Effect of Dexamethasone on Cell Proliferation of Neuroepithelial Tumor Cell Lines

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Abstract

The effect of glucocorticoid on cell proliferation, the expression of glucocorticoid receptor, and the relationship between inhibition of cell growth and apoptosis were investigated in four established neuroepithelial tumor cell lines (KNS42, T98G, A172, and U251MG). Glucocorticoid receptor expression was located in the cytoplasm of untreated cells, but translocated into nuclei after treatment with dexamethasone in KNS42, T98G, and A172 cells. U251MG did not express glucocorticoid receptors. Dexamethasone significantly inhibited the growth of KNS42 and T98G cell lines, at high concentrations in contrast to growth stimulation at low concentration. Dexamethasone inhibited proliferation of A172 cell line at all concentrations from 10^{-4} M to 10^{-7} M. These were prevented by RU38486, a specific glucocorticoid antagonist. Apoptosis did not occur in any cell lines after dexamethasone treatment. There was no response to glucocorticoid by U251MG cells. Dexamethasone treatment of neuroepithelial tumor cells expressing glucocorticoid receptors causes translocation into the nucleus to modulate cell proliferation upon binding of different concentrations of dexamethasone in vitro. Dexamethasone inhibits proliferation of some neuroepithelial cell lines, not by glucocorticoid-induced apoptosis. The bimodal potential of glucocorticoid to stimulate or suppress proliferation of neuroepithelial tumor cells expressing glucocorticoid receptor must be considered in clinical trials.

Key words: dexamethasone, glucocorticoid receptor, apoptosis, neuroepithelial tumor cell

Introduction

Dexamethasone is a synthetic glucocorticoid that is frequently used in the pre- and postoperative treatment of brain tumor patients. The actions of glucocorticoid in the central nervous system are largely mediated through the regulation of gene expression and protein synthesis, which modulate neurotransmitter synthetic enzymes, monoamine receptor-coupled second messenger systems, and protein phosphorylation systems in target cells. Dexamethasone can regulate cell growth in neuroepithelial tumors, but the mechanism is unknown. This study investigated the mechanism of medication and involvement of glucocorticoid receptors in cell growth after treatment with dexamethasone.

Materials and Methods

I. Cell culture

The human glioblastoma cell lines T98G, A172 were provided by the Japanese Cancer Research Resources Bank, Tokyo), the human astrocytoma cell line KNS42 (gift of Dr. I. Takeshita, Kyushu University, Fukuoka), and the human astrocytoma cell line U251MG (provided by the Japanese Cancer Research Resources Bank) were cultured in 100 mm dishes with RPMI1640 medium containing 10% fetal bovine serum, kanamycin monosulfate (100 mg/l), and L-glutamine (2932.3 mg/l). Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Dexamethasone was stored in ethanol at -80°C and diluted in culture medium just before use. RU38486 (generous gift from Roussel Uclaf Pharmaceuticals, Romainville, France) was also stored in ethanol at -80°C and diluted in culture medium before use (10^{-5} M) to prevent dexamethasone binding to glucocorticoid receptors. Only ethanol was added to the control culture medium. The final concentration of ethanol was 0.1%.

II. Effect of dexamethasone on cell growth

1.0 x 10⁶ cells/ml were dispensed into wells of 96-well plates in 100 ml of RPMI1640 medium. After 2-
3 days of culture, the medium was replaced with medium containing different concentrations of dexamethasone (10⁻⁴ M, 10⁻⁵ M, 10⁻⁶ M, or 10⁻⁷ M) and cultured for 3-5 days. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) stock solution was prepared at a concentration of 5 mg/ml phosphate buffered saline (PBS). 10 ml of MTT stock solution was added to each well and then wells were further incubated at 37°C for 6 hours. Subsequently, 150 ml isopropanol containing 0.04N HCl was added and the absorbance of each well was measured using an enzyme immunoreader (which automatically subtracts non-specific background from available data) at 495 nm. Cells growth was expressed as a percentage of mean control absorbance ± SEM, following subtraction of the mean absorbance of background. Examinations were performed three times for each sample.

