Growth suppression and astrocytic differentiation of glioma cells by interleukin-1

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The effect of recombinant human interleukin-1 (rHuIL-1) derivatives on human glioma cell lines was examined in vitro. Five glioma cell lines, U-251 MG, U-373 MG, U-87 MG, A-172, and T98G, were incubated in medium containing 1% fetal calf serum and various concentrations of different types of rHuIL-1: OCT-43 (rHuIL-1β), OCT-7000 (rHuIL-1α), and OCT-8000 (rHuIL-1α). The high-affinity IL-1 receptors were expressed in the U-251 MG and U-373 MG cell lines, and rHuIL-1 was found to suppress cell growth and to induce morphological differentiation of these cell lines. Growth inhibition occurred in a dose-dependent manner in concentrations of rHuIL-1 ranging between 1 and 100 ng/ml. Interestingly, HuIL-1 induced a transient growth of glioma cells shortly after administration, then suppressed cell growth with accompanying elongation of cytoplasmic processes. This unique process of transient growth suppression following growth stimulation was parallel to the efficacy of bromodeoxyuridine uptake in the rHuIL-1-treated cells. Concomitantly, accumulation of glial fibrillary acidic protein and cyclic adenosine monophosphate contents was observed in four glioma cell lines. Continuous HuIL-1 treatment for longer than 30 days elicited irreversible astrocytic terminal differentiation. These results indicate that IL-1 is an effector on the growth regulation of glioma cells, resulting in astrocytic differentiation in vitro.

KEY WORDS · interleukin-1 · glioma · glial fibrillary acidic protein · differentiation · cyclic adenosine monophosphate

INTERLEUKIN (IL)-1 is a cytokine with various biological effects, such as a regulatory effect on immune function and hematopoiesis. It is secreted not only by stimulated macrophages but also by a variety of other cell types, including glial cells in the central nervous system (CNS). The effect of IL-1 on the stimulation of host antitumor immune mechanisms has been previously reported. In addition, the growth of some tumors, such as malignant melanomas, was inhibited by IL-1 in vitro and in vivo. It has also been shown that IL-1 stimulates hematopoietic stem cells and induces the production of several hematopoietic growth factors, including granulocyte-macrophage colony-stimulating factor (CSF), granulocyte CSF, and IL-6. Thrombocytopenia and granulocytopenia by IL-1 are thought to be effective in treating patients with myelosuppression caused by radiotherapy and chemotherapy. We have previously reported that recombinant human IL-1β (rHuIL-1β) derivatives were partly effective in preventing chemotherapy-linked myelosuppression in patients with malignant brain tumors and in improving symptoms from damage caused by the chemotherapy. On the other hand, CSF's and IL's stimulate cell growth in vitro in some cell types, including several solid tumor cells as well as hematopoietic tumor cells. Although clinical trials of IL-1 administered to patients bearing malignant tumors have already been started, the effect of these cytokines on brain tumors has not been conclusively determined as yet. In order to assess the effect of IL-1, we examined the response of five human glioma cell lines to rHuIL-1 in vitro by IL-1 receptor binding assay, colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, flow cytometry for bromodeoxyuridine (BUDR) uptake assay and cell-cycle analysis, enzyme-linked immuno-
Effect of interleukin-1 on glioma cells

sorbent assay (ELISA) for glial fibrillary acidic protein (GFAP), and radioimmunoassay for cyclic adenosine monophosphate (cAMP).

Materials and Methods

Preparation of Recombinant Human IL-1

Three types of rHuIL-1 were used in the study. The first type, OCT-43, is a site-specific mutant of rHuIL-1β with serine in place of cystein at position 71. It consists of 153 amino acids with a molecular weight (MW) of 18 kD and an isoelectric point of 6.9. The specific activity of OCT-43 was 5 × 10^7 IU/mg protein, as determined by the A375S1 melanoma cell growth inhibition assay.22 The second type, OCT-7000, is a mutant of rHuIL-1α with asparaginic acid in place of asparagine at position 36 and serine in place of cystein at position 141. It consists of 159 amino acids with an MW of 18.5 kD and an isoelectric point of 5.0. The specific activity of OCT-7000 was 1.2 × 10^8 IU/mg protein. The third type, OCT-8000, is also a mutant form of rHuIL-1α with deletion of 14 amino acids in the N-terminal of OCT-7000.23 It consists of 145 amino acids with an MW of 17 kD and an isoelectric point of 4.8.

