Human Recombinant Interleukin-1 β -mediated Growth Inhibition of Cultured Malignant Glioma Cells

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Abstract

The effect of human recombinant interleukin-1 β (hrIL-1 β) on tumor growth was studied in eight glioma cell lines. hrIL-1 β inhibited growth in all cell lines, but to varying extents. Two cell lines were suppressed by 0.5 ng/ml hrIL-1 β , and three cell lines required 20 ng/ml. hrIL-1 β also induced morphological changes and increased F-actin contents. hrIL-1 β -treated cells demonstrated multipolar shapes and numerous processes with a greater number of cell-cell contacts 24 hours after treatment. Fluorescence microscopy revealed that these processes contained a large amount of polymerized F-actin. These results suggest that hrIL-1 β -mediated growth inhibition may be related to the differentiation of glioma cells.

Key words: malignant glioma, interleukin-1, actin, growth inhibition

Introduction

The cytokine interleukin-1 (IL-1) is produced by various cells including microglia⁶⁾ and glioma cells,^{4,5)} and displays a wide range of biological activities related to immunological response and cell proliferation. IL-1 causes growth inhibition in several malignant tumor cell lines such as A375,^{13,19)} K562,¹⁵⁾ L929,¹⁹⁾ and M1,²⁰⁾ but promotes growth in human astrocytoma U373.¹²⁾ No other glioma cell lines have been investigated.

Recently, clinical trials have assessed the effect of human recombinant IL-1 β (hrIL-1 β) on malignant tumors including glioma.^{10,11)} Clinical inhibition was reported in malignant tumors, especially glioma, mammary carcinoma, and malignant lymphoma of the skin. These clinical results correlated with *in vitro* responses for mammary carcinoma and lymphoma.^{18,22)} IL-1 β caused clinical inhibition of glioma, but growth promotion *in vitro*.¹²⁾ Here, we report a study of the direct effect of hrIL-1 β on glioma cell proliferation to clarify the discrepancy in clinical and *in vitro* responses.

Materials and Methods

Five human glioblastoma cell lines were obtained from patients in the Huntington Memorial Hospital, Pasadena, Cal., U.S.A.: HBR9T#2, HBR84T, HBR98T, O1T, and O9T. Two established human glioblastoma cell lines were provided by Dr. Higashi, Department of Neurosurgery, Okayama University Medical School, Okayama: T98G and KC-PC. One human glioblastoma cell line was obtained from a patient in Ryukyu University Hospital: RBR17T. The glial origin of cells was demonstrated by glial fibrillary acidic protein staining or standard histological criteria for cultured glial cells. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 (Flow Laboratories, Coasta Mesa, Cal., U.S.A.) supplemented with 5% fetal bovine serum (FBS) (Flow Laboratories), neomycin (50 mg/l), and amphotericin B (2.5 mg/l). Cell lines were cultured in humidified air containing 5% CO₂ at 37°C.

Growth inhibition was assessed by the ³H-thymidine incorporation assay. Glioma cell cultures were washed and adjusted to 1.0 $\times$ 10⁴ cells/200 μ l in DMEM/F12 supplemented with 5% FBS. This suspension was placed in 96-well, flat-bottomed

plates at 200 μ l per well. The plates were incubated for 48 hours to allow recovery from trypsinization. The medium was then changed to fresh medium containing several dilutions of hrIL-1 β (0.5–16 ng/ml) (Otsuka Pharmaceutical Co., Tokyo). After 32 hour culture with hrIL-1 β , cells were pulsed with 0.2 mCi/well of 3 H-thymidine (2 Ci/mmol; Du Pont/NEN Research Products, Wilmington, Del., U.S.A.) for a further 16 hours. The cells were harvested with a cell harvester, and the radioactivity incorporated measured by scintillation counter. Percentage growth inhibition was calculated by:

$$100 - \frac{\text{cpm in target cells in medium with hrIL-1}\beta}{\text{cpm in target cells in medium alone}} \times 100$$

Effect on doubling time was also evaluated. $0.5-2 \times 10^5$ cells were seeded in 35 mm tissue culture plates. Forty-eight hours later, the medium was changed with fresh medium, with or without hrIL-1 β (10 or 20 ng/ml). Forty-eight hours later, the medium was renewed. Cells were resuspended three times by brief treatment with an enzyme cocktail (0.1% trypsin, 0.02% ethylene glycol-bis[β -aminoethyl ether]-N,N,N',N'-tetra-acetic acid, 5% polyvinylpyrrolidone) and counted with a hemocytometer. The trypan blue dye exclusion method showed that viability was always greater than 95%.

