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Tumor Necrosis Factor-α Gene Transfer Augments Anti-Fas Antibody-mediated Apoptosis in Human Glioma Cells

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To effectively induce apoptosis in human glioma cells, we tried to transfer the tumor necrosis factor (TNF)-α gene into glioma cells to produce TNF-α locally in these cells. The stable transfectants of three glioma cells (U251-SP, U251-MG, and T98G) were resistant to exogenous TNF-α, but their cell surface expression of the Fas antigen was dramatically enhanced by about 10 to 100-fold as compared with untransfected glioma cells exposed to exogenous TNF-α. The Fas antigen is a transmembrane cytokine receptor protein of the nerve growth factor/TNF receptor superfamily. Although the untransfected glioma cells tested were resistant to anti-Fas antibody-mediated apoptosis, the TNF-α gene-transfected glioma cells exhibited high susceptibility to anti-Fas antibody-mediated apoptosis. Thus, TNF-α gene transfer combined with anti-Fas antibodies may be useful for the treatment of malignant glioma.

Key words: TNF-α — Gene transfer — Glioma — Apoptosis — Anti-Fas antibody

Malignant glioma is one of the most lethal tumors arising in the central nervous system. Because the tumor cells aggressively infiltrate into the normal brain tissue and/or disseminate into the subarachnoid space, the patients have a poor prognosis with an average survival of less than 2 years even if they receive multimodality treatment including surgery and post operative adjuvant therapy. Therefore, we need to develop a new form of therapy that can inhibit the growth and invasion of glioma cells. As a new approach for the treatment of malignant glioma, we have been developing a cytokine gene therapy using liposomes since 1988.[1-6]

Tumor necrosis factor (TNF)-α is a 17 kDa polypeptide,[7,8] which is produced mainly by monocytes and/or macrophages. This cytokine is an immunostimulant and an important mediator of host resistance to many infectious agents and malignant tumors.[9] We administered recombinant TNF-α to 20 patients with malignant gliomas and confirmed that it was more effective than the ordinary treatments.[10] However, it remained unsatisfactory. Also, TNF-α is known to be a cytokine that signals apoptosis in a variety of cell types. In addition, complementary DNAs for the TNF receptors have recently been molecularly cloned, and subsequent molecular studies have led to the identification of two distinct receptors, the 55 kDa and the 75 kDa TNF receptors.[11,12] The two receptors are related to each other and belong to a family of receptors that includes nerve growth factor receptor (NGFR) and T-cell antigen OX40. The Fas antigen also belongs to this family, and is a 45 kDa cell surface protein with a single transmembrane domain.[13] TNF, lymphotoxin (LT) or anti-Fas antibody stimulates corresponding receptors and induces apoptosis in some types of cells. However, almost all human glioma cell lines have a low susceptibility to apoptosis induced by TNF-α or anti-Fas antibody alone. Thus, we investigated the combined effects of the members of TNF/NGFR families that can induce apoptosis. Here, we show that glioma cells transfected with the TNF-α gene have a high susceptibility to anti-Fas antibody-mediated apoptosis, even though the untransfected glioma cells were resistant to anti-Fas antibody-mediated apoptosis.

MATERIALS AND METHODS

Materials Recombinant human TNF-α was provided by Asahi Chemical Industry Co., Ltd., Tokyo. Its specific activity was 2.2×10^6 U(JRU)/mg protein. Anti-Fas antibodies (IgG and IgM) and FITC-labeled goat anti-mouse IgG monoclonal antibody were purchased from Medical and Biological Laboratories Co., Ltd., Nagoya. In this experiment, we used two plasmids. The first was pcDVTNF-α that contains the SV40 early promoter and the coding sequence of TNF-α. It was constructed by Asahi Chemical Industry Co., Ltd. The second was pSV2neo that has an SV40 promoter and the neomycin phosphotransferase gene, an intracellular enzyme that activates G418. It was constructed by Toray Industries Inc., Tokyo.

Cell culture Three human glioma cell lines, U251-SP, U251-MG, and T98G, were used. The cells were maintained in Eagle's minimum essential medium supple-

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mented with 10% fetal calf serum, 0.1 m*M* non-essential amino acids, 5 m*M* L-glutamine, and antibiotics (streptomycin, 100 μg/ml; penicillin, 100 U/ml).

Transfection To prepare stable transfectants of the TNF-α gene and/or neomycin-resistance gene-transfected glioma cells, pcDVTNF-α, and/or pSV2neo (0.3 μg/ml of DNA; 15 nmol/ml of lipids) were transfected into glioma cells by liposomes.[14] Selection with G418 (500 μg/ml) was started 48 h later and the cells were incubated for 14 days. The growth characteristics of these cells were the same as those of the untreated glioma cells (data not shown).

