Jpn. J. Cancer Res. 86, 95–100, January 1995

Reinforced Cytotoxicity of Lymphokine-activated Killer Cells toward Glioma Cells by Transfection of the Killer Cells with the γ-Interferon Gene

Masaaki Mizuno, Jun Yoshida,[1] Toru Takaoka and Kenichiro Sugita

Department of Neurosurgery, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466

Lymphokine-activated killer (LAK) cells generated from peripheral blood lymphocytes incubated with recombinant interleukin-2 were transfected with the human γ-interferon (HuIFN-γ) gene by means of liposomes having a positive charge on their surface. The cells secreted significant amounts of HuIFN-γ (reaching more than 5 U/ml) into the culture medium. The HuIFN-γ produced by the cells induced intercellular adhesion molecule-1 (ICAM-1) and enhanced the expression of Fas antigen on the surface of human glioma cells. Also, LAK cells transfected with HuIFN-γ gene exhibited reinforcement of cytotoxicity toward human glioma cell lines (U251-MG and SK-MG-1). Furthermore, the reinforcement was significantly quenched by anti-ICAM-1 and/or anti-TNF-α monoclonal antibody.

Key words: ICAM-1 — γ-Interferon — Gene transfection — Lymphokine-activated killer cell — Glioma

Lymphokine-activated killer (LAK) cells generated from peripheral lymphocytes with interleukin-2 (IL-2) are known to have a selective cytotoxic effect against malignant neoplastic cells. For induction of these antitumor effects in LAK cells, adhesion reactions between LAK cells and their target cells are required. Adhesion is mediated by a multiple receptor-ligand system involving four families of related proteins; the immunoglobulin superfamily, the integrin family, the cadherin family, and the selectins. In the central nervous system, intercellular adhesion molecule-1 (ICAM-1), which is a cell-surface glycoprotein that belongs to the immunoglobulin superfamily, is most closely related to two adhesion proteins; neural cell adhesion molecule (NCAM)[1] and myelin-associated glycoprotein (MAG).[2] ICAM-1 is found on various cells including leucocytes, fibroblasts, endothelial and epithelial cells; and it is the adhesive ligand for lymphocyte function-associated antigen-1 (LFA-1).[3–5] ICAM-1 expression can be upregulated by pro-inflammatory cytokines such as interleukin-1 (IL-1), γ-interferon (IFN-γ), and tumor necrosis factor-α (TNF-α).[6,7] Recently it was reported that IFN-γ or TNF-α can induce and enhance ICAM-1 expression on the surface of human glia or glioma cells.[8,9]

Here, we examined the expression or alteration of surface antigen on human glioma cells and investigated whether the cytotoxicity of LAK cells against human glioma cells would be augmented, when human IFN-γ (HuIFN-γ) gene was transfected into LAK cells and HuIFN-γ was continuously produced by the cells.

[1] To whom requests for reprints should be addressed.

MATERIALS AND METHODS

Glioma cell lines Human glioma cell lines, U251-MG and SK-MG-1, were used. The cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum, 2 m*M* non-essential amino acids, 5 m*M* L-glutamine, and antibiotics (streptomycin, 100 μg/ml; penicillin, 100 U/ml).

Lipids and reagents Positively charged lipid, N-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride (TMAG), was purchased from Sogo Pharmaceutical Co., Ltd., Tokyo; dilauroyl phosphatidylcholine (DLPC), from Sigma Chemical Co., St. Louis, MO; and dioleoyl phosphatidylethanolamine (DOPE), from Avanti Polar Lipids, Inc., Pelham, AL.

Recombinant IL-2 was provided from Takeda Pharmaceutical Co., Ltd., Osaka. Natural HuIFN-γ was provided by Ohtsuka Pharmaceutical Co., Ltd., Tokyo. It had a specific activity of 5.0×10^6 U/mg protein. G418 (Geneticin) was purchased from GIBCO Laboratories, Grand Island, New York.

