Liposomal transfection of human γ-interferon gene into human glioma cells and adoptive immunotherapy using lymphokine-activated killer cells

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The authors evaluated the effect of liposomal transfection of human γ-interferon (HuIFN-γ) gene into human glioma cells and lymphokine-activated killer (LAK) cells, alone and in combination. An HuIFN-γ gene inserted in a eukaryotic expression vector was entrapped in liposomes bearing positive surface charges. Liposomal gene transfection induced production of HuIFN-γ and its secretion in culture medium of human glioma cell lines (SK-MG-1 and U-251 MG). At 4 days after transfection, the cells produced 10 to 50 U/ml of HuIFN-γ in the medium, whereby the major histocompatibility complex (MHC) class I and II antigens, as well as intercellular adhesion molecule-1 (ICAM-1), were induced on the glioma cell surface. The growth-inhibiting effect of transfection-induced HuIFN-γ was much stronger in comparison with control cultures exposed to 500 U/ml of exogenously added HuIFN-γ. In addition, 20% to 40% growth inhibition was obtained in the glioma cells when they were treated with LAK cells alone at a 5:1 ratio of effector to target cells. Liposomal transfection of HuIFN-γ gene into human glioma cells combined with immunotherapy using LAK cells was more effective than either technique alone. The reinforcement of growth inhibition in the case of combined therapy was quenched by anti-ICAM-1 monoclonal antibody, but not by anti-MHC class I or II monoclonal antibodies. These results suggest that the combined effect of liposomal transfection of HuIFN-γ gene plus LAK cells into human glioma cells is a potentially useful therapy for malignant glioma, and that the mechanisms of the reinforcement of growth inhibition are closely related to the expression of ICAM-1 on the glioma cell surface.

KEY WORDS • liposomal gene transfection • gamma-interferon • lymphokine-activated killer cell • intercellular adhesion molecule • glioma

Patients with malignant glioma have a poor prognosis despite the combined use of surgery, irradiation, chemotherapy, and a variety of immunotherapies. These patients are known to show decreased circulatory immune function and suppression of the tumor-specific immune response. In order to increase host immune function and inhibit tumor growth, many kinds of cytokines, such as interferons, tumor necrosis factors, and interleukins, have been used. In particular, intravenously or intrathecally administered human β-interferon (HuIFN-β) has been given to patients with malignant glioma since 1980 and its usefulness has been confirmed.3,10,14

Like HuIFN-β, human γ-interferon (HuIFN-γ) also has a variety of biological responses in both host and tumor cells. In brain tumors, HuIFN-γ has been reported to inhibit in vitro growth of glioma cell lines,15 to activate the specific cytotoxicity of lymphocytes in the presence of interleukin-2 (IL-2),1 and to increase the major histocompatibility complex (MHC) surface antigens and some kinds of cell adhesion molecules.8,9 The MHC antigens and cell adhesion molecules are essential in the control of immune responses functioning in the recognition of antigens by T lymphocytes.11 Alterations of the expression of MHC antigens or cell adhesion molecules may result in altered biological behaviors.

On the other hand, lymphokine-activated killer (LAK) cells generated by culturing peripheral blood lymphocytes with IL-2 can lyse a wide variety of tumor cells. Some investigators have reported that IFN-γ-potentiated LAK cell cytotoxicity,2,7,21 but this remains controversial.24,25 In the present experiments, we studied the growth-inhibiting effect of in vitro transfection of glioma cells with HuIFN-γ gene entrapped in the liposomes and also evaluated the usefulness of combining liposomal transfection and adoptive immunotherapy using LAK cells.
HuIFN-γ and LAK cells in human glioma

Materials and Methods

Cell Lines

Cells of the SK-MG-1 and U-251 MG human glioma cell lines were used in this study. The cells were maintained in Eagle’s minimum essential medium supplemented with 10% fetal calf serum, 2 mM nonessential amino acids, 5 mM L-glutamine, and antibiotic agents (streptomycin, 100 μg/ml, and penicillin, 100 U/ml).

Preparation of Liposome-Entrapped Plasmids

Liposome-entrapped plasmids were prepared by an improved procedure of the reverse-phase evaporation method. The liposomes were prepared using the positively-charged lipid N-(α-trimethylammonio-acetyl)-dioleoyl-D-glutamate chloride (TMAG), dilauroyl phosphatidylcholine (DLPC), and dioleoyl phosphatidyl-ethanolamine (DOPE), and were composed in a molar ratio of 1:2:2 as described in our previous papers. The genes were introduced into the SK-MG-1 cells by using a liposome-entrapped plasmid. This was constructed from HindIII-BglII, large fragments of pSV dihydrofolate reductase (pSVdhfr), HuIFN-γ complementary deoxyribonucleic acid (cDNA), and deoxyo-ligonucleotides.

