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RESEARCH LETTER

Increase in proliferation rate and normalization of TNF-α secretion by blockage of gene transfer–induced apoptosis in lymphocytes using low-dose cyclosporine A

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Efficient gene transfer of lymphocytes is extremely difficult. We have shown previously that induction of apoptosis may play a role in the gene transfer resistance of lymphocytes. Anti-CD3 antibody can be used as a surrogate for receptor-mediated gene transfer in T lymphocytes. However, anti-CD3 antibody has been shown to be the causative agent of apoptosis in receptor-mediated gene transfer. In this study, we show that blockage of apoptosis by addition of low-dose cyclosporine A can lead to normalization of elevated TNF-α secretion and to a significant increase in the proliferation rate of transfected lymphocytes. In contrast, this had no negative effect on cytotoxic activity of immunologic effector cells called cytokine-induced killer cells. Therefore, blockage of apoptosis should have an impact on the use of lymphocytes transfected with cytokine genes as immunologic effector cells in cancer gene therapy protocols. **Cancer Gene Therapy (2000) 7, 1411–1413**

Key words: Apoptosis; T lymphocytes; gene transfer; cyclosporine A.

Lymphocytes are attractive vehicles for cytokine delivery. However, most gene transfer methods are either toxic or have low efficiency when used to transfect lymphocytes. Recently, receptor-mediated gene transfer into T lymphocytes *via* the binding of DNA/CD3 antibody particles to the CD3/T-cell receptor (TCR) complex has been shown to transfect T cells efficiently.[1] However, we have shown that cytokine-induced killer (CIK) cells transfected *via* nonviral methods mediate apoptosis and secrete large amounts of TNF-α. This was due to the use of anti-CD3 antibody in receptor-mediated gene transfer.[2] In this study, we investigated if anti-CD3 antibody-induced apoptosis in CIK cells could be blocked by the addition of low-dose cyclosporine A. Cyclosporine A forms immunophilin–drug complexes in the lymphocytic cytoplasm, which inhibits dephosphorylation of NFAT by calcineurin and thus blocks IL-2 and TNF-α production.[3] We subsequently studied the use of cyclosporine A in blocking apoptosis to determine the effects on proliferation rate, TNF-α secretion, and cytotoxic activity of anti-CD3 antibody-treated CIK cells.

MATERIALS AND METHODS

CD3 receptor-mediated transfection of CIK cells

CIK cells were transfected using CD3 receptor-mediated gene transfer as recently described.[4] Briefly, a complex is formed containing inactivated adenovirus, streptavidin–polylysine, the plasmid pCEP, and an anti-CD3 antibody, coupled with polylysine. Together with this transfection complex, 2×10^6 cells/mL were incubated for 4 hours in serum-free Opti-MEM medium (Gibco BRL, Berlin, Germany). For the blocking of apoptosis, various amounts (0.5, 1, 1.5, 2 μM) of cyclosporine A (Sigma, Deisenhofen, Germany) were added to anti-CD3 antibody-treated CIK cells.

Flow cytometric analysis of apoptosis

Determination of apoptosis was performed as described before.[2] In brief, 2.5×10^5 cells/mL were incubated with 1.5 μg/mL annexin V marked with FITC (Boehringer, Ingelheim, Germany) and 0.5 μg/mL propidium iodide (Sigma) for 30 minutes at room temperature and then