Plasmids In this study, we used two different plasmids, pSVIFN-γ and pSV2neo. pSVIFN-γ is an SV40-derived expression vector containing the HuIFN-γ gene. This plasmid was constructed by Toray Industries, Inc., Tokyo. The other plasmid, pSV2neo, was used for the selection of transfectants; it has an SV40 promoter and the gene of neomycin phosphotransferase, an intracellular enzyme that inactivates G418.

Antibodies An anti-ICAM-1 monoclonal antibody was purchased from British Bio-technology Products, Ltd., Oxford, UK. An anti-Fas monoclonal antibody and FITC-labeled goat anti-mouse IgG monoclonal antibody

were purchased from Medical and Biological Laboratories Co., Ltd., Nagoya. An anti-TNF-α monoclonal antibody was provided by Asahi Chemical Industry Co., Ltd., Tokyo. One microgram of the antibody can quench 100 U of TNF-α. Neither anti-ICAM-1 nor anti-TNF-α monoclonal antibody suppressed the growth of glioma cells.

Preparation of liposomes with entrapped plasmids Liposomes with entrapped plasmids were prepared by an improved version[10] of the reverse-phase evaporation method as described in our previous papers.[11, 12] The liposomes were composed of TMAG:DLPC:DOPE in a molar ratio of 1:2:2.

Generation of LAK cells Peripheral blood lymphocytes (PBLs) were obtained from healthy allogeneic donors. Heparinized peripheral blood was diluted with phosphate-buffered saline (PBS) and PBLs were separated by Ficoll-Paque gradient centrifugation. The PBLs were washed three times with PBS and finally resuspended in complete RPMI1640 medium containing 10% fetal calf serum, 2 m*M* L-glutamine, antibiotics (streptomycin, 100 μg/ml; penicillin, 100 U/ml), and 10 U/ml of recombinant human IL-2. For this experiment, we used LAK cells from 5-day cultures.

Gene transfection into LAK cells HuIFN-γ gene and neomycin-resistance gene (or the latter only) were transfected into LAK cells by means of liposomes. At first, liposomes with entrapped pSV2neo only or with both plasmids (pSVIFN-γ and pSV2neo) were prepared to obtain neo-LAK cells (LAK cells transfected with pSV2neo) or IFN-γ-neo-LAK cells (LAK cells transfected with both pSVIFN-γ and pSV2neo). The concentration of liposomes was adjusted to 15 nmol/ml of lipids (0.3 μg/ml of DNA) in the RPMI1640 medium. LAK cells were incubated with liposomes of each of the above types at 37°C for 48 h in a humidified atmosphere of 5% CO_2 and 95% air. Thereafter, neo-LAK and IFN-γ-neo-LAK cells were selected for 14 days in RPMI 1640 medium containing 400 μg/ml of G418 and 10 U/ml of recombinant IL-2.

Determination of HuIFN-γ For the detection of HuIFN-γ secreted into the medium, we used the Centocor gamma interferon radioimmunoassay kit purchased from Centocor, Inc., Malvern, PA.

Detection of surface antigens on human glioma cells by fluorescence-activated cell sorter (FACS) analysis To measure cell-surface expression of ICAM-1 and Fas antigen on human glioma cells, the cells were removed from the culture plates. The cells (1×10^5) were resuspended in 25 μl of a 1:100 dilution of anti-ICAM-1 monoclonal antibody or 1:50 dilution of anti-Fas monoclonal antibody in the complete medium containing 10% fetal calf serum. Incubation was for 30 min on ice and the cells were washed with PBS, and then incubated with a 1:20 dilution of FITC-labeled goat anti-mouse IgG monoclonal antibody for 30 min on ice. Thereafter, the cells were washed with PBS three times and resuspended in 0.5 ml of PBS. Fluorescence was quantitated using an Epics profile (Coulter Corporation).