Gene Transfection

Two milliliters of SK-MG-1 or U-251 MG cell suspensions was placed in 7.5 x 10⁶ cells/ml culture medium in each well of Falcon No. 3042 plates and incubated at 37°C for 24 hours in a humidified atmosphere of 5% CO₂ and 95% air. After the liposomes or exogenous HuIFN-γ (500 U/ml, titer of 1 x 10⁹ U/ml and specific activity of 3.0 x 10⁷ U/mg of protein) were added to the medium, the solution was incubated at 37°C for 16 hours. Then the medium containing the liposomes was replaced with fresh medium containing no liposomes while the medium containing exogenous HuIFN-γ was not replaced, and the incubation was continued for up to 80 hours. The culture medium was collected at 2, 4, 6, and 9 days after mixing with liposomes, and the level of HuIFN-γ in the medium was measured by radioimmunossay. A viable cell count was performed at the same time.

Detection of MHC Antigens and Intercellular Adhesion Molecule-1

Expression of MHC antigens was detected by immunohistochemical study. Cells grown on coverslips were fixed in cold acetone. Mouse anti-MHC class I and II (human leukocyte antigen (HLA)-DR) monoclonal antibodies were applied first for 1 hour, followed by a 30-minute incubation with anti-mouse immunoglobulin (IgG) monoclonal antibody conjugated with horseradish peroxidase. The enzymatic activity was revealed using 0.015% H₂O₂ and 0.4% diaminobenzidine as substrate. Expression of MHC antigens in the glioma cells was studied at 2, 4, 6, and 9 days after adding exogenous HuIFN-γ or liposome-entrapped pSVIFN-γ.

Expression of intercellular adhesion molecule-1 (ICAM-1) was measured using fluorescence-activated cell sorter analysis. The cultured cells (1 x 10⁵) were harvested and resuspended in 25 μl of 1:100 dilution of anti-ICAM-1 monoclonal antibody in the complete medium containing 10% fetal calf serum. The cells were incubated for 30 minutes on ice, washed with phosphate-buffered saline (PBS), then incubated for 30 minutes on ice with a 1:20 dilution of fluorescein isothiocyanate-labeled goat anti-mouse IgG monoclonal antibody. Thereafter, the cells were washed with PBS three times and resuspended in 0.5 ml PBS. Fluorescence was quantitated using the EPICS profile software.

Generation of LAK Cells

Peripheral blood lymphocytes were obtained from healthy allogeneic donors. Heparinized peripheral blood was diluted 1:1 with PBS, and lymphocytes were separated by Ficoll-Paque gradient centrifugation. The cells collected at the gradient interface, designated as peripheral blood lymphocytes, were washed three times with PBS, then resuspended in complete medium consisting of RPMI 1640 containing 10% fetal calf serum, 2 mM L-glutamine, antibiotic agents (streptomycin, 100 μg/ml, and penicillin, 100 U/ml), and IL-2 (10 U/ml). The peripheral blood lymphocytes were activated to generate LAK cells by Day 5 of incubation.

Combined Application of HuIFN-γ Gene Transfection and LAK Cells

At first, we applied either exogenous HuIFN-γ (500 U/ml) or liposome-entrapped pSVIFN-γ (15 nmol/ml of lipids; 0.6 μg/ml of DNA) to each well. For the former treatment, glioma cells were incubated for 4 days without medium changes; for the latter, the cells were incubated with liposomes for 16 hours and the medium was then replaced with fresh medium containing no liposomes, after which the incubation was continued for up to 80 hours. The culture medium in both treatments was collected 4 days after adding reagents, and the level of HuIFN-γ in the medium was measured. At the same time, the cells were counted and LAK cells were applied at a 5:1 ratio of effector to target cells. After an additional 2 days of incubation, the results of combined application were evaluated.

* TMAG obtained from Sogo Pharmaceutical Co., Ltd., Tokyo, Japan; DLPC supplied by Sigma Chemical Co., St. Louis, Missouri; DOPE obtained from Avanti Polar Lipids, Inc., Pelham, Alabama; pSVIFN-γ plasmid constructed by Toray Industries, Inc., Tokyo, Japan.
† Natural HuIFN-γ obtained from Ohtsuka Pharmaceutical Co., Ltd., Tokyo, Japan.
‡ Mouse anti-MHC and anti-mouse IgG monoclonal antibodies obtained from Dakopatts, Glostrup, Denmark.
§ Anti-ICAM-1 monoclonal antibody obtained from British Biotechnology Products, Inc., Oxford, England; goat anti-mouse IgG monoclonal antibody supplied by Medical and Biological Laboratories Co., Ltd., Nagoya, Japan.
¶ EPICS software obtained from Coulter Corp., Hialeah, Florida.
TABLE 1
Production of HuIFN-γ in cultured glioma cells transected with pSVIFN-γ*