Cells were also analyzed by flow cytometry. 5×10^5 cells were seeded in 25 cm² tissue culture dishes. Forty-eight hours later, the medium was changed to medium with or without hrIL-1 β (10 or 20 ng/ml). Forty-eight hours later, the cells were resuspended with the enzyme cocktail and fixed for 30 minutes in 2% cold paraformaldehyde. After washing with phosphate buffered saline (PBS), cells were permeabilized by 0.25% lysophosphatidyl choline (Sigma Chemical Co., St. Louis, Mo., U.S.A.) for 5 minutes. Permeabilized cells were then incubated for 45 minutes at 4°C with fluorescein isothiocyanate (FITC)-conjugated phalloidin (Sigma Chemical Co.) in the dark. The cells were rewashed and suspended in PBS for analysis by flow cytometer (Ortho

Cytoron; Ortho Diagnostic Systems K.K., Tokyo). Most experiments used 5×10^4 cells for analysis. To determine the changes in relative F-actin content due to polymerization of F-actin (stress fibers) commonly observed in normal cells,²³⁾ the mean channel intensities of hrIL-1 β -treated cells were compared with untreated cells.

Fluorescence microscopic analysis used glioma cells grown on slides and cultured with or without hrIL-1 β (10 or 20 ng/ml) for 48 hours. The slides were rinsed briefly in PBS and fixed, and cells permeabilized as above were then incubated for 45 minutes at 4°C with FITC-conjugated phalloidin in the dark. After rinsing in PBS, coverslips were mounted on slides and observed using an inverted microscope (Nikon, Tokyo) with epifluorescence optics.

Data are expressed as mean $\pm$ SD and compared, where appropriate, with an unpaired Student's *t*-test. The data presented are representative of experiments performed in triplicate or greater.

Results

Table 1 shows that hrIL-1 β at 10 or 20 ng/ml prolonged the doubling time of all human glioma cell lines. The 3 H-thymidine incorporation assay also demonstrated growth inhibition. Figure 1 shows that five cell lines (O1T, O9T, HBR9T#2, HBR84T, and HBR98T) demonstrated a maximum inhibition of 3 H-thymidine uptake with only 0.5 ng/ml of hrIL-1 β . One cell line (T98G) demonstrated dose-dependent inhibition of 3 H-thymidine uptake. Two cell lines (KC-PC and RBR17T) showed only slight inhibition even at hrIL-1 β concentrations of 16 ng/ml. The data for these two cell lines were almost identical, with growth inhibition percentages for O1T and HBR84T of 77.0 and 66.7%, respectively, causing marked changes in doubling time. In contrast, inhibition of KC-PC and RBR17T were 20.2 and 31.2%, respectively, which left the doubling time unchanged in 10 ng/ml of hrIL-1 β .

Flow cytometry demonstrated changes in relative

Table 1 Doubling time (hrs) of glioma cells cultured with or without hrIL-1 β

	T98G	KC-PC*	RBR17T* (15)	O1T (12)	O9T (9)	HBR9T#2* (13)	HBR84T (10)	HBR98T (14)
Untreated	24.1	15.9	33.2	84.3	80.6	21.6	28.6	31.9
hrIL-1 β -treated	25.9	18.5	39.3	141.1	121.1	23.8	49.1	50.5

Numerals in parentheses indicate passage nos. Values are means of triplicate assays. SD was always $\pm 10\%$. All times were significantly longer compared with untreated controls ($p < 0.05$). *These three cell lines were treated with 20 ng/ml of hrIL-1 β , as 10 ng/ml had no effect on cell growth.

Fig. 1 hrIL-1 β -mediated growth inhibition of eight glioma cell lines. Results are mean of four or six cultures. SD was always $\pm 10\%$. $\circ$: HBR84T, $\bullet$: HBR98T, $\odot$: HBR9T#2, $\triangle$: O1T, $\blacktriangle$: O9T, $\square$: KC-PC, $\blacksquare$: T98G, $\times$: RBR17T.

Fig. 2 F-actin histograms for O1T (left) and HBR84T (right). Profiles for untreated cells shown by dotted line and hrIL-1 β -treated cells by solid line.

F-actin content as mean channel intensity in the fluorescence histograms (Fig. 2). Mean channel intensities increased significantly compared with unexposed control cells except for KC-PC. Results reflected the growth inhibition assay; changes in O1T and HBR84T were prominent, but not in KC-PC and RBR17T (Table 2).

Fluorescence microscopy showed profound hrIL-1 β -induced alterations in cell morphology, especial-

ly in O1T and HBR84T. HBR84T appeared epithelioid with short or no processes, but transformed to a multipolar shape with numerous processes and more cell-cell contacts 24 hours after hrIL-1 β treatment. O1T had a polymorphic appearance, but became mainly bipolar (Fig. 3). Processes contained much polymerized F-actin and an increased number of stress fibers appeared in the cytoplasm.