Cell Cultures

Five human glioma cell lines (U-251 MG, U-373 MG, U-87 MG, A-172, and T98G) were used in the experiments.24 All cell lines were maintained in monolayer cultures under standard culture conditions (37°C, 5% CO2) in Eagle’s minimum essential medium (EMEM) with nonessential amino acids, sodium pyruvate, penicillin, streptomycin, and 10% fetal calf serum (FCS). The four glioma cell lines in exponential growth phase in EMEM containing 10% FCS were harvested by a brief treatment with 0.1% trypsin supplemented with 0.05% ethylenediamine tetra-acetic acid (EDTA), suspended in EMEM with 1% FCS, and seeded into 90-mm Falcon dishes with or without rHuIL-1. The cells were divided every 6 or 7 days and maintained for up to 30 days. The cells were counted using the trypan blue exclusion method and observed via an inverted microscope.

Interleukin-1 Binding Assay

OCT-7000 was iodinated in a 1.5-ml tube coated with 2.5 mg of 1,3,4,6-tetrachloro-3,6-diphenylglycoluril dissolved with 50 µl chloroform.22 After the chloroform dried, 3 µg of OCT-7000 was reacted with 500 µCi of Na[125]I at 0°C. The [125]I-labeled IL-1α (OCT-7000) was separated from free [125]I by elution with a 0.1% bovine serum albumin (BSA)-phosphate-buffered saline (PBS) solution on a PD-10 column. The specific activity of this [125]I-labeled IL-1α was 109,458 cpm/mg. The U-251 MG and U-373 MG cells were incubated in flat-bottomed six-well plates at a density of 1 × 10^6 cells/well for the binding assay, 0 to 500,000 cpm of [125]I-labeled IL-1α with or without unlabeled OCT-7000 (0.5 to 1 µg/well) in a total volume of 200 to 500 µl of the medium containing 1% FCS was added to each well and incubated for 120 minutes at 4°C. The cells were lysed with 500 µl of 1% sodium dodecyl sulfate (SDS) and 0.2 N NaOH, then counted using an automatic well gamma system.25 The saturation curves generated for the IL-1 binding site were analyzed to determine the dissociation constant (Kd) and the maximum number of binding sites.

MTT Assay

For the MTT assay, cells in complete medium containing 10% FCS were trypsinized and seeded into flat-bottomed 96-well microtiter plates with medium containing 1% FCS (1 × 10^4 cells/well). After 6 hours of incubation at 37°C, fresh medium containing OCT-43, OCT-7000, or OCT-8000 (1 to 100 ng/ml) was added to each well. The effect of rHuIL-1 on cell growth was determined on Day 5 of incubation by monitoring the number of metabolically active cells using the MTT assay as described elsewhere.26 Briefly, 40 µl of MTT solution (2.5 mg/ml) was added to each culture well, the plates were incubated at 37°C for 4 hours, and MTT formazans were inspected microscopically. Then, 100 µl of a 10% SDS-0.1 N HCl solution was added to each well. After incubation overnight, absorbance at 570 nm was measured using a microplate reader.

The time course of the growth of U-251 MG and U-373 MG cells incubated with rHuIL-1 was also investigated. Cells were incubated in EMEM supplemented with 1% FCS in flat-bottomed 96-well microtiter plates (1 × 10^6 cells/well) in the presence of 10 ng/ml of OCT-43, OCT-7000, or OCT-8000. Control cells were incubated in complete medium with 1% FCS and no IL-1. On Days 2, 4, and 6 of incubation, cell growth was analyzed by the MTT assay as described above.

The results of the MTT assays were expressed as follows: relative growth index = absorbance at 570 nm of rHuIL-1-treated cells + absorbance at 570 nm of control cells. All assays were carried out in triplicate.