III. Expression of glucocorticoid receptor

Cell pellets obtained from cultured cells were washed twice with PBS, pH 7.4 for 5 minutes and lysed with 200 ml of phenylmethylsulfonyl fluoride buffer consisting of 1% NP 40, 0.1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM phenylmethane sulfonyl fluoride, and 0.2 U/ml aprotinin. Cell pellets were homogenized with a microwave homogenizer at 4°C for 2 minutes and the lysates were centrifuged at 15,000 rpm at 4°C for 20 minutes. Supernatants were collected, mixed with 100 ml of sodium dodecyl sulfate sample buffer, incubated at 100°C for 5 minutes, and rapidly cooled on ice. Cell lysates were subjected to denaturing electrophoresis on 10% polyacrylamide gels and then transferred to nitrocellulose membranes. Membranes were processed for Western blot analysis using a 1:200 dilution of primary antibody (polyclonal rabbit anti-human glucocorticoid receptor antibody; Affinity BioReagents, Inc., Golden, Colo., U.S.A.) followed by incubation with biotinylated goat anti-rabbit IgG at a dilution of 1:400 for 30 minutes, in avidin-biotin complex (1:200) for 30 minutes. Signals were detected by the Amersham ECL system (Amersham Japan, Tokyo).

IV. Localization of glucocorticoid receptor

KNS42, T98G, A172, and U251MG cells were cultured on cover glasses in RPMI1640 medium. Samples were prepared in three 25-mm dishes for each cell line: One sample as a control antibody negative sample, and two samples for antibody staining with or without 10⁻⁶ M dexamethasone treatment 2 hours before. Cover glasses were washed twice with PBS for 10 minutes and fixed in 4% paraformaldehyde, pH 7.2 for 10 minutes at 4°C. After fixation, cover glasses were washed twice with PBS for 5 minutes and permeabilized in 0.3% Triton X-100 for 20 minutes. After PBS wash, cells were incubated in 3% normal goat serum in PBS for 20 minutes to reduce nonspecific background staining. Cover glasses were incubated overnight at 4°C with an anti-glucocorticoid receptor antibody (polyclonal rabbit anti-human glucocorticoid receptor antibody; Affinity BioReagents, Inc.) at a concentration of 5 mg/ml, then in biotinylated goat anti-rabbit IgG at a dilution of 1:400 for 1 hour, in avidin-biotin complex (1:200) for 30 minutes, and in a diaminobenzidine hydrogen peroxide solution for 15 minutes.

V. Effect of dexamethasone on cell apoptosis

1 x 10⁵ cells were collected from 100-mm dishes, centrifuged, and washed twice with PBS at 3-5 days after treatment with dexamethasone with and without RU38486. The cell pellets were lysed in 100 ml of buffer consisting of 10 mM Tris-HCl buffer, 10 mM ethylenediaminetetra-acetic acid (EDTA) (pH 8.0), and 0.5% Triton X-100. After 4 minutes rotation at 4°C, the cell lysates were centrifuged at 16,000 rpm for 20 minutes at room temperature. The supernatant containing ribonucleic acid (RNA) and fragmented deoxyribonucleic acid (DNA) was collected in a 1.5 ml tube and incubated at 37°C for 60 minutes with 5 ml ribonuclease A (10 mg/ml) to digest RNA. Five ml of proteinase K (10 mg/ml) was added and incubated at 37°C for 60 minutes, and the DNA was precipitated by 20 ml 5 M NaCl and 120 ml isopropanol at -20°C overnight. The lysates were centrifuged at 16,000 rpm for 20 minutes and the pellets were collected, air dried, and dissolved in 20 ml of Tris-EDTA buffer. Samples were electrophoresed on a 2% agarose gel with Tris-acetate, EDTA buffer. Fragmented DNA was visualized with ethidium bromide staining. This method was modified to exclude high molecular weight DNA when extracting fragmented DNA.

