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The Cytocidal Activity of OK-432-activated Mononuclear Cells against Human Glioma Cells is Partly Mediated through the Fas Ligand/Fas System

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We have been applying an adoptive immunotherapy protocol to patients with malignant brain tumors using OK-432-activated peripheral blood mononuclear cells (OK-MCs). In order to elucidate the mechanism of OK-MCs' cytotoxicity, we examined the expression of Fas ligand mRNA in OK-MCs and the cytocidal activity of these cells against a human glioma cell line, T98G which expresses a high level of Fas. The expression of Fas ligand mRNA was low in non-treated peripheral blood mononuclear cells and was elevated by treatment with OK-432, irrespective of the dose employed. Apoptosis of T98G cells induced by OK-MCs was unequivocally inhibited by the pretreatment of T98 G cells with ZB4 monoclonal antibody, which binds to Fas and blocks the binding of Fas ligand to Fas. These data indicate that the cytotoxic activity of OK-MCs via apoptosis seems to be at least partly mediated by the Fas ligand/Fas system. Adoptive immunotherapy using the Fas ligand/Fas system could be a new treatment modality for human malignant brain tumors.

Key words: Adoptive immunotherapy — Fas — Fas ligand — Glioma — OK-432

Fas/APO-1 (CD95) is a type I membrane protein that mediates apoptosis when ligated with anti-Fas monoclonal antibody (Fas mAb).[1] Molecular cloning of Fas cDNA has shown that Fas belongs to the tumor necrosis factor (TNF)/nerve growth factor receptor superfamily.[2] Recently, Fas ligand, which binds to Fas, has been purified from a cytotoxic T lymphocyte (CTL) hybridoma.[3] It is a membrane-type cytokine belonging to the TNF family[4,5] and is expressed in activated T cells stimulated by phorbol myristate acetate, ionomycin, ConA, anti-CD3, and high concentrations of interleukin (IL)-2.[6,7] Recently, Kägi *et al.*[8] demonstrated that the Fas ligand/Fas system plays a crucial role in all T-cell mediated cytotoxicity as well as in perforin pathways.

Previous studies have shown that lymphokine-activated killer (LAK) cells are capable of killing a wide variety of autologous and allogeneic tumor cells *in vitro*.[9] Although several clinical trials with intratumoral infusion of LAK cells and IL-2 through an Ommaya's catheter/reservoir system have been reported, they were not as effective as expected.[10,11]

OK-432 (Picibanil) is an immunomodulatory agent prepared from an attenuated strain of *Streptococcus pyogenes*.[12] When peripheral blood mononuclear cells (PBMCs) are incubated with OK-432 for 24 h, they acquire cytolytic activity against human tumor cells.[13] Because OK-432 induces killing activity earlier than IL-2, the risk of bacterial contamination in the culture medium is expected to be lower. OK-MCs produce several kinds of cytokines including IFN-α, IFN-β, IFN-γ, TNF, IL-1, IL-2 and tumor growth-inhibitory factor in both experimental animals and humans.[13–16] The cytolytic activity is thought to be due to not only cell-mediated, but also cytokine-mediated mechanisms. These data suggest that OK-MCs might be more favorable for clinical application than LAK cells.

We have been applying an adoptive immunotherapy regimen using OK-MCs to treat patients with malignant brain tumors. OK-MCs appear to consist of both activated T cells and natural killer (NK) cells.[17] It was recently shown that NK cells also express Fas ligand and possess the capability of killing target cells expressing Fas, such as virus-infected cells or tumor cells.[18,19] These facts led us to speculate that the Fas ligand/Fas system might be involved in the cytotoxicity of OK-MCs.