<table>
<thead>
<tr>
<th>Agent</th>
<th>Human Glioma Cell Line</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SK-MG-1</td>
</tr>
<tr>
<td>untreated (control) cells</td>
<td>not detected</td>
</tr>
<tr>
<td>empty liposomes</td>
<td>not detected</td>
</tr>
<tr>
<td>liposome-entrapped pSVIFN-γ</td>
<td>41.3 ± 5.5 U/ml</td>
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</tbody>
</table>

* Data are taken from six experiments and expressed as mean ± standard deviation. HuIFN-γ = human γ-interferon; pSVIFN-γ = SV40-derived expression vector with HuIFN-γ gene insertion.

**Growth Inhibition**

Inhibition of gene transfection and/or LAK cell growth was evaluated by determining the number of viable cells, expressed as the number of trypan blue-excluding cells counted in a hemocytometer. The inhibition (% cytotoxicity) of gene transfection and/or LAK cell growth was determined with the following formula:

\[
\text{% cytotoxicity} = 1 - \frac{\text{number of cells in target well}}{\text{number of cells in control well}} \times 100.
\]

**Results**

Production of HuIFN-γ in Glioma Cells Transfected With pSVIFN-γ

When glioma cells were transfected with pSVIFN-γ by means of liposomes and incubated for 4 days, HuIFN-γ was detected via radioimmunoassay in the media of both SK-MG-1 and U-251 MG cells. The HuIFN-γ was detected at 2 days after transfection and reached a maximum after 4 days. Thereafter, it gradually decreased and was not detected at all by 9 days after transfection. The maximum level of HuIFN-γ in the medium was 41.3 ± 5.5 U/ml (mean ± standard deviation) in SK-MG-1 cells and 19.3 ± 6.1 U/ml in U-251 MG cells (Table 1). On the other hand, HuIFN-γ was not detected in the culture medium in either SK-MG-1 or U-251 MG cells when empty liposomes were added to the medium.

Expression of MHC Antigens or ICAM-1 on Glioma Cells

The MHC class I and II (HLA-DR) antigens were negative at the first passage of culture, but could be induced to express MHC antigens for 2 to 6 days after adding more than 50 U/ml exogenous HuIFN-γ. This was confirmed by immunohistochemical staining. Similarly, the antigens could be detected on glioma cell surfaces during the same time periods when liposome-entrapped pSVIFN-γ was added to the medium, even though the level of HuIFN-γ in the medium was less than 50 U/ml.

Expression of ICAM-1 was detected by fluorescence-activated cell sorter analysis. It was not expressed in either SK-MG-1 or U-251 MG cells when the cells were untreated. However, it was detected on glioma cell surfaces when liposome-entrapped pSVIFN-γ was added to the medium and the cells were incubated for 4 days (Fig. 1).

Growth Inhibition

Empty liposomes did not significantly suppress the growth of either SK-MG-1 or U-251 MG cells; however, liposome-entrapped pSVIFN-γ suppressed cell growth remarkably. Compared with the growth-inhibiting effect of exogenous HuIFN-γ (500 U/ml), the effect of gene transfection was obviously stronger and was cytotoxic (Fig. 2), although the level of HuIFN-γ in the culture medium was very low (Table 1).

The glioma cells were cultured for 4 days after adding exogenous HuIFN-γ or transfection of its gene. Four days after the reagents were added, the culture medium was collected and the number of viable cells counted. Thereafter, LAK cells were applied at an effector-to-target cell ratio of 5:1. After an additional 2 days of incubation, the growth-inhibiting effect was evaluated by determining the number of viable cells. As shown in Fig. 3, glioma cells transfected with pSVIFN-γ were suppressed by LAK cells more efficiently than those incubated with exogenously added HuIFN-γ. The combined therapy of HuIFN-γ gene transfection followed by adoptive immunotherapy using LAK cells resulted in a synergistic growth-inhibiting effect in both SK-MG-1 and U-251 MG cells. The reinforcement of growth inhibition in the case of the combined therapy was partially quenched by anti-ICAM-1 monoclonal antibody (Fig. 4), but not by anti-MHC class I or class II monoclonal antibody (data not shown).

**Discussion**

Recently, a number of biological response modifiers have emerged as potentially useful agents in patients with malignant glioma. In 1980, intravenous or intra- or intrathecal administration of IFN's was introduced for the treatment of malignant glioma following both in vitro and in vivo demonstrations of its efficacy. Interferons have a definite direct growth-inhibiting effect on glioma cells and modulate the expression of the cell surface antigens, including MHC antigens and cell adhesion molecules, thus providing a potential regulatory mechanism for local immune reactivity. Gamma-interferon in particular has very strong immunoreactivities.