Results

I. Effect of dexamethasone on cell growth

The effect of dexamethasone on cell growth was investigated as the relationship between the concentration of dexamethasone and the absorbance of cells measured in the MTT assay (Fig. 1). Dexamethasone at concentrations between 10⁻⁷ M and 10⁻⁵ M significantly promoted the growth of KNS42 and T98G cells compared to untreated cells (p < 0.05). However, dexamethasone at a concentration of 10⁻⁴ M inhibited the growth of all cell lines (p < 0.05). Dexamethasone inhibited growth in A172 cells.
at all concentrations \( p < 0.05 \). No modulation of cell growth by dexamethasone was observed in U251MG cells.

RU38486 prevented the effects of dexamethasone in KNS42, T98G, and A172 cells. There was no significant difference between untreated cells and cells treated with dexamethasone or RU38486 at each concentration (Fig. 2).

II. Expression of glucocorticoid receptor

Western blot analysis of the neuroepithelial tumor cell lines (KNS42, T98G, A172, and U251MG) showed glucocorticoid receptor protein bands of approximately 97 kDa in T98G, A172, and KNS42 cells, but not in U251MG cells (Fig. 3).

III. Localization of glucocorticoid receptor

Immunocytochemical analysis showed that KNS42, T98G, and A172 cells had clear positive glucocorticoid receptor staining in the cytoplasm. There was strong nuclear staining in dexamethasone-treated cells (Fig. 4), in contrast to weak
Fig. 4 Photomicrographs of cultured neuroepithelial tumor cells stained by the immunoperoxidase technique with anti-human glucocorticoid receptor antibody. Note the intense nuclear staining and absence of cytoplasmic staining in KNS42 (B), T98G (D), and A172 (F) cells treated with dexamethasone. In contrast, cytoplasmic staining and weak nuclear staining is observed in KNS42 (A), T98G (C), and A172 (E) untreated cells. Original magnification x 200.
nuclear staining in untreated cells. When the primary antibody was omitted, neither cytoplasmic nor nuclear staining was seen in the presence or absence of dexamethasone. No staining was visible in the nucleus or cytoplasm in U251MG cells.

IV. Effect of dexamethasone on cell apoptosis

DNA fragmentation was examined by detection of a nucleosomal ladder on 2% agarose gels after treatment with only dexamethasone or dexamethasone and RU38486. No characteristic pattern of DNA fragmentation was observed in KNS42, T98G, A172, or U251MG cells in the presence or absence of dexamethasone.

Discussion

Our experiments showed that low dose dexamethasone at concentrations of $10^{-7}$ M to $10^{-5}$ M promoted growth of KNS42 and T98G cells whereas high dose dexamethasone at a concentration of $10^{-4}$ M caused growth inhibition. All doses of dexamethasone at concentrations from $10^{-7}$ M to $10^{-4}$ M inhibited growth of A172 cell. These effects were all inhibited by RU38486, suggesting that glucocorticoid has a significant role in cell growth regulation.

Investigation of the expression and localization of glucocorticoid receptor in KNS42, T98G, and A172 cells showed that most receptors were located in the cytoplasm of KNS42, T98G, and A172 cells. After treatment with dexamethasone, glucocorticoid-receptor complex was translocated into the nucleus as previously reported in other cell lines. We also found that U251MG had no glucocorticoid receptors and dexamethasone had no effect on cell growth as shown previously. All these results suggest that the glucocorticoid-receptor complex is involved in growth regulation in cell lines expressing glucocorticoid receptor.

The bimodal process of proliferation in KNS42, T98G, and A172 cells is very interesting. Our experiments cannot exclude the existence of an inhibitor for the glucocorticoid-receptor complex in the cytoplasm or nucleus. However, there is no reported evidence for such inhibitors. Also, such an inhibitor might be expected to cause the effect of dexamethasone to appear dose-dependently at higher concentrations, not as the observed bimodal pattern. Therefore, this hypothetical inhibitor would have no involvement with cell proliferation. We also found that the mechanism of inhibition of cell growth with dexamethasone in KNS42, T98G, and A172 cell lines was not programmed cell death, as reported in human leukemia cells and thymocytes.

