Jpn. J. Cancer Res. 85, 750–755, July 1994

Transfection-induced Tumor Necrosis Factor-α Increases the Susceptibility of Human Glioma Cells to Lysis by Lymphokine-activated Killer Cells: Continuous Expression of Intercellular Adhesion Molecule-1 on the Glioma Cells

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To develop more effective adoptive immunotherapy, we transfected the human tumor necrosis factor-α (TNF-α) gene into human glioma cells (U251-SP), which were used as target cells. TNF-α is known to increase both the expression of intercellular adhesion molecule-1 (ICAM-1) on the surface of glioma cells and the susceptibility of glioma cells to lymphokine-activated killer (LAK) cell cytolysis. We compared the expression of ICAM-1 induced by TNF-α generated by the TNF-α gene-transfected cells with that induced by exogenously added TNF-α. When the TNF-α gene was transfected into U251-SP cells, the expression of ICAM-1 was detected on the cell surface from 3 days after the transfection and continued until at least 9 days. In contrast, it was expressed only transiently in the case of exogenously added TNF-α. Also, the cytolytic activity of LAK cells induced by transfection-induced TNF-α was significantly stronger than that induced by exogenously added TNF-α. The increased susceptibility was quenched by anti-ICAM-1 monoclonal antibody. These data indicated that continuous expression of ICAM-1 induced by TNF-α gene transfection of glioma cells resulted in higher cytolytic activity of LAK cells.

Key words: Lymphokine-activated killer cell — Gene transfection — TNF-α — ICAM-1 — Glioma

The central nervous system has a unique environment, in that the brain parenchyma is devoid of lymphatics. The possibility that lymphocyte infiltration of brain tumors represents an insufficient or aborted immune response has been pointed out by several investigators.[1,2] Furthermore, gliomas, particularly of the highest grade, glioblastoma multiforme, secret potent immunosuppressive factors, for example, transforming growth factor-β (TGF-β).[3,4] This environment makes the effective immunotherapy of brain tumors difficult. In fact, clinical experience with biological response modifiers (BRMs) such as interferon (IFN),[5] tumor necrosis factor (TNF),[6] and monoclonal antibodies,[7] and immune cells involving lymphokine-activated killer (LAK) cells[8,9] and tumor-infiltrating lymphocytes (TILs)[10] with antitumor activity, has been unfavorable. Recently we and several other investigators have developed new approaches to modified adoptive immunotherapy of brain tumors.[11,12] For effective immunotherapy, the interaction requires the recognition of specific or non-specific antigens to be coordinated with increased cell-cell adhesion. TNF-α and IFN-γ produced *in vitro* by activated lymphocytes increase the expression of intercellular adhesion molecule-1 (ICAM-1) on the surface of glioma cells.[13] ICAM-1 has recently been shown to be a natural ligand for lymphocyte function-associated antigen-1 (LFA-1), a leucocyte surface antigen.[14,15] Both ICAM-1 and LFA-1 are thought to participate in LAK cell-mediated cytotoxicity. However, in *in vitro* experiments, the expression of ICAM-1 induced by cytokines such as TNF-α and IFN-γ, rapidly disappears if they are removed from the medium. It may disappear even faster *in vivo* than *in vitro*, considering that the half life of TNF-α in blood is as short as about 20 min.[16] The situation may be similar intrathecally. It is important to maintain an effective concentration of the cytokines continuously for the success of immunotherapy. In this study, we examined whether the expression of ICAM-1 on the surface of glioma cells can be maintained for a long period by TNF-α gene transfection into glioma cells. We also investigated the alteration of the susceptibility of glioma cells transfected with the TNF-α gene to LAK cell cytolysis.

MATERIALS AND METHODS

Glioma cell lines Cells of a U251-SP human glioma cell line were used. The cells were cultured 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 and penicillin, 100 U/ml).

Plasmid and cytokines pcDVTNF-α, a human TNF-α gene inserted into an SV40-derived expression vector, was used. This plasmid was constructed by Asahi Chemical Industry Co., Ltd., Tokyo. Recombinant human TNF-α (specific activity, 2.2×10^6 U/mg protein) was

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obtained from Asahi Chemical Industry Co., Ltd. Recombinant interleukin-2 (IL-2) was obtained from Takeda Pharmaceutical Co., Ltd., Osaka.

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. Anti-ICAM-1 antibody was purchased from British Biotechnology Products Ltd., Oxford, UK.

Preparation of liposomes with entrapped pcDVTNF-α An improved procedure[17, 18] of the reverse-phase evaporation method[19] was used to entrap plasmids in liposomes. The liposomes were composed of TMAG:DLPC:DOPE in a molar ratio of 1:2:2. Empty liposomes did not significantly suppress the growth of glioma cells.