Flow Cytometric Analysis of BUdR Labeling Index

The influence of rHuIL-1 on the cell cycle and on the BUdR labeling index of human glioma cells was analyzed by flow cytometry.27 The U-251 MG and U-373 MG glioma cells were incubated for 6 days on 90-mm Falcon dishes (10^4 cells/dish) in complete medium with 1% FCS with or without 10 ng/ml of OCT-43. A final concentration of 10 µM BUdR was added to each dish 30 minutes before harvest. The cells were rinsed twice with PBS and trypsinized as described above, then fixed and chilled with 70% ethanol for a minimum of 30 minutes. The cells were resuspended in 2 N HCl to denature the deoxyribonucleic acid (DNA) and neutralized with 0.1 N sodium tetaborate, followed by a 30-minute reaction at room temperature using a 1:100 dilution of fluorescein isothiocyanate (FITC)-conjugated anti-BUdR monoclonal antibody. The procedure continued with the cells being rinsed twice with PBS containing 1% FCS and 0.05% sodium azide, incubated with 100 µl of 1 mg/ml ribonuclease for 20 minutes at 37°C, and stained with 5 µg/ml of propidium iodide. Flow cytometry of these cells was performed on a FACScan® and the bivariate BUdR/DNA (FITC/prodipium iodide) distributions were displayed in contour plots for analysis of the cell cycle and labeling index (the percentage of DNA synthesizing cells).28 All assays were performed twice in independent experiments.

* OCT-43, OCT-7000, and OCT-8000 kindly provided by Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan.
† U-251 MG cells kindly provided by Dr. Takakura, Tokyo University, Tokyo, Japan; U-373 MG cells supplied by American Type Culture Collection, Rockville, Maryland; A-172 and T98G cells obtained from the Japanese Cancer Research Resources Bank, Tokyo, Japan.
‡ Automatic well gamma system, Model ARC-300, manufactured by Aloka, Tokyo, Japan.
§ MTT supplied by Kanto, Tokyo, Japan.
¶ FACScan flow cytometer manufactured by Becton-Dickinson, Sunnyvale, California.
Measurement of GFAP

Changes in the expression of GFAP by rHuIL-1 in U-251 MG and U-373 MG cell lines were analyzed by ELISA. After 6 days of incubation in flat-bottomed 96-well microtiter plates, cells treated with 10 ng/ml of OCT-43, OCT-7000, or OCT-8000 were lysed with 100 μl of a lysis buffer containing 0.5% Triton X-100, 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, and 1 mM phenylmethyl sulfonylfluoride. After 16 hours of incubation at 4°C in the lysis buffer added to an equivalent volume of PBS, a sample was aspirated from each well and each well of the plate was blocked overnight with PBS containing 2.5% BSA and 0.1% sodium azide. The cells were washed once with PBS containing 0.05% Tween, followed by a 12-hour reaction at 4°C using a 1:500 dilution of mouse anti-GFAP monoclonal antibody. The cells were then washed four times with PBS-Tween, reacted using a 1:1000 dilution of peroxidase-conjugated goat anti-mouse immunoglobulin for 2 hours at room temperature, and washed again four times with PBS-Tween. A substrate solution was prepared by dissolving 250 mg of orthophenylenediamine dihydrochloride in 100 ml of a 100-mM phosphate buffer (pH 6.0) to which 50 μl of a 50% H2O2 solution was added; 100 μl of this solution was added to each well of the assay plate immediately after preparation. After incubation with the substrate solution for 15 minutes at room temperature, 100 μl of 2 N H2SO4 was added to each well and absorbance at 492 nm was measured using a microplate reader. Comparison between wells was accomplished using the following rate: GFAP expression per cell = absorbance at 492 nm of ELISA absorbance at 570 nm of MTT assay in the equivalent condition of culture. All assays were carried out in duplicate.

Cyclic AMP Assay

The cAMP content of four human glioma cell lines (U-251 MG, U-373 MG, A-172, and T98G) was assayed. On Day 6 of incubation in 90-mm Falcon dishes, ethanol was applied to control cells and cells treated with 10 ng/ml of OCT-43, then the cells were scraped off with a rubber policeman. The ethanol extracts were assayed via radioimmunoassay using the 3H-cAMP assay kit. All assays were carried out in duplicate.