Determination of human TNF-α The concentration of human TNF-α in the medium was determined by an enzyme-linked immunoassay as described in a previous paper.[15]

Detection of Fas antigen The presence of the Fas antigen was detected by using a fluorescence-activated cell sorter (FACS). The glioma cells (1×10^5) were harvested and resuspended in 25 μl of a 1:100 dilution of an anti-Fas antibody (IgG). The mixture was incubated for 30 min on ice and then the cells were washed with 1.5 ml of phosphate-buffered saline (PBS). Next, the cells were incubated with a 1:20 dilution of FITC-labeled goat anti-mouse IgG monoclonal antibody for 30 min on ice. Finally, the cells were washed three times with PBS and resuspended in 0.5 ml of PBS. Fluorescence was quantified using an EPICS instrument (Coulter Corporation, Hialeah, FL).

Agarose gel electrophoresis Cells were incubated for 3 days in the medium containing an anti-Fas antibody. The cells were harvested, washed with PBS and then lyzed in NTE buffer (150 m*M* NaCl, 50 m*M* Tris-HCl (pH 8.0), 10 m*M* EDTA) containing 0.5% SDS and 0.2 mg/ml proteinase K). After incubation overnight at 37°C, DNA was extracted with phenol/chloroform twice and precipitated with ethanol. The extracted samples were incubated for 2 h in TE buffer (10 m*M* Tris-HCl (pH 8.0), 1 m*M* EDTA) containing 0.1 mg/ml of RNase A. The samples were loaded on 2% agarose gel in the presence of 0.5 mg/ml of ethidium bromide.

Growth inhibition A glioma cell suspension in culture medium (2 ml; 2.5×10^4 cells/ml) was placed in each well of a 6-well plate (Falcon #3046) and incubated at 37°C for 24 h in a humidified atmosphere of 5% CO_2 and 95% air. The cells were then treated with an appropriate concentration of anti-Fas antibody (IgM). They were cultured for 3 days, and the cell growth was evaluated by

Table I. Concentration of TNF-α Secreted into the Medium from TNF-α Gene-transfected Glioma Cells

	Stable transfectants		
	U251-SP	U251-MG	T98G
Control	0.2>	0.2>	0.2>
neoR gene-transfected	0.2>	0.2>	0.2>
TNF-α gene-transfected	48.8±7.9	32.4±5.2	30.3±4.2

The values are the concentrations of TNF-α (U/ml) in the medium at 6 days after inoculation and are expressed as the mean and standard deviation (SD) of four experiments.
neoR: neomycin-resistant.

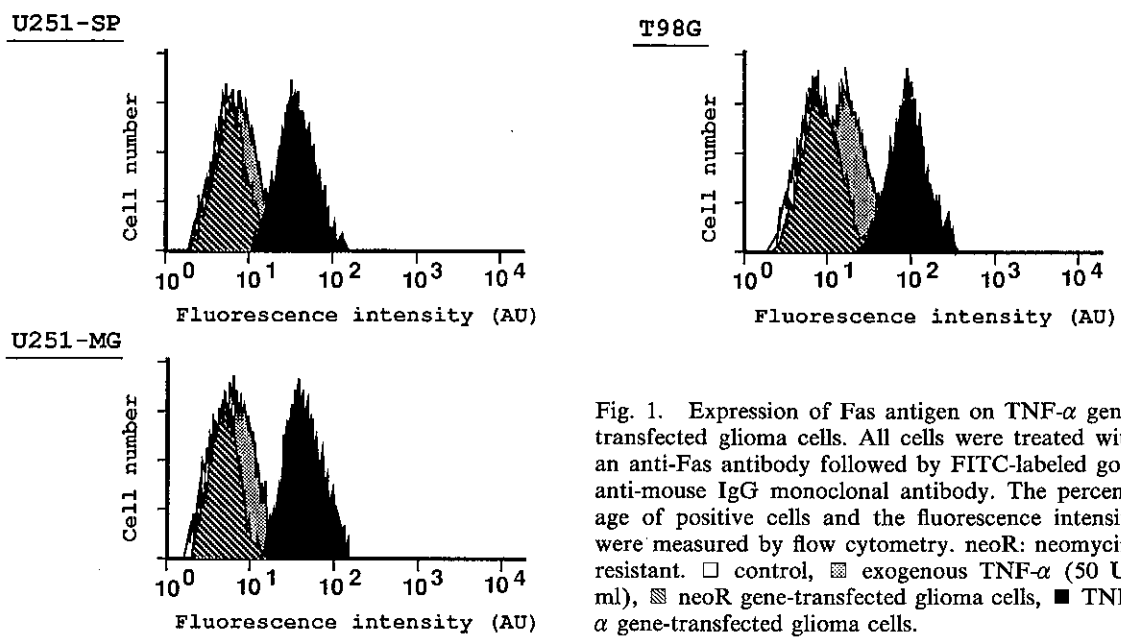

Fig. 1. Expression of Fas antigen on TNF-α gene-transfected glioma cells. All cells were treated with an anti-Fas antibody followed by FITC-labeled goat anti-mouse IgG monoclonal antibody. The percentage of positive cells and the fluorescence intensity were measured by flow cytometry. neoR: neomycin-resistant. □ control, ▨ exogenous TNF-α (50 U/ml), ▧ neoR gene-transfected glioma cells, ■ TNF-α gene-transfected glioma cells.

counting the number of trypan blue-excluding cells in a hemocytometer.