Generation of LAK cells Heparinized blood was obtained from healthy allogeneic donors. Mononuclear cells were separated by standard Ficoll-Hypaque gradient centrifugation. Cells collected at the gradient interface were washed twice in phosphate-buffered saline (PBS) and finally resuspended in RPMI 1640 medium containing 10% fetal calf serum, 2 m*M* L-glutamine, antibiotics (streptomycin, 100 μg/ml and penicillin, 100 U/ml) and 10 U/ml of recombinant human IL-2. For this experiment, we used LAK cells after culture for 5 days.

Determination of the amount of TNF-α The amount of TNF-α in the medium was measured by an enzyme-linked immunoassay with a lower detection limit of 0.2 U/ml.[20]

Flow-cytometric analysis Target cells were harvested with 0.05% EDTA and aliquoted at a concentration of 1×10^6 cells/tube. The cells were resuspended in 100 μl of a 1:100 dilution of anti-ICAM-1 monoclonal antibody in the complete medium containing 10% fetal calf serum. After incubation for 45 min at 4°C, the cells were washed with PBS twice and were resuspended in 20 μl of a 1:32 dilution of FITC-conjugated goat anti-mouse IgG monoclonal antibody (Medical and Biological Laboratories Co., Ltd., Nagoya). After incubation for 45 min at 4°C, the cells were washed with PBS twice and were resuspended in 1 ml of PBS again. The samples were analyzed immediately on a flow cytometer (EPICS Profile, Coulter Corporation).

Lytic activity Two milliliters of glioma cell suspension in culture medium (15×10^4/2 ml) was placed in each well of a 6-well plate (#3046; Falcon, NJ) and incubated at 37°C for 24 h in a humidified atmosphere of 5% CO_2 and 95% air. Then our original liposomes (15 nmol/ml of lipid; 0.3 μg/ml of DNA) or 40 U/ml of exogenous TNF-α was added to the culture medium and incubation was continued at 37°C for 24 h. The medium was replaced with fresh medium, and the cells were further incubated for a definite time. The treated cells were harvested with 0.05% EDTA. The cells were pelleted by centrifugation and the pellet was resuspended in 500 μl of medium. For a pellet of 5×10^6 cells, 37 MBq/100 μl of ^{51}Cr was added and the mixture was incubated at 37°C for 45 min. After having been washed with medium three times, the cells (5×10^4/100 μl medium) were added to each well of a U-bottomed 96-well plate (#25850, Corning). Thereafter the effector cells (LAK cells) were added at 20:1 effector/target (E/T) ratio, and the plates were incubated for 4 h at 37°C. After the incubation, supernatant (100 μl) was collected from each well and counted in a gamma counter.

Specific lysis was calculated according to the following formula:

$$\%\ \text{cytotoxicity} = (\text{experimental cpm} - \text{spontaneous cpm})/(\text{total cpm} - \text{spontaneous cpm}) \times 100.$$

Effect of anti-ICAM-1 monoclonal antibody on the increase of susceptibility to LAK cell cytolysis induced by TNF-α ^{51}Cr-labeled glioma cells were treated with exogenous TNF-α or liposomes with entrapped pcDVTNF-α for a definite time, then incubated with anti-ICAM-1 monoclonal antibody (final concentration, 10 μg/ml) for 30 min at room temperature. These cells were washed with PBS three times, then effector cells (LAK cells) were added to each well at 20:1 E/T ratio and the % cytotoxicity was calculated as mentioned above.

RESULTS

Detection of TNF-α in the medium The human glioma cells, U251-SP, were cultured for 24 h in the presence of 40 U/ml of exogenous TNF-α and thereafter the medium

Table I. Levels of TNF-α Detected in the Medium

	Days			
	1	3	5	9
Exogenous TNF-α	37.5±2.2	<0.2	<0.2	<0.2
Liposome-entrapped pcDVTNF-α	<0.2	42.3±3.1	44.6±3.9	38.7±2.9

The values are means and SD of 3 experiments and represent level of TNF-α in the medium (U/ml).

was replaced with fresh medium. After the medium exchange, TNF-α was not detected in the medium. In contrast, when the cells were similarly cultured in the presence of liposomes with entrapped pcDVTNF-α and thereafter the medium was replaced with fresh medium, transfection-induced TNF-α, which was produced by the cells transfected with pcDVTNF-α, was detected continuously in the medium up to 9 days after the medium exchange (Table I). The expression of TNF-α reached a peak 5 days after the medium exchange and decreased gradually thereafter.