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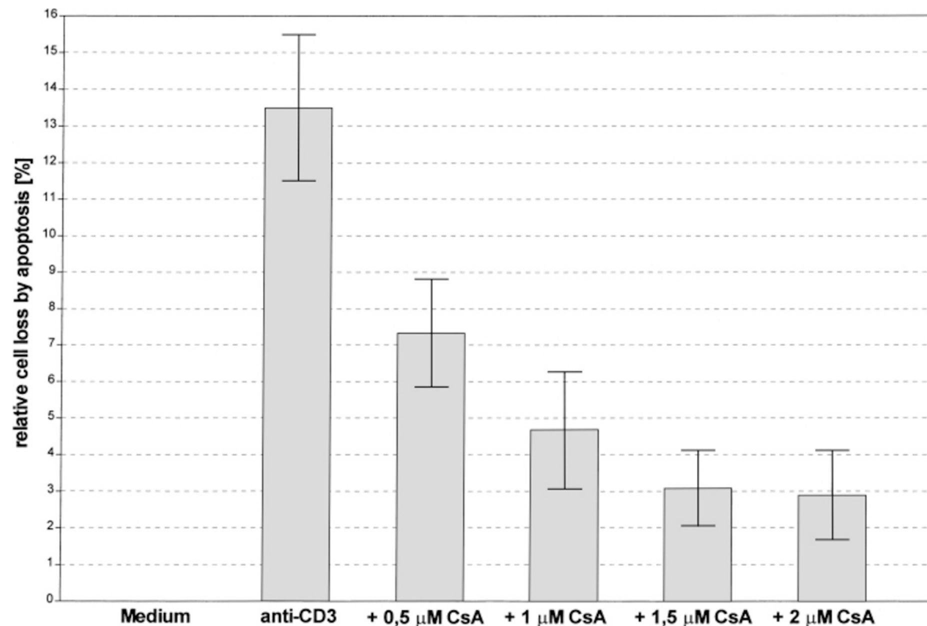

Figure 1. Blockage of CD3-induced apoptosis by addition of various amounts of cyclosporine A. CIK lymphocytes were incubated with anti-CD3 antibody with and without addition of various amounts of cyclosporine A as indicated. Anti-CD14 was used as a negative control antibody (medium control). After incubation, apoptosis was determined by annexin V and propidium iodide stain. The figure represents data from 10 experiments. Data are presented as mean ± SEM.

analyzed using a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany).

TNF-α enzyme-linked immunosorbent assay

TNF-α levels were determined by an enzyme-linked immunosorbent assay kit (Boehringer, Mannheim, Germany).

^{51}Cr release assay

^{51}Cr release assays were performed as reported elsewhere.[4] Primary target cells were derived from CML patients using Ficoll density gradient centrifugation. After 4 hours of incubation,cells were harvested and the supernatant was analyzed in a gamma counter (Biogamma II, Beckham, Fullerton, CA).

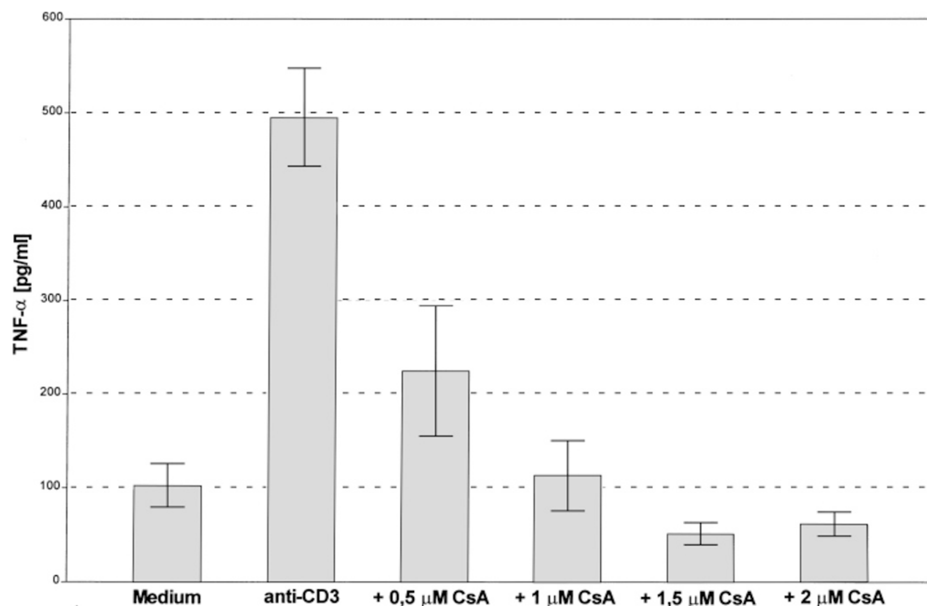

Figure 2. Secretion of TNF-α after addition of anti-CD3 antibody to CIK lymphocytes. CIK lymphocytes were incubated with anti-CD3 antibody with or without addition of various amounts of cyclosporine A as indicated. TNF-α secretion was determined as described under *Materials and methods.* Results shown represent data from six separate experiments. Data are shown as mean ± SEM.