Cytotoxicity (6 h) toward glioma cells Cytotoxicity (6 h) was determined by means of the standard ^{51}Cr release assay using U251-MG or SK-MG-1 as target cells. They were labeled with 3.4 MBq ^{51}Cr for 1 h at 37°C, and washed three times before use. For the assay, 5×10^3 targets and 25×10^3 effectors (effector/target (E/T) ratio of 5/1) were placed in 96-well microplates (Falcon, #3075). Supernatants were harvested after a 6 h incubation and counted in a gamma counter. The value of % cytotoxicity was calculated by using the following equation:

$$\%\ \text{cytotoxicity (6 h)} = (\text{experimental counts} - \text{spontaneous counts})/(\text{total counts} - \text{spontaneous counts}) \times 100$$

Cytotoxicity (96 h) toward glioma cells U251-MG and SK-MG-1 glioma cells (2×10^4/ml) were inoculated in 24-well plates (Falcon, #3047) and incubated for 24 h. Then neo-LAK and IFN-γ-neo-LAK cells were applied to cultured glioma cells at the E/T ratio of 5/1. After 96 h of incubation, the number of trypan blue-excluding cells was counted in a hemocytometer, and the value of % cytotoxicity was calculated from the following equation:

$$\%\ \text{cytotoxicity} = 1 - (\text{number of live target cells / total number of target cells}) \times 100$$

Effect of anti-ICAM-1 and/or anti-TNF-α monoclonal antibodies U251-MG and SK-MG-1 glioma cells were incubated for 24 h in 24-well plates (Falcon, #3047). neo-LAK and IFN-γ-neo-LAK cells were applied to cultured glioma cells at the E/T ratio of 5/1 and simultaneously anti-ICAM-1 (10 μg/ml) and/or anti-TNF-α (5 μg/ml) monoclonal antibodies were also added. After 96 h of incubation, the viable cells were counted by trypan blue staining. The value of % cytotoxicity was calculated by using the same equation as mentioned above.

RESULTS

Production of HuIFN-γ in LAK cells transfected with its gene In order to confirm the production of HuIFN-γ in LAK cells transfected with its gene (IFN-γ-neo-LAK cells), we measured HuIFN-γ secreted into the culture medium. After 96 h of incubation, the level of HuIFN-γ in the medium was 5.21 ± 0.48 (mean$\pm$SD) U/ml, which was significantly higher than the level in the medium of neo-LAK cells, measured simultaneously (1.38 ± 0.20 U/ml) (Table I). The levels presented in Table I were the maximum ones.

Alteration of surface antigens on human glioma cells by HuIFN-γ produced by LAK cells transfected with its gene (IFN-γ-neo-LAK cells) The alteration of surface antigens on human glioma cells was analyzed by FACS, when HuIFN-γ produced by IFN-γ-neo-LAK cells was added to cultured glioma cells. As surface antigens, ICAM-1 and Fas antigen were examined. ICAM-1 was not expressed spontaneously on the surface of human glioma cells, U251-MG and SK-MG-1. However, when the cells were treated with HuIFN-γ produced by IFN-γ-neo-LAK cells, ICAM-1 was induced on the surface of the cells. The minimum dose of HuIFN-γ, which was able to induce ICAM-1 on the surface of human glioma cells, was 2 U/ml. ICAM-1 was not detected until 6 h after the cytokine treatment, but thereafter it was detected and then reached its maximum after 72 h. Fig. 1A showed ICAM-1 expression in the case of U251-MG cells. On the other hand, Fas antigen was expressed on the surface of glioma cells even when the cells were completely untreated (Fig. 1B). When the cells were treated with HuIFN-γ (5 U/ml) produced by IFN-γ-neo-LAK cells, the expression of Fas antigen was markedly enhanced.

Table I. Level of HuIFN-γ in the Medium

	96 h	
PBLs	<0.1	
LAK	1.24±0.23	
neo-LAK	1.38±0.20	*
IFN-γ-neo-LAK	5.21±0.48	*

The cells were incubated for 96 h and then the amount of HuIFN-γ in the medium was measured by radioimmunoassay. The level of HuIFN-γ is expressed in terms of U/ml medium. n=6. Significant difference between the indicated values: *; $P<0.01$.