Flow-cytometric analysis of ICAM-1 expression We examined the effect of exogenous and/or transfection-induced TNF-α on the expression of ICAM-1 in U251-SP cells. The results are shown in Fig. 1. Untreated cells did not express ICAM-1 on their surface. On the other

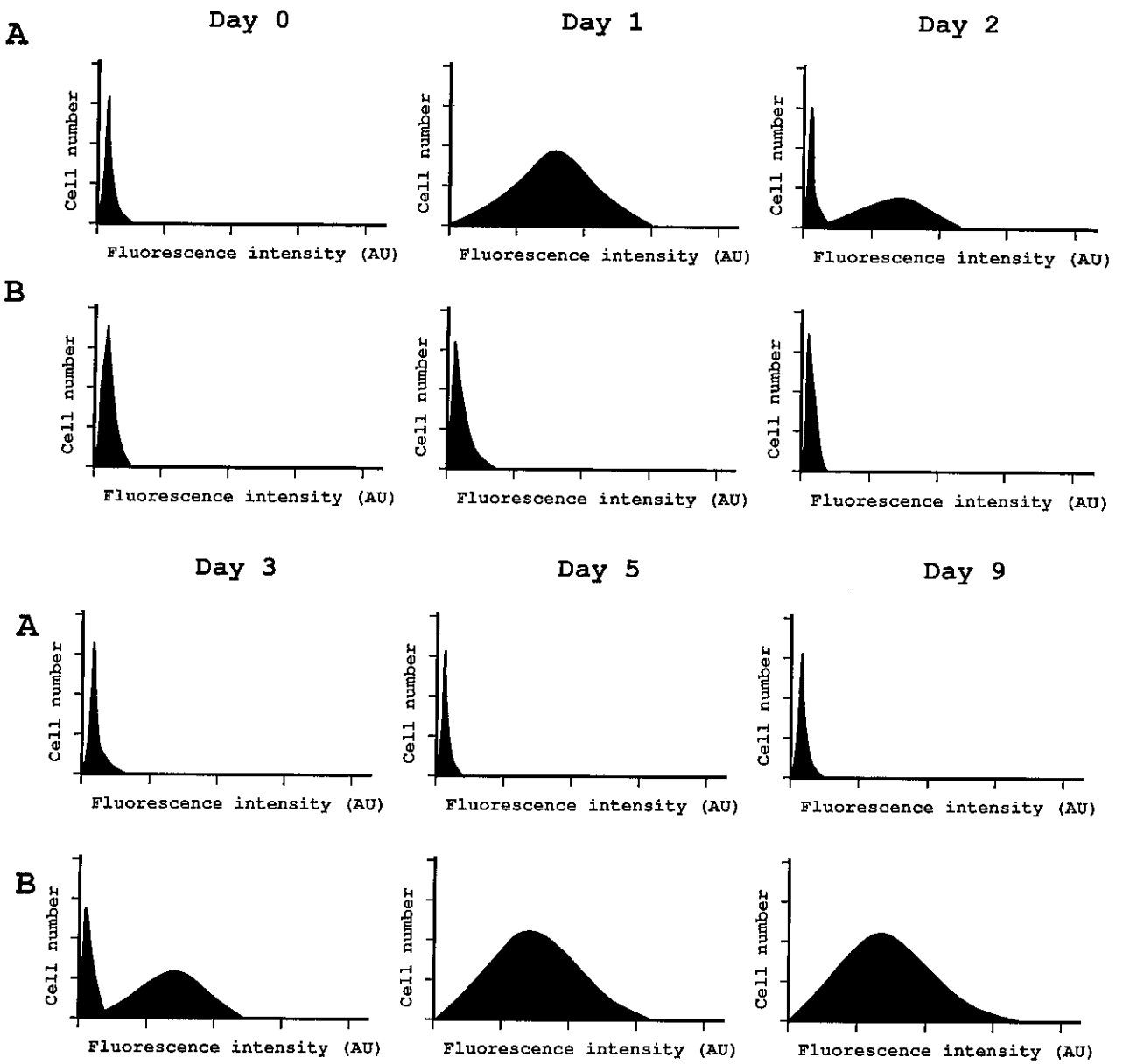

Fig. 1. A: Expression of ICAM-1 on U251-SP cells treated with 40 U/ml of TNF-α. The number of positive cells and fluorescence intensity were measured by flow cytometry. B: Expression of ICAM-1 on U251-SP cells treated with liposomes with entrapped pcDVTNF-α. AU, arbitrary units.

Table II. Alteration of Percent Cytotoxicity in LAK Cells Treated with Exogenous TNF-α and Liposome-entrapped pcDVTNF-α

	Days			
	1	3	5	9
Untreated	39.9±2.1	40.9±2.0	40.7±2.1	40.1±3.4
Exogenous TNF-α	49.5±1.7	39.7±2.1	41.8±1.9	40.6±4.5
Liposome-entrapped pcDVTNF-α	38.2±2.5	45.9±1.2	50.5±1.2	52.9±1.0

The values are means and SD of 3 experiments and represent percent cytotoxicity of LAK cells. Exogenous TNF-α, 40 U/ml; liposomes with entrapped pcDVTNF-α, 0.3 μg of DNA/ml and 15 nmol lipids/ml.

