Response of C-6 Glioma to Isoproterenol and Papaverine In Vivo Depends on β-Adrenergic Receptor Density

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Rat C-6 glioma serves as an experimental model for human glioma. C-6 glioma cells carried to high culture passages in medium containing 10% fetal bovine serum when injected in vivo are unresponsive to treatment with the β-adrenergic agonist isoproterenol and the phosphodiesterase inhibitor papaverine. When C-6 glioma cells are kept in culture in serum-containing medium, β-adrenergic receptor density falls and, concomitantly, ability to accumulate cyclic adenosine monophosphate in response to stimulation with catecholamines declines. Responsiveness to treatment in vivo with a β-adrenergic agonist was restored when C-6 glioma cells were cultured in serum-free defined medium prior to systemic injection into rats. Culturing of C-6 glioma cells in serum-free medium significantly increases the number of β-adrenergic receptors when compared with C-6 glioma cells grown in serum-containing medium.

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New approaches to the therapy of human gliomas are needed. Glial tumors share certain properties with normal neural tissues, such as receptors for neurotransmitters, and it may prove possible to take advantage of this fact in designing treatment strategies. C-6 glioma, an experimental brain tumor of rats induced with *N*-methyl-nitrosourea, has been widely used as a model to study human gliomas [1]. C-6 glioma cells produce S-100 protein, an acidic protein that has been shown to be present in fibrillary astrocytes. C-6 glioma cells possess the enzyme 2′,3′-cyclic nucleotide 3-phosphohydrolase (CNP), a membrane-associated enzyme considered to be a marker for myelin and oligodendroglia [13, 14]. Cells from this tumor possess receptors for several neurotransmitters on their surfaces, the most extensively studied at present being the β-adrenergic receptor. In vitro stimulation of β-adrenergic receptors by β-adrenergic agonists activates adenylate cyclase and increases the intracellular content of cyclic adenosine monophosphate (cAMP) in C-6 glioma cells [2, 7, 11]. cAMP is involved in cell growth, proliferation, and differentiation [6]. The cAMP system is thought to be defective in malignant cells. Many malignant cell lines have been shown to contain a smaller-than-normal amount of cAMP [8], which may be a factor in preventing tumor cells from remaining in the quiescent stage of the cell cycle. We showed previously that treatment of C-6 glioma–bearing rats with the β-adrenergic agonist isoproterenol and with the cAMP phosphodiesterase inhibitor papaverine inhibits growth of this tumor in vivo [3].

The density of β-adrenergic receptors on the surface of C-6 glioma cells falls when the tumor cells are carried through several passages in culture in medium containing serum, and receptor density continues to decline as the number of passages increases [4]. In the present work we show that C-6 glioma loses in vivo responsiveness to treatment with a β-adrenergic agonist after the tumor cells are passed serially in vitro in a medium containing serum. We further show that if such C-6 glioma cells, shown to be unresponsive to in vivo treatment, are recultured in vitro in defined serum-free medium, the number of β-adrenergic receptors on the cell surfaces increases; when transferred back into rats, the recultured cells once again become responsive to treatment with a β-adrenergic agonist.

Methods

C-6 Glioma Cultures

C-6 glioma cells were obtained from the American Type Culture Collection and maintained in culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and L-glutamine (10 ml of 200 mM solution per 500 ml of medium) in a humidified atmosphere of 5% carbon dioxide at 37°C. Cells carried in this medium lose β-adrenergic receptors as the passage number increases.

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To increase the number of β-adrenergic receptors on glioma cells from high passage, cells were cultured in serum-free defined medium. Serum-free DMEM was supplemented with insulin, 5 μg/ml; transferrin, 5 μg/ml; linoleic acid, 5 μg/ml, complexed with 1 mg/ml fatty acid–free bovine serum albumin; and fibroblast growth factor, 10 ng/ml. The medium was changed every four to seven days. Cells were plated into culture flasks that had been pretreated with 5% FBS and subsequently washed.

Assay for β-Adrenergic Receptor

For the β-adrenergic receptor assay, cells were plated at 2×10^6 cells per flask (25 cm^2 surface area) and cultured in medium containing 10% FBS or in defined serum-free medium. Cells from three separate cultures containing FBS were assayed for β-adrenergic receptor on the second, third, and fourth days of culturing. Cells from cultures containing defined medium were assayed on days 2, 4, 7, and 11 from two cultures, and on days 2, 4, 8, and 10 from one culture. C-6 glioma cells grown in medium containing 10% serum reached confluence on day 4. Cells in serum-free defined medium grew more slowly than those in serum-containing medium and reached confluence between days 9 and 11.