Results

Expression of the IL-1 Receptor

Binding of 125I-labeled interleukin (IL)-1α to U-251 MG and U-373 MG cells was rapid, even at 4°C. The IL-1 binding site was saturable when U-251 MG or U-373 MG cells were incubated with increasing concentrations of 125I-labeled IL-1α, and the data were linear when plotted on Scatchard coordinates (Fig. 1). The saturation curves revealed a single type of high-affinity IL-1 receptor (Kd = 33.6 pM for U-251 MG cells and 44.3 pM for U-373 MG cells), which was of a moderately high density (307 sites/cell for U-251 MG cells and 2110 sites/cell for U-373 MG cells).

Effect of rHuIL-1β on Glioma Cell Growth

In order to examine the effects of rHuIL-1 on glioma cells, growth of the five different glioma cell lines was assessed for different doses of rHuIL-1. Cell growth was determined by MTT assay after treatment with rHuIL-1 at various concentrations (1 to 100 ng/ml) for 5 days. The results showed that the growth of all five cell lines was suppressed in a dose-dependent manner (Fig. 2). At a dose of 10 ng/ml, rHuIL-1 suppressed cell growth to approximately 50% of that for nontreated cells on Day 6 of culture. Notably, rHuIL-1 transiently stimulated cell growth for the first 24 hours, followed by a dramatic inhibitory effect on cell growth thereafter (Fig. 3). After passage of the four glioma cell lines, no transient growth promotion was observed, and the growth inhibitory effects of IL-1 reached a plateau of approximately 50%, then continued upward until Day 30.

DNA Synthesis and Cell Growth Suppression

The cycle of U-251 MG and U-373 MG human glioma cells treated with 10 ng/ml of rHuIL-1β was compared to that of control cells on Days 1, 2, 4, 6, and 30 by flow cytometry. The glioma cells incubated in 1% FCS in the absence of IL-1 gradually accumulated in the G0/G1 phase. The addition of rHuIL-1β did not enhance this tendency of G0/G1 arrest. Recombinant HuIL-1β affected the cell cycle very little, including the mean (± standard deviation) percentage of S-phase cells (29.0% ± 1.0% for rHuIL-1β-treated U-251 MG cells vs. 31.0% ± 1.0% for control cells, and 46.3% ± 1.0% for IL-1-treated U-373 MG cells vs. 45.2% ± 1.0% for rHuIL-1β-treated U-373 MG cells vs. 45.2% ±

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"*3H-cAMP assay kit provided by Amersham Corp., Arlington Heights, Illinois."
Effect of interleukin-1 on glioma cells

The synthesis of DNA in rHuIL-1-treated glioma cell lines was examined by measuring the uptake of BUdR. As shown in Fig. 5, DNA synthesis activity was stimulated significantly in the rHuIL-1β-treated U-251 MG cell line within 1 or 2 days after administration. Following this event, continued treatment up to 30 days with rHuIL-1β markedly suppressed the DNA synthesis, especially in the U-251 MG cell line.

Morphological Differentiation of Glioma Cell Lines

Morphological differentiation of glioma cell lines was elicited by exposure to rHuIL-1. Stellate and multiple thin cytoplasmic processes with many knotty structures were characteristic for the rHuIL-1β-treated glioma cell lines, similar to that of normal astrocytes (Fig. 6). When the glioma cell lines reached confluence in the presence of rHuIL-1, the growth of these cells was contact-inhibited. On the other hand, the cells in control cultures without rHuIL-1 were overcrowded and piled up with no apparent morphological differentiation. Morphological differentiation was first observed at 24 hours after administration of rHuIL-1 in culture. Longer exposure (at least 30 days) with rHuIL-1 appeared to situate the cells in terminal differentiation since morphological differentiation was maintained even after the cytokine was removed. Persistence of multipolar cytoplasmic processes was observed for at least 7 days and cell growth did not recur in the absence of rHuIL-1β.
Elevation of Cyclic AMP Levels in Glioma Cell Lines

Cyclic AMP in each human glioma cell line treated with 10 ng/ml of rHulL-1β for 6 days was quantified by radiommunoassay. The content of cAMP in IL-1 treated morphologically differentiated cells was approximately two or three times greater than in the controls (Table 1).