Our experiments found no remarkable change in cell count after treatment with dexamethasone at concentrations from $10^{-7}$ M to $10^{-4}$ M. This result shows that dexamethasone did not induce necrosis or apoptosis but just suppressed the proliferation of A172 cells at concentrations within the physiological range (from $10^{-8}$ M to $10^{-7}$ M) and higher. Therefore, low dose administration of dexamethasone could retard A172 cell proliferation. In contrast, dose-dependent increase of KNS42 and T98G cell growth was found at dexamethasone concentrations of $10^{-7}$ M to $10^{-5}$ M, but cell numbers sharply decreased at the concentration of $10^{-4}$ M, and some cultured cells were visible at the surface of medium in dishes. Therefore, this process was not due to apoptosis induced by dexamethasone or over proliferation. Leukemia cells undergo glucocorticoid-induced apoptosis dose-dependently from $10^{-8}$ M to $10^{-4}$ M, but a concentration of $10^{-4}$ M dexamethasone is $10^3$ times that of physiological range and is likely to damage cultured cells. We conclude that the inhibition obtained after high dose treatment of KNS42 and T98G cells was probably not due to receptor mediation, but necrotic changes, resulting from cell membrane changes. A substantial dose of dexamethasone would be necessary to achieve an anti-tumor effect against KNS42 and T98G cells. These results suggest that the glucocorticoid-receptor complex acts differently in the regulation of gene expression and molecular synthesis related to cell proliferation in individual cell lines. The complex might turn on acceleration of cell growth-related gene in KNS42 and T98G cells, but suppress of cell growth-related gene in A172 cells.

High-dose treatment has achieved an anti-neoplastic effect in a patient with a glioma expressing glucocorticoid receptor, and a bimodal response to glucocorticoid in primary cultured astrocytic cells obtained during surgery and established astrocytic tumor cell lines, HU197, D384, or WF, which all express glucocorticoid receptor. The bimodal potential to stimulate or suppress proliferation of neuroepithelial tumor cells expressing glucocorticoid receptor must be considered in clinical trials of glucocorticoid by selecting the appropriate concentration (from $10^{-8}$ M to $10^{-7}$ M). Further research is necessary to analyze the mechanism of the gene site that mediates the regulation of cell growth by glucocorticoid-receptor complex in individual cell lines to explain the heterogeneous response in vitro and to examine whether dexamethasone can regulate tumor cell proliferation in vivo.
References


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Commentary

The authors reported the effects of dexamethasone on the cell proliferation of neuroepithelial tumor lines expressing glucocorticoid receptors. Interestingly, the effects of dexamethasone on the proliferation of tumor cells depended on its concentration in the culture supernatants. Cell proliferation was enhanced at low concentration of dexamethasone whereas it was inhibited at high concentration. They suggested that this bimodal effect of dexamethasone might be attributed to an inhibitor of glucocorticoid receptor complex in the cytoplasm or nucleus. We previously reported that suramin, a non specific antagonist for growth factors, showed bimodal actions on the proliferation of human glioma cells. Similarly to dexamethasone, suramin promoted the proliferation of cells at low concentration and inhibited it at high concentration. We further investigated the mechanism of intracellular signaling caused by suramin. In the cells treated with a low concentration of suramin, activity of MAP kinase was higher than those treated with high concentration. MAP kinase is one of the regulators of cell growth. I agree with the authors on the point that dexamethasone may cause unwanted ef-
fects on cell growth during administration or when the plasma level of dexamethasone decreases after one-course treatment, and that glioma cells which responded to dexamethasone by such mechanism may grow unexpectedly.

Reference


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This very interesting paper revisits an issue that was the subject of intense controversy many years ago when the first large clinical trials for the treatment of primary malignant brain tumors were undertaken. At that time the role of glucocorticoid administration in the management of patients with primary malignant brain tumors was not understood, and several trials were designed using radiotherapy, chemotherapy, and the prolonged administration of corticosteroids as part of a protocol. Unfortunately, at that time it appeared that there was no survival advantage in the patients given supplemental glucocorticoids. In later trials it was recognized that it was nearly impossible to control for the use of steroids, as so many of the patients who were aggressively treated required intermittent or sometimes continual management with glucocorticoid in order to control radionecrosis or cerebral edema associated with primary malignant brain tumors.