RESULTS

Production of TNF-α in TNF-α gene-transfected glioma cells The TNF-α gene-transfected glioma cells secreted TNF-α into the medium. The levels of TNF-α in the medium after incubation of the cells for 6 days are shown in Table I. The level of TNF-α in the medium was 48.8±7.9 U/ml in U251-SP cells, 32.4±5.2 U/ml in U251-MG cells and 30.3±4.2 U/ml in T98G cells. These levels (Table I) were the maximum ones obtained in these experiments. On the other hand, TNF-α was not detected in the medium of either the untreated glioma cells or the neomycin-resistance gene-transfected glioma cells.

Expression of Fas antigen and susceptibility of glioma cells to anti-Fas antibody-mediated apoptosis The glioma cells evaluated already expressed the Fas antigen on their surface, even before the cells were treated (Fig. 1). However, these cells had a low susceptibility to anti-Fas antibody-mediated apoptosis. However, expression of the Fas antigen was augmented dramatically in the TNF-α gene-transfected glioma cells, by about 10- to 100-fold as compared to the untransfected cells (Fig. 1). These cells had a high susceptibility to anti-Fas antibody-mediated apoptosis. In these cases, we examined nuclear DNA of the cells and confirmed DNA fragmentation or "laddering" (Fig. 2). Fig. 3 shows the anti-Fas antibody-mediated apoptosis. The growth of the glioma cells was inhibited by the anti-Fas antibody in a concentration-dependent manner when more than 1.0 μg/ml of the antibody was added to the cells. However, there was no significant difference among the untreated, exogenous TNF-α-treated, and neomycin-resistance gene-transfected cells. In contrast, the growth of the TNF-α gene-transfected cells was significantly inhibited even when less than 1.0 μg/ml of anti-Fas antibody was added to the cells. This result indicated that TNF-α gene transfer augmented the susceptibility of glioma cells to anti-Fas antibody by 10- to 100-fold as compared to the untreated as well as the untransfected cells.

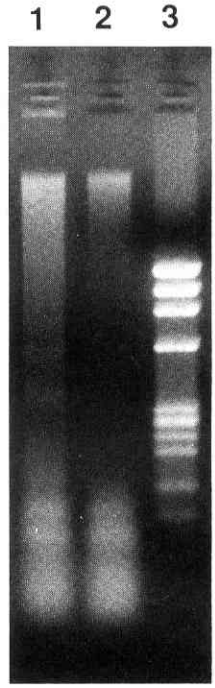

Fig. 2. Gel electrophoresis of nuclear DNA of glioma cells (U251-SP) exposed to anti-Fas antibody. The glioma cells were incubated for 3 days in the presence of anti-Fas antibody (1.0 μg/ml). Lane 1, TNF-α gene-transfected glioma cells exposed to anti-Fas antibody (1.0 μg/ml); lane 2, untransfected glioma cells exposed to anti-Fas antibody (1.0 μg/ml); lane 3, molecular markers (PhiX174). Similar results were obtained in the cases of U251-MG and T98G cells.

DISCUSSION

Apoptosis is morphologically distinguishable from necrosis, which occurs during accidental cell death caused by physical or chemical agents. Apoptosis mediates programmed cell death and can sometimes be seen during the regression of tumors. Many factors can induce apoptosis, including TNF-α, anti-Fas antibody, ceramide and some types of anticancer drugs. However, the relationship between Fas-dependent apoptosis and TNF-α-induced cytotoxicity has remained obscure. Weller *et al.* reported that Fas gene transfer and combination immunotherapy using anti-Fas antibodies and cytokines may overcome the anti-Fas antibody resistance of Fas-negative human malignant glioma cells, which may represent subpopulations within single gliomas or form a separate subgroup of human malignant gliomas.[16] They demonstrated that TNF-α augmented the Fas-mediated apoptosis of Fas-transfected glioma cells by enhancing the expression of the Fas antigen on the cell surface. Our experiments also demonstrated that TNF-α enhanced the expression of the Fas antigen, but only by 2- or 3-fold at most. In addition, the glioma cells treated with TNF-α did not show altered susceptibility to anti-Fas antibody. On the other hand, TNF-α gene transfer to the glioma cells dramatically enhanced the expression of the Fas antigen by about 10- to 100-fold, and induced high susceptibility to anti-Fas antibody-mediated apoptosis. In addition, we detected the transmembrane form of TNF-α on the glioma cells transfected with TNF-α gene (in preparation). Recently, Grell *et al.* have shown that the transmembrane form of TNF is a much stronger ligand