Statistical analysis

A Wilcoxon matched pairs test was used in analyzing statistical significance. A P value $<.05$ was considered significant. Data are presented as mean±SEM.

RESULTS

Blockage of apoptosis after transfection of lymphocytes

Because the induction of apoptosis and the decrease of viable immunologic effector cells may be an obstacle if these cells are to be used for therapy, we tested if we could block apoptosis induction by the addition of cyclosporine A. Unmodified CIK cells show apoptosis in 5.6±1%. In CD3 receptor-mediated gene transfer, the relative apoptotic cell loss increased to 13.5±2% (P=.002). Using cyclosporine A at a concentration of 2 μM, it was possible to decrease the amount of apoptosis to 2.9±1% (P=.002). This effect was dose-dependent (Fig 1). Addition of cyclosporine A also increased the proliferation rate of transfected lymphocytes and restored the absolute cell count to levels comparable to untransfected controls (data not shown).

Secretion of TNF-α after transfection of lymphocytes

Because TNF-α plays a crucial role in apoptosis induction, we measured the TNF-α secretions before and after transfection of CIK cells. Cells without addition of anti-CD3 antibody produced low amounts of TNF-α (102±24 pg/mL/10^6 cells). In contrast, addition of anti-CD3 antibody produced high amounts of TNF-α (494±53 pg/mL; P=.03). Addition of cyclosporin A led to a significant reduction of TNF-α secretion (51±13 pg/mL; P=.03). Reduction of TNF-α secretion was dose-dependent (Fig 2).

Cytotoxicity of CIK cells after addition of cyclosporine A

Because cyclosporine A reduces the amount of apoptosis and the secretion of TNF-α after CD3 receptor-mediated gene transfer, we investigated the influence of cyclosporine A on the cytotoxic activity of CIK cells. Cytotoxic activity was determined using a ^{51}Cr release assay with primary CML cells as targets. Addition of up to 2 μM cyclosporine A had no significant influence on the cytotoxic activity of CIK cells (data not shown).

DISCUSSION

Genetically modified T lymphocytes are being considered as potent effectors for adoptive transfer immunotherapy of cancer. CD3 receptor-mediated gene transfer is an efficient method for transfection of T lymphocytes and cell mortality is relatively low, compared to other nonviral gene transfer methods.[1] However, in a previous study,[2] we have shown that nonviral transfection methods can lead to induction of apoptosis in lymphocytes and increased secretion of TNF-α. TNF-α is known to cause apoptosis in CD8$^+$ T cells *via* the p75 TNF receptor (TNFR-2, CD120b).[5] CIK cells express the p75 TNF receptor and the CD3/TCR complex. In CIK cells, the TCR complex plays a minor role in mediating the cytotoxic activity. We reasoned that the induction of apoptosis after CD3 receptor-mediated gene transfer might be mediated *via* a form of apoptotic cell loss called activation-induced cell death. Therefore, we attempted to block this pathway by adding a low dose of cyclosporine A, inhibiting calcineurin-dependent signals without being cytotoxic. Here, we show that induction of apoptosis can be blocked by the addition of cyclosporine A. There are other derivates of cyclosporine A that are not immunosuppressive, which could be used instead of cyclosporine A. However, at low doses of cyclosporine A being used here, no decrease in cytotoxic activity of activated lymphocytes was seen. Blockage of apoptosis led to a significant increase in the proliferation rate of transfected lymphocytes. Furthermore, TNF-α secretion, which is elevated after addition of anti-CD3 antibodies, was completely normalized by the addition of cyclosporine A. Therefore, addition of cyclosporine A should be effective in preventing induction of apoptosis in CD3 receptor-mediated transfection of lymphocytes without interfering with the cytotoxic activity. Blockage of apoptosis should have an impact on the use of lymphocytes exogenously transfected with cytokine genes as immunologic effector cells in cancer gene therapy protocols.

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