Cytotoxicity toward glioma cells As shown in Table II, the values of % cytotoxicity (6 h) of IFN-γ-neo-LAK cells toward U251-MG and SK-MG-1 glioma cells were 35.9% and 29.2%, respectively, which represented a slight increase in cytotoxicity as compared with that of neo-LAK cells (20.3% and 16.1% in U251-MG and SK-MG-1 cells, respectively). The values of cytotoxicity of IFN-γ-neo-LAK cells toward glioma cells at 96 h of incubation (80.3% and 92.4% for U251-MG and SK-MG-1 cells, respectively) were much greater than those at 6 h of incubation (35.9% and 29.2% for U251-MG

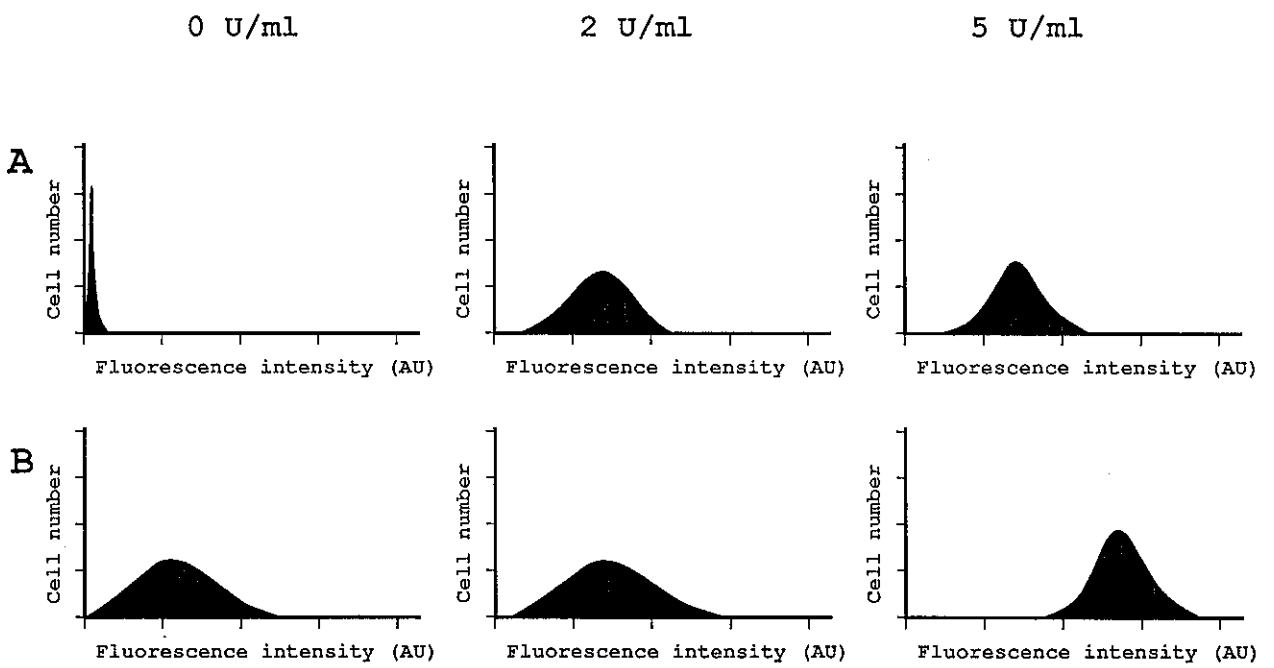

Fig. 1. Expression of ICAM-1 (A) and Fas antigen (B) induced on human glioma cells (U251-MG) by HuIFN-γ produced by LAK cells transfected with HuIFN-γ gene (72 h incubation). Cells were grown for 72 h in the medium containing the appropriate amount of HuIFN-γ (0, 2, and 5 U/ml). All cells were treated with mouse anti-ICAM-1 or anti-Fas monoclonal antibody followed by FITC-labeled goat anti-mouse IgG monoclonal antibody. The percentage of positive cells and fluorescence intensity were measured by flow cytometry.