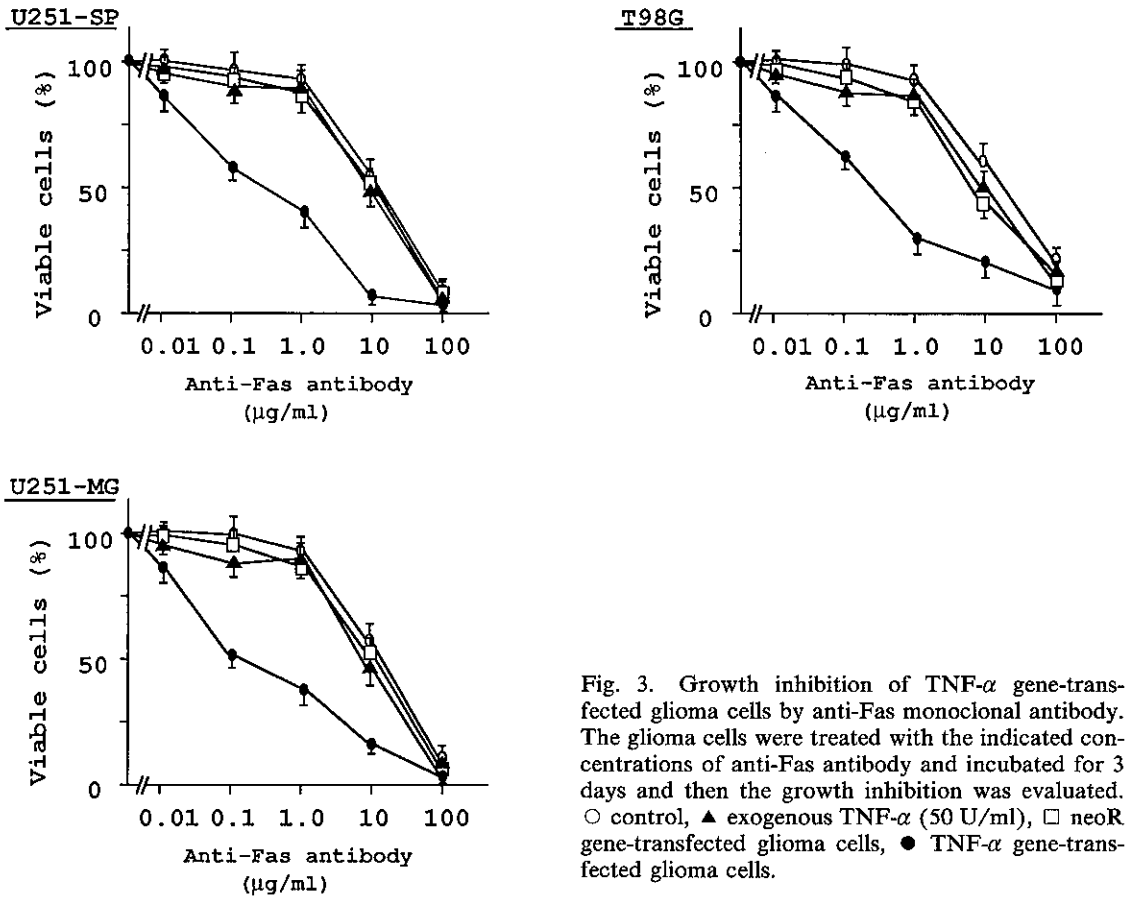

Fig. 3. Growth inhibition of TNF-α gene-transfected glioma cells by anti-Fas monoclonal antibody. The glioma cells were treated with the indicated concentrations of anti-Fas antibody and incubated for 3 days and then the growth inhibition was evaluated. ○ control, ▲ exogenous TNF-α (50 U/ml), □ neoR gene-transfected glioma cells, ● TNF-α gene-transfected glioma cells.

of the type II TNF receptor than soluble TNF.[17] These findings suggest that TNF-α gene transfer may induce stronger cytotoxic action on glioma cells that are resistant to TNF-α alone. Also, Wong and Goeddel have reported that anti-TNF receptor antibody and Fas antibody synergistically kill tumor cells.[18] The anti-Fas antibody seems to have potential for the treatment of cancer.

Further, we have succeeded with *in vitro* and *in vivo* gene transfer using liposomes tagged with a monoclonal antibody against a glioma-associated antigen (immunoliposomes).[1-6] Thus, TNF-α gene transfer using immunoliposomes and immunotherapy with anti-Fas antibodies may prove clinically useful against gliomas.

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