In order to elucidate the relationship between OK-MCs-mediated cytotoxicity and the Fas ligand/Fas system, we have studied the expression of Fas ligand mRNA in OK-MCs and determined the cytotoxicity of OK-MCs against T98G, a human glioma cell line which expresses a high level of Fas.[20]

MATERIALS AND METHODS

Production of OK-MCs Human peripheral blood was obtained from a healthy adult volunteer (K.T.). PBMCs were separated by gradient centrifugation using Lymphocyte Separation Medium (Litton Bionetics, MD). OK-432 (Picibanil, kindly provided by Chugai Pharmaceutical Co., Tokyo) is a lyophilized preparation of Su-Strain of *Streptococcus pyogenes A3* killed by treatment with both penicillin and H_2O_2. One Klinische Einheiten (KE) of OK-432 is equivalent to 0.1 mg of lyophilized preparation. PBMCs were suspended at 2×10^5 cells/ml in

serum-free RPMI 1640 containing 0.25, 0.125, and 0.025 KE of OK-432, respectively, and cultured for 24 h at 37°C in a 5% CO_2, 95% air, water-saturated atmosphere. After culture, OK-MCs were collected by centrifugation and washed with phosphate-buffered saline (PBS).

mRNA purification and reverse transcription-polymerase chain reaction (RT-PCR) Poly (A) RNA was prepared from OK-MCs using a QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech, Uppsala, Sweden) and was reverse-transcribed with a First-Strand cDNA Synthesis Kit (Pharmacia Biotech). PCR amplification of the cDNA was performed with 50 pmol of each primer, 5 μl of 10× buffer (100 m*M* Tris-HCl pH 8.3, 500 m*M* KCl, 15 m*M* $MgCl_2$), 1 μl of 2.5 m*M* dNTP, and 2.5 U of Taq polymerase (Takara Biomedicals, Tokyo) in 50 μl of sterile distilled water overlaid by mineral oil. Specific Fas ligand primers were designed according to the previously published cDNA sequence[21] as follows: 5′ primer, GCCATGCAGCAGCCCTTCAATTAC; 3′ primer, GTGCTTCTCTTAGAGCTTATATAAGCC. We used amplified β-actin cDNA as an internal control. The conditions for PCR were 1 min at 94°C, 1.5 min at 55°C and 2.0 min at 72°C for 30 cycles. The PCR products were separated by electrophoresis on 1.0% agarose gel containing ethidium bromide and visualized under ultraviolet light.

Expression of Fas in cultured glioma cells The expression of Fas in T98G was examined by flow cytometry. T98G cells, obtained from the American Type Culture Collection (Rockville, MD), were maintained in minimum essential medium (MEM) supplemented with 7.5% gentamycin (Schering-Plough, Osaka), and 10% (vol/vol) heat-inactivated fetal bovine serum (Nalgene, Victoria, Australia) in a water-saturated 5% CO_2 atmosphere at 37°C. Cells suspended in PBS with 0.1% EDTA were incubated with 0.1 ml of PBS containing 50 ng/ml of IgG class anti-Fas mAb, UB2 (MBL, Nagoya), at 4°C for 60 min. Non-specific mouse IgG (Dako Japan, Kyoto) was applied to the sample instead of the primary antibody as a negative control. The cells were washed twice in PBS and incubated with FITC-conjugated goat anti-mouse IgG antibody (Vector Laboratories Inc., Burlingame, CA) at RT for 30 min. Finally, the cells were washed twice and analyzed using a FACScan (Becton-Dickinson, Mountain View, CA).

Effect of Fas mAb on T98G cells *in vitro* We used a commercially available IgM class of anti-Fas mAb (MBL) for the induction of apoptosis in T98G cells. The cells were incubated with 100 ng/ml of Fas mAb for up to 72 h. At the time indicated the cells were detached with 0.05% trypsin and 0.1% EDTA and suspended in PBS. Viable cells were determined by trypan blue dye exclusion. In order to inhibit Fas-mediated apoptosis, we used an anti-Fas mAb, ZB4 (MBL), which binds to Fas and inhibits Fas-mediated apoptosis. After pretreatment with ZB4 (500 ng/ml) for 1 h, cells were incubated for up to 96 h with 100 ng/ml of Fas mAb and viability was determined.