Table II. Growth Inhibition of Glioma Cells by IFN-γ-neo-LAK Cells

	% Cytotoxicity	
	6 h	96 h
A: U251-MG cells		
PBLs	0	0 (6.7)[a]
LAK	19.5±2.8	19.9±3.4 (5.4)
neo-LAK	20.3±3.3	23.1±4.1 (5.2)
IFN-γ-neo-LAK	35.9±2.4	80.3±6.8 (1.3)
B: SK-MG-1 cells		
PBLs	0	0 (6.2)[a]
LAK	15.9±2.2	20.8±1.9 (4.9)
neo-LAK	16.1±3.6	23.7±3.9 (4.7)
IFN-γ-neo-LAK	29.2±2.9	92.4±7.2 (0.5)

The cells were incubated with human glioma cells for 6 or 96 h, and then the growth inhibition rates were evaluated by ^{51}Cr-release assay (6 h) or by counting of the viable cells in a hemocytometer (96 h). The values are given as percentage growth inhibition. n=6.
a) Mean of 6 experiments expressed as cell number $\times 10^{-4}$.

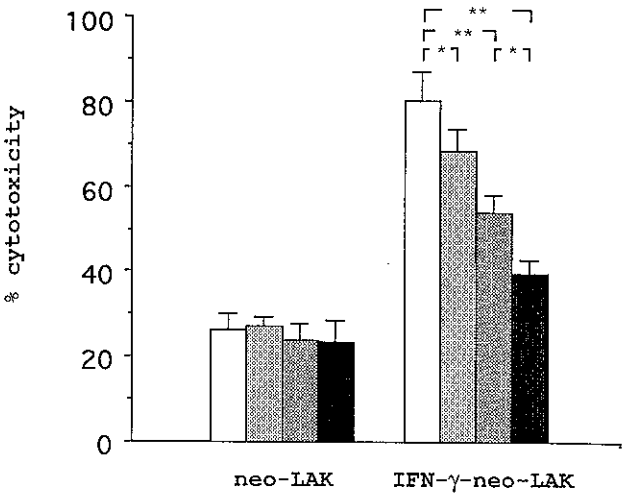

Fig. 2. Combined effect of anti-ICAM-1 and anti-TNF-α monoclonal antibodies. Human glioma cells (U251-MG) and neo-LAK or IFN-γ-neo-LAK cells were incubated for 96 h with anti-ICAM-1 (10 μg/ml) and/or anti-TNF-α (5 μg/ml) monoclonal antibodies, and growth inhibition was evaluated by counting the viable cells in a hemocytometer. □; control (normal mouse IgG, 10 μg/ml), ▨; anti-TNF-α monoclonal antibody (5 μg/ml), ▩; anti-ICAM-1 monoclonal antibody (10 μg/ml), and ■; anti-TNF-α (5 μg/ml) and anti-ICAM-1 (10 μg/ml) monoclonal antibodies. The values of mean and SD of 6 experiments are given. Similar results were obtained in the case of SK-MG-1 cells. Significant difference between the indicated values: *; $P<0.01$; **; $P<0.001$.

and SK-MG-1 cells, respectively) (Table II). On the other hand, the cytotoxicity of neo-LAK cells was almost the same as that at 6 h of incubation.

Effect of anti-ICAM-1 and/or anti-TNF-α monoclonal antibodies A relationship between ICAM-1 expression and the reinforcement of cytotoxicity in LAK cells transfected with HuIFN-γ gene was confirmed by experiments using the anti-ICAM-1 monoclonal antibody. When the anti-ICAM-1 monoclonal antibody (10 μg/ml) was added to the medium, the reinforcement of cytotoxicity of LAK cells transfected with HuIFN-γ gene was cancelled significantly, but not completely (Fig. 2). On the other hand, the reinforcement of cytotoxicity of LAK cells transfected with HuIFN-γ gene (IFN-γ-neo-LAK cells) was cancelled to a much greater degree than by the addition of anti-ICAM-1 monoclonal antibody alone when both anti-ICAM-1 and anti-TNF-α monoclonal antibodies were added to the medium (Fig. 2). In contrast, no effect was observed in the case of neo-LAK cells (Fig. 2).