Discussion

In 1987, Lachman *et al.*¹²⁾ reported that IL-1 promoted the growth of human astrocytoma U373 based on ^3H -thymidine incorporation assay. Kasahara *et al.*⁸⁾ later found that IL-1-mediated proliferation was inhibited by pretreatment with low concentrations of cholera toxin (< 10 ng/ml), and suggested that G protein-like molecules were involved in the early stages. They also demonstrated that Ca^{2+} mobilization and intracellular cyclic adenosine monophosphate level were not related to IL-1-mediated proliferation. IL-1 also acts as astrocyte growth factor both in developing and injured brains.⁷⁾ Clearly, IL-1 has no

Table 2 Flow cytometry analysis of F-actin content in glioma cells cultured with or without hrIL-1 β

	T98G	KC-PC*	RBR17T* (24)	O1T (17)	O9T (9)	HBR9T#2* (13)	HBR84T (19)	HBR98T (14)
Untreated	84.6 $\pm$ 0.2	62.6 $\pm$ 0.5	32.4 $\pm$ 0.7	74.7 $\pm$ 0.5	81.3 $\pm$ 1.4	72.3 $\pm$ 0.6	63.5 $\pm$ 1.0	65.1 $\pm$ 0.8
hrIL-1 β -treated	102.8 $\pm$ 0.5	62.1 $\pm$ 0.3	38.6 $\pm$ 0.2	86.7 $\pm$ 0.8	108.7 $\pm$ 0.6	82.6 $\pm$ 0.1	78.2 $\pm$ 0.7	84.1 $\pm$ 0.8

Numerals in parentheses indicate passage nos. F-actin contents are expressed by mean channel intensities, rounded off, and expressed as mean $\pm$ SD. Mean channel intensities were significantly greater compared with untreated controls ($p < 0.001$) except for KC-PC. *These three cell lines were treated with 20 ng/ml of hrIL-1 β , as 10 ng/ml had no effect on cell growth.

Fig. 3 A–D: Phase-contrast micrographs, showing morphological changes in O1T (A, B) and HBR84T (C, D). $\times 200$. E, F: F-actin distribution in O1T stained with FITC-conjugated phalloidin. $\times 200$. A, C, and E are untreated and B, D and F are hrIL-1 β -treated cells.

therapeutic value in patients with malignant glioma.

However, IL-1 has cytotoxic or cytostatic effects on other malignant cells. In 1987, Onozaki *et al.*²⁰⁾ reported growth inhibition (maximum, 50%) of mouse myeloid leukemic cell line (M1), possibly induced by terminal differentiation. In 1986, Lachman *et al.*¹³⁾ reported a cytotoxic effect on human melanoma cell line (A375). Endo *et al.*³⁾ suggested this cytotoxic effect to be related to a decrease in ornithine decarboxylase activity. In 1988, Masuda *et al.*¹⁶⁾ reported that mitochondrial manganese superoxide dismutase was induced by IL-1 in a human melanoma cell line (A325), which demon-

strates IL-1-mediated growth suppression. Mitochondrial manganese superoxide dismutase induction was also observed in fibroblast and peripheral monocytes, in which IL-1 promotes growth. These data suggest that this phenomenon is common to IL-1 responder cells.

These experimental data have encouraged clinical phase study in Japan.^{10,11)} Significant responses have been observed in malignant glioma patients, possibly due to systemically augmented host anti-tumor immune defense mechanisms.^{1,18)} Diffuse infiltration of leukocytes (CD45⁺), B cells, and natural killer cells (Leu7⁺) was observed in a biopsy specimen from a

malignant lymphoma patient treated with hrIL-1 β . Belardelli *et al.*¹¹⁾ reported a marked increase in survival time and inhibition of metastasis due to subcutaneous IL-1 β injection in mice with transplanted Friend leukemia cells. This response was suppressed by anti-T cell antibody. North *et al.*¹⁸⁾ also reported that the *in vivo* effect of IL-1 β did not occur in T cell-deficient mice.

Our study investigated the reasons for the discrepancy in *in vivo* and *in vitro* responses. The direct effect of hrIL-1 β on glioma cell proliferation showed all glioma cells to be inhibited. However, the extent of inhibition varied. KC-PC, RBR17T, and HBR9T#2 needed high concentrations (20 ng/ml) of hrIL-1 β before growth inhibition was induced, while O1T and HBR84T responded to low concentrations (0.5 ng/ml). The difference in response may be related to the number of hrIL-1 β receptors in these cells,³⁾ differences in intracellular second messenger, or heterogeneity in early passage.⁹⁾

The morphological changes involving numerous processes after hrIL-1 β treatment resembled the terminal differentiation of normal astrocytes induced by acidic fibroblast growth factor, which contains base sequences homologous to IL-1.²¹⁾ Polymerization of F-actin also occurred in hrIL-1 β -treated glioma cells. The mechanism of this growth inhibitory effect may be related to the differentiation of glioma cells. In 1990, Lichtor *et al.*¹⁴⁾ reported significant expression of messenger ribonucleic acid encoding for IL-1 β in a low-grade glioma but not in glioblastoma. They suggested that a decrease in IL-1 β may correlate with tumor progression. In 1991, Nitta *et al.*¹⁷⁾ reported tumor necrosis factor- α gene expression in five glioma cell lines after stimulation with 100 U/ml of IL-1 β . These phenomena may be also related to the growth inhibitory effect of IL-1 β .

This study showed that hrIL-1 β inhibits the growth of glioma cells *in vitro*, suggesting that hrIL-1 β has therapeutic value in glioma patients. A further study is required to investigate immunological modulation and changes in blood flow in the tumor when IL-1 is administered systemically, as little is known about its effects *in vivo*.^{1,2,18)}

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