Cytocidal activity of OK-MCs against T98G cells In order to analyze the cytocidal activity of OK-MCs against T98G, the T98G cells (1×10^5) were mixed with various numbers of OK-MCs (prepared from PBMCs treated with 0.25 KE of OK-432 for 24h) and incubated for up to 72 h. After 24, 48, 72 h incubation, the T98G cells were detached with 0.05% trypsin and 0.1% EDTA in PBS. Viable cells were determined by trypan blue dye exclusion. To investigate whether the Fas ligand expressed in OK-MCs is functional, the ZB4 mAb was used as described above.

***In situ* detection of DNA double-strand breaks** T98G cells (1×10^4) were incubated in chamber slides (Nunc, Inc., Naperville, IL) for 24 h and mixed with OK-MCs (4×10^5: E/T ratio=40). After 48 h incubation, T98G cells were washed with PBS three times and fixed with methanol for 10 min. DNA double-strand breaks were detected by a modified TUNEL method[22] using the "ApopTag" *in situ* apoptosis detection kit (Oncor, Gaithersburg, MD).

RESULTS

Expression of Fas ligand mRNA The Fas ligand mRNA was detected at a low level in non-treated PBMCs from a healthy adult. The amounts of amplified products were unequivocally elevated by OK-432 treatment, irrespective of the dose employed (Fig. 1). The nucleotide sequence of the PCR product was shown to correspond to that of the human Fas ligand by a dideoxy termination method (data not shown).

Expression of Fas and cytocidal effect of Fas mAb Flow cytometric analysis demonstrated that Fas was highly

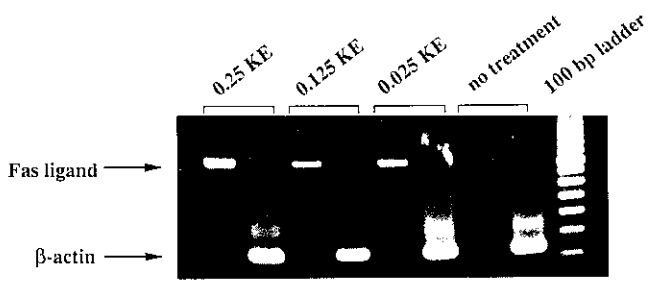

Fig. 1. cDNA obtained from OK-MCs after reverse transcription was amplified by PCR using specific Fas ligand primers. The amplified products were easily discernible after the treatment with OK-432. The amounts of amplified products were elevated by treatment with OK-432, irrespective of doses.

expressed in T98G cells (Fig. 2). When treated with Fas mAb (IgM), the number of viable cells was significantly decreased in a time-dependent manner. Normal mouse IgM, used as an isotypic control, showed no effect on the growth of T98G cells(data not shown). The cytocidal activity induced by Fas mAb (IgM) was blocked by the pretreatment of T98G with ZB4 antibody, although incubation of the cells with ZB4 antibody alone had no effect on cell growth (Fig. 3A).

Cytocidal effect of OK-MCs On the other hand, T98G cell viability when incubated with various numbers of OK-MCs (1×10^6, 4×10^6, 1×10^7) decreased in a dose- and time-dependent manner (Fig. 3B). Pretreatment of T98G cells with ZB4 partially inhibited the cytocidal

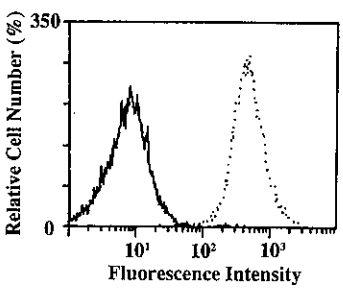

Fig. 2. Flow cytometric analysis of Fas expression in T98G cells. Dashed lines, Fas-positive cells; solid lines, control using nonspecific mouse IgG.

