2d) or 6 days (GMF 6d + 6d). The slender cell bodies become flattened, and cytoplasmic processes become shorter after withdrawal of GMF. Bar, 100 μm.

tumors that are induced by ethylnitrosourea in rats (27–29). NGF treatment of rats bearing implanted glioma cells leads to a decreased tumor growth and an increased survival time (30). GMF treatment of the athymic mice bearing inoculated glioma cells has also led to retardation of tumor growth (19). These findings suggest that “differentiation factors” such as NGF and GMF can be utilized for management of neurogenic tumors through control of growth and differentiation. NGF- and GMF-induced changes in growth and morphological characteristics of T9 glioma cells presented here appear to be an excellent...
system in which the action mechanisms of these factors can be studied. Such studies may further provide a basis for developing new modes of cancer therapy aimed at reverse transformation and tumor regression.

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REFERENCES

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