This scientific paper confirms the lack of an additional enhancing antitumor effect of dexamethasone. They showed that dexamethasone administration to malignant tumor cell lines did not produce an increase in apoptosis. They showed that at low concentrations dexamethasone could actually stimulate the growth of certain cell lines and did show that at appropriate dosages there was a degree of suppression of proliferation of tumor cells produced by dexamethasone.

In the opinion of this reviewer the effects of dexamethasone presented in this very interesting research should not alter our custom of using glucocorticoids for the clinical management of patients with malignant brain tumors. The effectiveness of steroids against the secondary effects of brain tumors is well proven, and no other agents are as successful in controlling cerebral edema or the effects of radiation necrosis. The question of whether a standard dose of steroids should be incorporated into multimodality glioma treatment protocols might be revisited, and the data provided in this paper would help to determine an appropriate dose recommendation.

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The authors have studied the effects of dexamethasone at various doses on neuroepithelial cell growth and apoptosis. No cell line underwent apoptosis. However, dexamethasone promptly reduced proliferation of A172 cells. Interestingly, treatment with dexamethasone led to translocation of the glucocorticoid receptor from the cytoplasm to the nucleus. They suggest that the glucocorticoid status of brain tumors may play a role in determining their response to treatment. All neurosurgeons use dexamethasone for their patients harboring malignant brain tumors, and for good reasons. However, whereas we all believe that dexamethasone impacts favorably on stabilizing the cerebral capillary endothelial cell preventing cerebral edema formation, the present study (and others previously published) indicate that steroids such as dexamethasone have pleotrophic effects. We must be aware of the growth inhibitory and potential apoptotic effects of steroids in the patients whose tumors we are treating. Finally, while the authors’ immunocytochemical data regarding the translocation of the glucocorticoid receptor to the nucleus appear convincing, it would have been confirmatory if they had shown by nuclear and cytoplasmic fractionation studies followed by Western blotting that this truly was the case.

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Kawamura et al. reported the bimodal effect of dexamethasone to stimulate or suppress proliferation of neuroepithelial tumor cells. This effect was blocked by RU38486, a specific glucocorticoid antagonist, and the translocation of glucocorticoid receptor after treatment with dexamethasone was clearly shown by immunocytochemistry. As the authors mentioned, certain inhibitors may modulate the effect of dexamethasone. In this experiment, the authors used the culture medium supplemented with 10% FCS which may contain various growth factors and inhibitors. Therefore, they might observe a different phenomenon by using a plain medium. In general, the effect of
Dexamethasone appears immediately, and continues for a relatively short period. Although the effect of dexamethasone was examined for only several days after the treatment, any possible late effect seems interesting as well. The authors concluded that cell death after dexamethasone treatment was not attributed to apoptosis as judged by DNA laddering. However, the DNA laddering pattern is one of the characteristic phenomena for apoptosis, and it is not suitable for detecting the early apoptotic process. If more sensitive and quantitative methods, such as annexin-5 staining or TUNEL techniques were employed, the early steps of apoptosis might be found. At any rate, further study regarding glucocorticoid receptor may provide a new strategy for glioma therapy in the future.

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The authors demonstrated the effect of dexamethasone (DEX) on cell proliferation of neuroepithelial tumor cell lines. Glucocorticoid (e.g., DEX) is frequently reported to induce apoptosis or change of cell growth in hematopoietic cells, but this is rare in neuroepithelial tumor cells. One reason is that neuroepithelial tumor cells present various responses to glucocorticoid treatment. For example, cell growth of some neuroepithelial tumor cells is inhibited by DEX (described in this paper) and promoted in others (e.g., U87MG: personal experimental result). Also, there are cases that the response is different according to the dose of DEX as in this paper. The authors call their characterizations “bimodal potential.” Therefore, the authors concluded that it is important to consider the response of neuroepithelial tumor cells to glucocorticoid in individual patients with brain tumors before clinical trials. I think that this evidence is a very important point in selection of effective treatments.

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