DISCUSSION

LAK cells can lyse a wide variety of neoplastic cells, but the molecular basis of target recognition by LAK cells is unknown. Recently it was reported that cell adhesion molecules were strongly related to the target recognition by LAK cells.[13, 14] In particular, the regulation of ICAM-1 seems to be an important issue, because there is recent evidence for a correlation between ICAM-1 expression and the cytotoxicity of LAK cells. Here, we studied whether ICAM-1 was induced on the surface of human glioma cells as target cells, and whether the cytotoxicity of LAK cells against human glioma cells was increased by transfecting HuIFN-γ gene into the LAK cells. LAK cells transfected with HuIFN-γ gene (IFN-γ-neo-LAK cells) continuously secreted HuIFN-γ into the medium and the produced HuIFN-γ induced the continuous expression of ICAM-1 on the surface of human glioma cells. Its expression was not detected until 6 h after HuIFN-γ produced by IFN-γ-neo-LAK cells was added to glioma cells, but thereafter it gradually increased up to 72 h and was then expressed continuously for at least 4 days. IFN-γ-neo-LAK cells exhibited more potent cytotoxic effects against human glioma cells than did non-transfected LAK cells or neo-LAK cells, especially at 96 h incubation. Also, the reinforcement of the cytotoxic effect in IFN-γ-neo-LAK cells was significantly, but not completely, quenched by anti-ICAM-1 monoclonal antibody. This fact indicated that the reinforcement of the cytotoxic effect in IFN-γ-neo-LAK cells was induced by not only the induction of ICAM-1 but also another mechanism. We thought that one possibility might be the enhancement of Fas antigen which was observed in this experiment. It has been reported that the

surface density of Fas antigen is enhanced by IFN-γ or TNF-α in normal and neoplastic cells[15] and that APO-1 (Fas antigen) is coregulated with ICAM-1 in some kinds of cells.[16] In the present study, we observed the same phenomenon in human glioma cells. In general, the induction/enhancement of ICAM-1 and/or Fas antigen by cytokines such as IFN-γ or TNF-α is transient and they rapidly decrease and disappear if cytokines are removed. Also, *in vivo*, many cytokines act at localized sites and are rapidly degraded after signal transduction. Accordingly, it is very difficult to maintain constant levels of cytokines at particular sites. However, it may become possible to induce/enhance the continuous expression of ICAM-1 and/or Fas antigen even *in vivo*, because IFN-γ-neo-LAK cells have the potential to produce more than 5 U/ml of HuIFN-γ continuously. This increases the cytotoxicity of IFN-γ-neo-LAK cells.

On the other hand, we reported in the previous paper that the major mechanism of the reinforcement of cytotoxicity in LAK cells transfected with TNF-α gene may depend on transmembrane-formed TNF-α (cell-associated TNF-α) expressed on the cell surface.[17] We could not detect transmembrane-formed TNF-α on the surface of LAK cells transfected with HuIFN-γ gene. However, it was thought that a small amount of TNF-α might be secreted into the medium and play an important role in the reinforcement of cytotoxicity in IFN-γ-neo-LAK cells, because the cytotoxicity of the cells was partially quenched by anti-TNF-α monoclonal antibody. Generally, activated lymphocytes produce several kinds of cytokines, such as IFN-γ, TNF-α, and IL-1, which would enhance the cytotoxicity by different mechanisms from each other. The combination of TNF-α and IFN-γ is known to inhibit synergistically the growth of neoplastic cells.[18–20] We speculate that a part of the enhanced cytotoxicity observed in IFN-γ-neo-LAK cells may be ascribed to the synergistic growth-inhibitory effect of the combination of TNF-α and IFN-γ produced by IFN-γ-neo-LAK cells. Modified adoptive immunotherapy using LAK cells transfected with HuIFN-γ gene may be very useful as a local therapy for patients with malignant glioma. However, further investigation of the cytotoxic mechanisms of LAK cells is needed first.

(Received July 15, 1994/Accepted October 12, 1994)

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