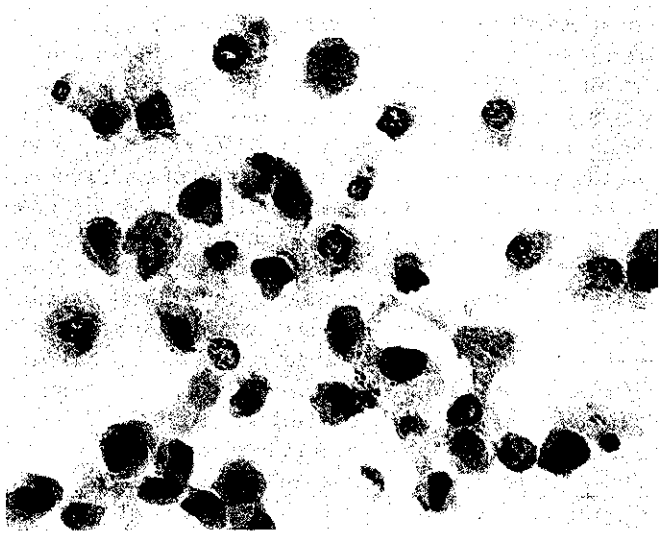

Fig. 4. Detection of DNA double-strand breaks *in situ*. The stained nuclei display specific apoptotic features, such as condensation and fragmentation of chromatin (counterstained with hematoxylin, original magnification × 300).