Expression of GFAP

The ELISA for the U-251 MG and U-373 MG human glioma cell lines using anti-GFAP monoclonal antibody revealed that expression of GFAP was significantly enhanced after treatment with rHulL-1β. The expression of GFAP in U-251 MG and U-373 MG cells treated with 10 ng/ml of rHulL-1α or rHulL-1β for 6 days, expressed as the ratio of absorbance at 492 nm in ELISA to absorbance at 570 nm in MTT assay, increased approximately 1.5- to fourfold in comparison with that of the control culture (Fig. 7).

Discussion

The initial step in the action of IL-1 is the binding of this cytokine to plasma membrane receptors. There are two types of IL-1 receptors: type 1 receptors are found on T cells, fibroblasts, and other cells; and type

<table>
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<tr>
<th>Cell Line</th>
<th>cAMP Content (pmol/mg protein)</th>
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<tr>
<td></td>
<td>Control cells (1% FCS)</td>
</tr>
<tr>
<td>U-251 MG</td>
<td>19.3 ± 2.8</td>
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<tr>
<td>U-373 MG</td>
<td>11.4 ± 0.9</td>
</tr>
<tr>
<td>A-172</td>
<td>12.1 ± 2.1</td>
</tr>
<tr>
<td>U-87 MG</td>
<td>7.2 ± 0.9</td>
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* rHulL-1β = recombinant human interleukin-1β; cAMP = cyclic adenosine monophosphate; FCS = fetal calf serum.

1 Measured by radiommunoassay; expressed as the mean ± standard deviation. Significance was assessed by an unpaired Student’s t-test. Each assay was carried out twice.
Effect of interleukin-1 on glioma cells

Fig. 6. Photomicrographs showing morphological differentiation of glioma cell lines induced by recombinant human interleukin-1β (rHuIL-1β). × 150. Glioma cell lines U-251 MG (A and B), U-373 MG (C and D), U-87 MG (E and F), and A-172 (G and H) were treated with (right) and without (left) 10 ng/ml of rHuIL-1β. Examination revealed morphological changes with stellate and multiple cytoplasmic processes typical for astrocytic differentiation in the presence of rHuIL-1β after 6 days (B and D) and 3 days (F and H), compared to no morphological differentiation in control cells treated with complete medium containing 1% fetal calf serum for 6 days (A and C) or 3 days (E and G).
2 receptors are found on monocytes, B cells, and neutrophils. It has been shown that IL-1α and IL-1β seem to bind both type 1 and type 2 receptors. Both types of IL-1 receptors are expressed in cells in the CNS. Although they appear to be located on neurons rather than glia, except in injured brain tissue, Gottschall, et al., reported that high-affinity IL-1 receptors were expressed in the human glial cell line U-87 MG. Our studies revealed that other human glioma cell lines, such as U-251 MG and U-373 MG, expressed a single type of specific, saturable, and high-affinity receptors of moderately high density. Cyclic AMP is the intracellular second messenger of type 2 IL-1 receptors and serine kinase is that of type 1 IL-1 receptors. The IL-1 receptors expressed on glioma cells seem to be type 2 based on the increase of cAMP contents by IL-1 in our study.

Animal studies and preliminary clinical studies have shown IL-1 to have two major biological effects: acceleration of hematopoietic recovery from cytopenia and an antitumor effect. Both effects are of considerable benefit in the therapy of patients with malignant tumors.

Some cytokines, such as interferons and tumor necrosis factor, were reported to have antitumor effects. These can be either direct or indirect with enhancement of host antitumor immune mechanisms. The direct effects include cytotoxic or cyostatic phenomena, induction of cell differentiation, and the effect of tumor vessels as seen in the in vivo results of tumor necrosis factor.

Interleukin-1 augments antitumor immune mechanisms such as natural killer cell and cytotoxic T lymphocyte activity. It also exerts direct cytostatic and cytotoxic effects on certain tumor cells in vitro and in vivo. In the current study, we examined the growth inhibitory effects of rHuIL-1 on five human glioma cell lines in vitro; the prolonged culture of human glioma cells in the presence of rHuIL-1 disclosed a cytostatic effect.