Table III. Effect of Anti-ICAM-1 Monoclonal Antibody

	Anti-ICAM-1(−)	Anti-ICAM-1(+)
Untreated	40.1±3.4	39.2±3.6
Exogenous TNF-α	53.5±2.0	40.8±4.8
Liposome-entrapped pcDVTNF-α	53.2±2.7	41.2±2.0

The values are means and SD of 3 experiments and represent percent cytotoxicity of LAK cells.

hand, the cells which had been treated with exogenous or transfection-induced TNF-α expressed ICAM-1 on their surface. However, the expression of ICAM-1 was transient in the case of exogenous TNF-α, and it was not detected at 48 h after the medium exchange. In contrast, the expression of ICAM-1 in the cells transfected with pcDVTNF-α was found 3 days after gene transfection and continued up to 9 days (Fig. 1). Thus, the expression of ICAM-1 paralleled the content of TNF-α in the medium.

Alteration of susceptibility of U251-SP cells to LAK cell cytolysis by exogenous or transfection-induced TNF-α When U251-SP cells were exposed to less that 50 U/ml of exogenous TNF-α, growth was not significantly inhibited, but their susceptibility to LAK cell cytolysis increased markedly as compared with that of the untreated cells (exogenous TNF-α on day 1 in Table II). However, the increased susceptibility disappeared rapidly when the medium was replaced with fresh medium and TNF-α in the medium was removed (exogenous TNF-α on day 3 in Table II). In contrast, U251-SP cells treated with liposomes with entrapped pcDVTNF-α exhibited an increase of susceptibility from 3 days to 9 days after treatment (Table II). The increased susceptibility of U251-SP cells to LAK cell cytolysis paralleled their expression of ICAM-1.

Effect of anti-ICAM-1 monoclonal antibody on the increase of susceptibility to LAK cell cytolysis by exogenous or transfection-induced TNF-α By using anti-ICAM-1 monoclonal antibody, we investigated whether the increased susceptibility to LAK cell cytolysis could be ascribed to the expression of ICAM-1 on target cells. The increased susceptibility to LAK cell cytolysis was quenched by anti-ICAM-1 monoclonal antibody in both exogenous and transfection-induced TNF-α treated U251-SP cells (Table III). It appears that ICAM-1 induced on U251-SP cells plays a role in the increased susceptibility of the glioma cells to LAK cell cytolysis.

DISCUSSION

The mechanisms by which LAK cells kill neoplastic cells are not known, but their antitumor activities are thought to be potentiated by cytokines and cell adhesion molecules.[21] Cytokines such as TNF-α, TNF-β, and IFN-γ, possess direct antitumor activity in addition to inducing the release of other cytokines from leucocytes. We have tried to develop cytokine therapy for brain tumors by utilizing cytokines with direct antitumor activity. On the other hand, it is also important to enhance immune response by increasing cell-cell adhesion. However, it was reported that lymphocytes in patients with brain tumors were not necessarily antigenically stimulated by tumor cells, and the host immune response to the tumors was very weak in many cases.[22]

We have shown here that transfection-induced TNF-α, which was produced by the cells transfected with the TNF-α gene, enhanced LAK cell cytolysis for a long period. TNF-α is known to increase the expression of ICAM-1 on the surface of glioma cells and the susceptibility of glioma cells to LAK cell cytolysis.[10, 23] We also confirmed that TNF-α treatment or TNF-α gene transfection induced the expression of major histocompatibility complex (MHC) class I and II antigens (data not

shown). The expression of ICAM-1 and MHC class I and II antigens was markedly detected on the surface of glioma cells exposed to exogenous TNF-α or transfection-induced TNF-α, whereas the untreated glioma cells showed no ability as antigen-presenting cells and the expression of ICAM-1 was not detected on their surface. Moreover, we confirmed that the susceptibility of glioma cells to LAK cell cytolysis was enhanced by the induction of ICAM-1.

Clinical immunotherapy of brain tumors has, to date, been relatively unsuccessful.[5, 24] This has partly been attributed to the rapid clearance of cytokines administered for the treatment of the tumors, so that on effetive concentration of the cytokines is not maintained. This problem could be overcome by serial administrations of exogenous TNF-α, or by transfecting the TNF-α gene into glioma cells. We adopted the latter approach, and found that the expression of ICAM-1 on the surface of glioma cells is maintained for a long period by the transfection-induced TNF-α. We used liposomes for the gene transfection, since they afford a high transfection efficiency and the possibility of targeting the genes to glioma cells selectively by using a monoclonal antibody which reacts specifically with a surface antigen of human glioma cells.[17, 18] This modified adoptive immunotherapy is an attractive approach for the treatment of brain tumors that have poor immunogenicity.

(Received September 9, 1993/Accepted March 14, 1994)

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