In this study, we investigated the alteration of cell surface antigens and the reinforced effect of cytotoxicity of LAK cells when HuIFN-γ gene was transected into glioma cells, which then continuously produced HuIFN-γ. As a result, HuIFN-γ produced in the cells by its gene transfection induced MHC antigens and ICAM-1 on the glioma cell surface. Antigens of MHC class I and II are essential in the control of immune response. Although LAK cells are cytotoxic to a wide variety of tumor cells (so-called "non-MHC-restricted cytotoxicity"), some reports showed that target susceptibility to natural killer and LAK cytotoxicity was inversely correlated with the target expression of MHC antigens. The relationship of HuIFN-γ, MHC antigens, and LAK cells is very complicated and difficult.
HuIFN-γ and LAK cells in human glioma

**SK-MG-1**

**U251-MG**

![Graphs showing expression of intercellular adhesion molecule-1 (ICAM-1) on human glioma cell surfaces. Expression of ICAM-1 was detected at 4 days after transfection by fluorescence-activated cell sorter analysis. AU = arbitrary units. A: Untreated control cells. B: Cells treated with liposome-entrapped SV40-derived expression vector with human γ-interferon gene insertion (pSVIFN-γ).](image)

The adoptive immunotherapy reported by Rosenberg and colleagues\(^8^,\text{,}^9^,\text{,}^1\) was effective in patients with advanced cancer, but several studies have reported that adoptive immunotherapy is not satisfactory because LAK cells may become trapped in the reticular formation and thus cannot reach the target cells. However, in our patients with malignant glioma, it has been possible to administer liposomes and/or LAK cells into the tumor cavity repeatedly through an Ommaya reservoir placed at surgery. The procedure seems to ensure long-term expression of the HuIFN-γ gene product in the patient. Moreover, we are studying the enhancement of LAK cytotoxicity with bifunctional antibodies, which are composed of anti-CD3 monoclonal antibody to trace. Our data indicated that induction of MHC antigens on the glioma cell surface was not related to the cytotoxicity of LAK cells, because the reinforcement of LAK cytotoxicity that was induced by HuIFN-γ gene transfection into the glioma cells was not neutralized by anti-MHC class I or class II monoclonal antibodies. On the other hand, ICAM-1, which belongs in the immunoglobulin superfamily, seems to be an important agent because the reinforcement of LAK cytotoxicity was partially canceled by anti-ICAM-1 monoclonal antibody.

**Fig. 2.** Graphs showing growth inhibition of human glioma cells by exogenous human γ-interferon (HuIFN-γ) or transfection of its gene. Open circles = untreated control cells; closed circles = empty liposomes (15 nmol/ml lipids); triangles = exogenous HuIFN-γ (500 U/ml); and squares = liposome-entrapped SV40-derived expression vector with HuIFN-γ gene insertion (pSVIFN-γ) (15 nmol/ml lipids, 0.6 µg/ml DNA). Each symbol represents the mean of six experiments. In both SK-MG-1 and U-251 MG cells, the growth-inhibiting effect of HuIFN-γ gene transfection was much stronger than that of 500 U/ml exogenous HuIFN-γ, to the extent of being cytoidal.

**Fig. 3.** Graphs showing cytotoxic activity of lymphokine-activated killer (LAK) cells against human glioma cells: A = LAK cells alone; B = exogenous human γ-interferon (HuIFN-γ, 500 U/ml) plus LAK cells; C = HuIFN-γ gene transfection plus LAK cells. The growth-inhibiting effect was enhanced when LAK cells were applied 4 days after the addition of 500 U/ml exogenous HuIFN-γ or transfection of its gene. The effect was synergistic in the case of HuIFN-γ gene transfection. Data are presented as the mean ± standard deviation of six experiments.
Fig. 4. Graphs showing the effect of anti-intercellular adhesion molecule-1 (ICAM-1) monoclonal antibody on lymphokine-activated killer (LAK) cell cytotoxicity against human glioma cells: A = LAK cells alone; B = exogenous human γ-interferon (HuIFN-γ, 500 U/ml) plus LAK cells; C = HuIFN-γ gene transfection plus LAK cells. Shaded columns represent no anti-ICAM-1 monoclonal antibody; open columns represent the application of anti-ICAM-1 monoclonal antibody (5 µg/ml). Data are presented as the mean ± standard deviation of six experiments. Statistical significance: * = p < 0.01; ** = p < 0.001.

and anti-G-22 monoclonal antibody that reacts specifically with a surface antigen (G-22) of human glioma. We have confirmed an increase in affinity of gene-transfected glioma cells and LAK cells using the bifunctional antibodies; details will be reported at a later date. These results suggest that combining liposomal transfection of HuIFN-γ gene into human glioma cells with the addition of LAK cells is a potentially useful therapy for malignant glioma.

References


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