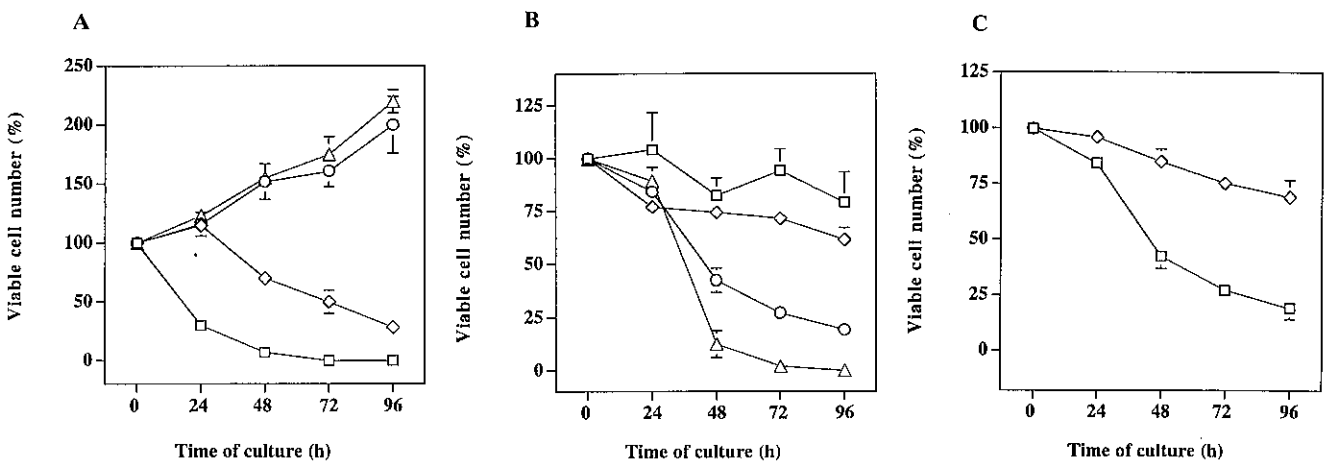

Fig. 3. Cytocidal activity of OK-MCs against Fas-expressing T98G cells. A, T98G cells were cultured with either anti-Fas IgM (100 ng/ml, □), ZB4 (500 ng/ml, ○) or both (◇). In the case of treatment with Fas mAb (IgM), the number of viable cells was significantly decreased in a time-dependent manner. The cytocidal activity induced by Fas mAb (IgM) was blocked by the pretreatment of T98G with ZB4 antibody. Treatment with ZB4 antibody alone had no growth-inhibitory effect on T98G as compared with non-treatment control (△). B, T98G cells were mixed with various numbers of OK-MCs and incubated for up to 72 h. ◇: OK-MCs, 1×10^6 (E/T ratio=10), ○: OK-MCs, 4×10^6 (E/T ratio=40), △: OK-MCs, 1×10^7 (E/T ratio=100), □: PBMCs, 4×10^6. The number of viable cells decreased in a dose- and time-dependent manner. C, The viable cell number of T98G treated with OK-MCs (□, 4×10^6, E/T ratio=40) decreased in a time-dependent manner, whereas the treatment with ZB4 (◇) caused partial inhibition of the cytocidal activity of OK-MCs. The activity is expressed as the mean of triplicate experiments.

activity of OK-MCs (Fig. 3C). These results indicate that the Fas ligand expressed in OK-MCs is functional and the cytotoxicity of OK-MCs is mediated at least partly by the Fas ligand/Fas system.

***In situ* detection of DNA double-strand breaks** The majority of T98G cells treated with OK-MCs for 48 h displayed nuclear changes consistent with apoptosis, such as fragmented nuclei and condensed chromatin (Fig. 4). These nuclei were clearly stained by the modified TUNEL method.

DISCUSSION

Recently, attention has been drawn to the role of the Fas ligand/Fas system in immunology. Recent investigations have clarified the physiological role of the Fas ligand/Fas system in the clonal deletion of autoreactive T cells.[23] The Fas ligand/Fas system was also found to be involved in CTL-mediated cytotoxicity. Interaction between CTL and the MHC-antigen complex induces synthesis of the Fas ligand on CTL. The Fas ligand expressed on the surface of CTL then binds to Fas on the target cell and induces apoptosis.[24] It has also been reported that Fas ligand mRNA is expressed in mouse NK cells and that the Fas ligand in NK cells exhibits cytotoxicity against Fas-expressing cells,[18] indicating that the cytotoxicity of NK cells is partly mediated through the Fas ligand/Fas system.

We previously reported that an IgM class anti-Fas mAb could induce apoptosis by activation of the sphingomyelin signaling system in human cultured glioma cells that express high levels of Fas.[20, 25] Furthermore, we have demonstrated by immunohistochemistry that Fas is expressed on biopsied glioma cells. The degree of Fas expression correlated with that of apoptotic cell death assessed by the modified TUNEL method.[22, 26] These results strongly suggest that the Fas ligand/Fas system may play an important role in apoptosis of human gliomas.

In parallel with these studies, we have been applying an immunotherapy regimen using OK-MCs to patients with malignant brain tumors. Clinical evaluation based on CT and MRI revealed that 4 of 11 (36.4%) patients showed evidence of tumor growth suppression following injection of OK-MCs.[27] However, no investigation regarding the apoptosis-inducing effects of OK-MCs has been performed.

In the present study, we have demonstrated that the expression of human Fas ligand mRNA is markedly increased in PBMCs following treatment with OK-432. This increase of Fas ligand mRNA was also observed in OK-MCs obtained from patients suffering from malignant brain tumors (data not shown). OK-MCs showed a clear cytocidal activity towards Fas-expressing T98G cells *in vitro*, though this cytotoxicity was inhibited by pretreatment with IgG class anti-Fas mAb, ZB4, which inhibits Fas-mediated apoptosis. These data suggest that the cytocidal activity of OK-MCs seems to be at least partly mediated by the Fas ligand/Fas system. It is unclear, however, whether Fas ligand in OK-MCs is the only major factor contributing to cytotoxicity in Fas-expressing T98G cells, or whether this may also be induced by some other cytokine(s) produced by OK-MCs. Interestingly, it has been reported that the expression of Fas is increased by the treatment with IFN-γ.[28] Therefore, the relationship between the Fas ligand/Fas system and other cytokines produced by OK-MCs should be further investigated. We expect that the induction of apoptosis through the Fas ligand/Fas system may lead to the development of a new anti-tumor strategy.

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