For the receptor assay, cells were scraped off the flask surface with a rubber policeman, washed in Hanks' balanced salt solution (HBSS) without Ca^{++} and Mg^{++} (HBSS: Gibco Laboratories, Grand Island, NY), and resuspended in 50 mM Tris-phosphate buffer (pH 7.5) at a cell concentration of 5×10^6 per milliliter. Cells were sonicated for 5 to 12 seconds to produce membrane fragments, and 0.2 ml of this solution was aliquoted into 12 × 75 mm glass tubes. The β-adrenergic agonist ^{3}H-dihydroalprenolol with specific activity of 49 Ci/nmol (New England Nuclear, Boston, MA) was added to each tube to produce a final concentration of 2.0 nM. Half the tubes were incubated with 10 mM propranolol hydrochloride, a β-adrenergic antagonist (a gift from Ayerst Laboratories, New York, NY), specifically to block β receptors prior to incubation with ^{3}H-dihydroalprenolol. Tubes were incubated at room temperature for 10 minutes and then quickly filtered through Whatman GF/C 2.4 μm glass microfiber filters at 4°C. Each filter was washed twice with 4 ml of cold phosphate-buffered saline. The filters and trapped tissue were solubilized with 0.5 ml of NCS tissue solubilizer (6 N) for at least 16 hours at room temperature. Five milliliters of scintillation fluid (toluene plus 4% Omnifluor) was added, and samples (in duplicate or triplicate) were counted in a Beckman scintillation counter at 47% efficiency. Specific binding of the ^{3}H-dihydroalprenolol to the β receptor was calculated by subtracting the number of nonspecific counts not displaced by propranolol from the number of total counts. Results were expressed as the number of specific counts per milligram of protein. Protein determination was done using Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA).

C-6 Glioma in Vivo

C-6 glioma cells grown in serum-containing medium or in defined medium were removed from the flasks using a rubber policeman, and viable cells were counted, resuspended in HBSS, and injected subcutaneously between the scapulae in 2- to 4-day-old Wistar/Furth rats at a dose of 2×10^3 cells per animal. Seven litters (65 rats) were injected with cells grown in defined medium, and eight litters (65 rats) with cells grown in serum-containing medium. Each litter was divided into experimental and control groups. From the day of cell implantation, experimental animals were injected with the β-adrenergic agonist isoproterenol (isoproterenol hydrochloride, Winthrop Laboratories, Division of Sterling Drug, New York, NY) at a dose of 2 μg per gram of body weight per day in three divided doses, and with papaverine (papaverine hydrochloride, Eli Lilly and Co, Indianapolis, IN) at a dose of 20 μg per gram of body weight per day in two divided doses. Control rats were injected three times a day with saline solution. Rats were killed 17 or 18 days after tumor inoculation, and the tumors were excised and weighed.