Upon exposure to some agents, glioma cells differentiate in vitro. Agents such as retinoids and interferon-β, as well as the environment around the cell such as the extracellular matrix, induce differentiation of glioma cells. The effects of these agents on the differentiation of glioma cells have been evaluated by morphological observations and by quantitative studies of GFAP and cAMP.

Glioblastoma is a glioma-specific protein expressed in astrocytes and astrocytomas. Jacque, et al., examined GFAP in human brain tumors and found that the GFAP content in gliomas correlated well with the malignancy of the gliomas. In astrocytomas, the amount of GFAP is higher than in normal white matter whereas glioblastomas contain a low amount of GFAP. Recently, Rutka and Smith reported that GFAP-negative human astrocytoma cells transfected with GFAP complementary DNA formed elongated processes in culture and demonstrated decreased cellular proliferation and tumorigenicity. In our experiments, the expression of GFAP antigen was enhanced by rHuIL-1. This finding suggests that IL-1 induces astrocytic differentiation of undifferentiated glioma cells.

Cyclic AMP is the intracellular second messenger of hormones and cytokines. It is associated with cellular differentiation and various cell functions. The intracellular cAMP level in undifferentiated glioma cells is lower than in differentiated glial cells. Thus, the increase of cAMP glioma cells exhibited in our study is indicative of IL-1-induced differentiation of these cells.

The transient IL-1-induced growth promotion followed by inhibition observed in the current study is suggestive of the need for one division of stimulated cells in order for differentiation to occur. The minimal cell-cycle changes and the decrease in the BUdR labeling index may reflect a decrease in the velocity of DNA synthesis. Our findings suggest that IL-1 has no cytotoxic effect on glioma cells but that it induces cell differentiation.

In the CNS, IL-1 is produced and secreted by glial cells, mainly microglia cells, and stimulates astroglial proliferation in fetal and injured brain tissue. Glial, et al., found that tissue levels of IL-1 were elevated in the acute phase of brain injury and during normal early brain development. The histological detection of IL-1 in the CNS and glial tissues has been reported. Interleukin-1 may be the glial stimulatory factor in astroglial proliferation. Lachman, et al., documented the growth-promoting effect of recombinant IL-1 on the U-373 MG glioma cell line. These results do not conflict with our observations because the H-
Effect of interleukin-1 on glioma cells

Thymidine incorporation assay in their study was performed after only 48 hours of incubation. Our investigation suggests that IL-1 stimulates glioma cells and may stimulate fetal astrocytes, and that it does not induce cell proliferation but rather induces differentiation followed by growth inhibition.

We previously reported preliminary clinical trials of rhU1L-1/3 in the treatment of chemotherapy-induced myelosuppression in patients with malignant brain tumors. Both granulocytopenia and thrombocytopenia were controlled in patients who had received nitrosourea and other chemotherapeutic agents in combination with rhU1L-1/3. It may be possible to administer relatively high doses of chemotherapeutic agents if rhU1L-1/3 is given simultaneously. In our earlier clinical study, we detected no direct antitumor effects of IL-1. Systemically administered IL-1 may not reach intracranial tumors and the total amount of IL-1 given to the patients who had received the chemotherapeutic agents was not enough to inhibit the growth of their gliomas. Cytokines act in their native state by autocrine or paracrine mechanisms, and the local administration of the antitumor cytokines is recommended. It has been shown that intracerebroventricular infusion of IL-1 rapidly decreases the peripheral cellular immune response in rats. A pyrogenic action, the major adverse effect of IL-1, may be enhanced by intracranial administration. Some molecular biological methods against these adverse effects on intracranial administration of IL-1 may enable the effective use of IL-1 in the treatment of the patients with malignant gliomas.

Conclusions

We found that IL-1 derivatives inhibit the proliferation of glioma cells and induce astrocytic differentiation in vitro. Interleukin-1 is an agent that may induce glial differentiation of glioma cells in vivo and may be useful in the treatment of malignant gliomas, not only as an agent to treat myelosuppression but also as a direct antitumor agent.

Acknowledgments

We express our appreciation to Drs. Y. Hira and S. Nakai, Otsuka Pharmaceutical Co., Ltd., Takushima, Japan, for valuable discussions.

References


J. Neurosurg. / Volume 81 / September, 1994