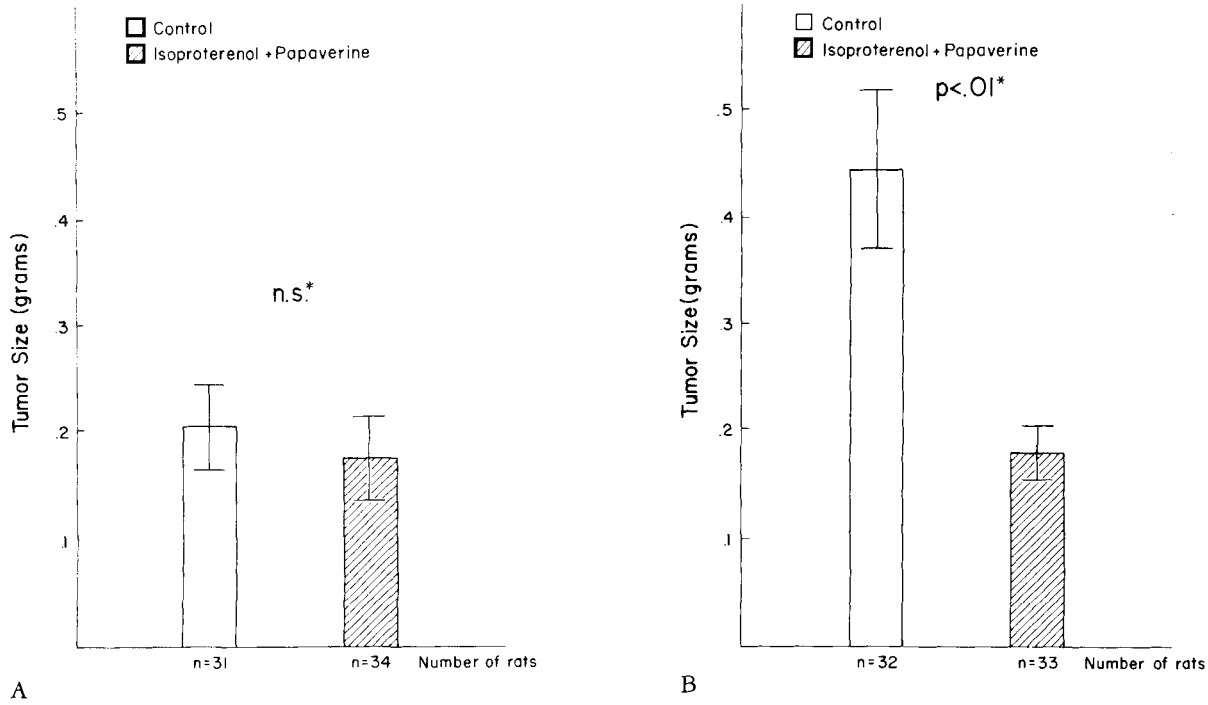
Results

C-6 glioma cells grown in defined serum-free medium had a slower rate of growth than glioma cells grown in medium containing 10% FBS. C-6 glioma cells grown in defined medium reached confluence between days 9 and 11; cells grown in serum-containing medium reached confluence on day 4. β-Adrenergic receptor density on C-6 glioma cells grown in defined serum-free medium was greater than that observed on cells grown in 10% FBS. The number of specific counts on cells cultured with FBS was 2,188 ± 298 CPM per milligram of protein (nine measurements from three experiments), and on cells cultured in serum-free defined medium 3,497 ± 362 CPM per milligram of protein (twelve measurements from three experiments), the difference significant at $p < 0.01$ (Student t test).

After several passages in culture medium containing 10% serum, C-6 glioma cells, when grown in vivo, were no longer sensitive to treatment with isoproterenol and papaverine. The mean tumor weight (± standard error of the mean) in rats injected with C-6 glioma cells cultured in medium with 10% serum and treated with isoproterenol and papaverine was 206 ± 39 mg (n = 31) and was not significantly different from that in controls treated with saline (176 ± 39 mg; n = 34). When C-6 glioma cells were grown in defined serum-free medium and then injected into newborn rats, the mean tumor weight in control animals was 444 ± 74 mg (n = 32). The mean tumor weight in animals treated with isoproterenol and papaverine was 182 ± 26 mg (n = 33). This difference was highly significant ($p < 0.01$) (Figure).

Discussion

C-6 glioma cells have surface β-adrenergic receptors. Activation of such receptors by a β-adrenergic agonist elicits a rise in intracellular cAMP [7]. Similar effects can be obtained with prostaglandin E_1, dibutyryl cAMP, and phosphodiesterase inhibitors [10, 12]. The



Effect of isoproterenol and papaverine treatment on the growth of C-6 glioma in rats. (A) Tumors from cells grown in medium containing 10% fetal bovine serum. (B) Tumors from cells grown in serum-free defined medium. Vertical bars show standard error of the mean. Asterisks indicate significance by Student t *test.*

presence of β-adrenergic agonists in the culture medium causes malignant C-6 glioma cells to assume the morphological features of mature astrocytes [9].

We showed previously that treatment of rats bearing C-6 glioma with the β-adrenergic agonist isoproterenol and the phosphodiesterase inhibitor papaverine significantly slows growth of this tumor [3]. After serial passages in culture, C-6 glioma cells, when injected in vivo, lost the ability to respond to treatment with adrenergic agonists. Lack of response to treatment in vivo with the β agonist was most likely related to a decrease in the number of β-adrenergic receptors on the C-6 glioma cells.

High-passage C-6 glioma cells cultured in serum-containing medium are known to have fewer β-adrenergic receptors than do cells from low passages, and they concomitantly lose the ability to accumulate cAMP when stimulated with catecholamines [4].

To restore responsiveness to treatment, we cultured unresponsive C-6 glioma cells in serum-free defined medium; following this treatment the number of β-adrenergic receptors increased. It has been documented that culturing C-6 glioma cells in the absence of serum increases the number of β-adrenergic receptors over that found in cells grown in serum. This increase results from a rise in the number of binding sites per cell. No change in affinity of the receptor for the ligand was found. Accumulation of cAMP in response to β-adrenergic stimulation in C-6 cells grown in defined conditions was significantly higher than in cells grown in medium containing FBS [4, 5]. We found that sensitivity of C-6 glioma in vivo to β-adrenergic stimulation depends on the number of β-adrenergic receptors on the cell surface. C-6 glioma cells, when grown through several passages in culture in medium containing 10% serum, become unresponsive to treatment with isoproterenol and papaverine when grown in vivo. When animals bearing tumors from C-6 glioma cells grown in defined medium were treated with isoproterenol and papaverine, growth of these tumors was significantly suppressed. Suppression of growth was not related to maturation of the tumor cells in defined medium, because tumors from control animals injected with cells maintained in defined medium were even larger than tumors from control animals injected with cells maintained in medium containing 10% serum.

Our work indicates that it is possible to slow growth of the C-6 glioma tumor of the rat in vivo by taking advantage of specific neuropharmacological properties of this tumor. We hope that this therapeutic approach, geared as it is to take advantage of the surface receptor properties of the tumor, will also prove applicable to the treatment of human gliomas.

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