Adoptive Immunotherapy with Murine Tumor-specific T Lymphocytes Engineered to Secrete Interleukin 2

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Abstract

Adoptive immunotherapy of cancer is not widely studied, although it has been proposed as a promising strategy for cancer gene therapy. One of the major obstacles to this approach is the difficulty in introducing cytokine genes efficiently into T lymphocytes. In this report, we developed an adoptive immunotherapy model with murine tumor-specific cytotoxic T lymphocytes. By using an adenoviral vector, we achieved up to 100% gene transduction of murine T lymphocytes. Treatment of mice with the cytotoxic T lymphocytes genetically modified to produce interleukin 2 resulted in reduction of tumor metastasis and longer survival from intracerebral tumor death, providing a hopeful strategy for treatments of human cancers.

Introduction

Several approaches are being pursued to develop the gene therapy of cancer (1—5). Along with vaccination protocols using tumor cells genetically modified to express cytokines (6—9), adoptive immunotherapy is another promising approach (10—12). Transduction of TIL (13) with cytokine genes could make these cells destroy tumors more effectively. Although several cytokine genes have been proposed for use in TIL, the adoptive approach is not widely studied (5). Research progress of this approach has been hampered by our inability to introduce foreign genes efficiently into T lymphocytes. It has remained unknown which cytokines are beneficial for cancer therapy when they are expressed in CTL. In this report, using newly developed recombinant adenoviral vectors, we have achieved highly efficient gene transfer into tumor-specific CTL. Expression of the IL-2 gene in the CTL enhanced their antitumor activity in vivo.

Materials and Methods

Tumor Cell Lines and Animals. B16F10, a metastatic subline of murine melanoma B16, was obtained from Dr. Glenn Dmanoff at the Whitehead Institute for Biomedical Research, Cambridge, MA. B16 and B16F10 cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 2 mM l-glutamine. Female C57BL/6 × DBA/2 F1 (hereafter called BDF2F1) mice, purchased from Charles River Japan, Atsugi, Japan, were used at the age of 6 to 8 weeks.

Preparation of TIL. Preparation of TIL was done as described (14—16) with some modifications. Freshly digested B16 melanoma tumor was suspended at 5 × 106 cells/ml in CM at 4°C. Cells were mixed with an equal volume of anti-CD8-conjugated immunomagnetic beads at 1 × 107/ml and incubated for 2 h at 4°C. Beads with attached cells were pelleted, washed three times with cold CM, suspended at 1 × 107 beads/ml in CM, plated in 24-well tissue culture plates, and incubated at 37°C in 5% CO2. By day 1, the beads, which had separated from the cells, were pelleted and discarded. All cultures were stimulated on day 1 with 2 × 105 irradiated (10,000 rads) tumor cells and 1 × 106 irradiated (3,000 rads) normal mouse splenocytes per well. The in vitro stimulation was repeated every 7 to 14 days. Cultures were split when confluent and were replated at 2 × 106 cells/ml in fresh CM. Cultures received fresh CM every 2 to 3 days.

Irradiation of Cells. Tumor cells and splenocytes were irradiated with a HITACHI MBR-1505R X-ray generator.

Flow Cytometry. Flow cytometry of cells was performed with a FACScan (Becton-Dickinson). The anti-mouse CD3 monoclonal antibody MAb1442 was purchased from Chemical International, Inc. Hybridoma cell lines which produce anti-mouse CD8 (53-6.72; ATCC TIB105), anti-mouse CD4 (GK1.5; ATCC TIB207), anti-mouse NK (PK136; ATCC HB191) were purchased from the ATCC. Ascites fluids containing monoclonal antibodies were prepared as described (17).

IL-2 Bioassay. IL-2 bioassay was done as described (18). Briefly, CTL were plated in 96-well tissue culture plates at 1 × 104 cells/well in a final volume of 0.2 ml growth medium supplemented with various concentrations of IL-2. After incubation for 20 h, cells were pulsed with 1 μCi [3H]thymidine/well for well and incorporation of the isotope was measured 4 h later.

Cytotoxicity Assay. Cytotoxicity against B16 melanoma cells were assessed using β-galactosidase enzyme assay. First, B16 cells were marked with a reporter lacZ gene by retroviral infection. Approximately 90% of the cells expressed β-galactosidase. 3 × 104 B16 cells were cocultured with various numbers of the TIL for 18 h in 24-well plates. Then the detached tumor cells were discarded and the β-galactosidase activity of the B16 cells which remained adhesive on the plates was assayed by the method described (19). The percentage of the detached cells were calculated as

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\% \text{of detached cells} = 1 - \frac{\text{data of well without effector cells}}{\text{experimental data}}\times 100
\]

51Cr Release Assay. CTL-mediated cytotoxicity against YAC-1 lymphoma cells (ATCC; TIB160) and P815 mastocytoma cells (ATCC; TIB64) was measured by the standard 4-h 51Cr release assay as described (20). The anti-mouse anti-CD3 monoclonal antibody from the hybridoma 145-2C11 (ATCC: CRL1975) was used at a final concentration of 1/4000 dilution of ascites.

Retrovirus-mediated Gene Transduction. To introduce a marker gene into cultured cells, we used pSV2/MPGlamZ (6, 21), which produces the replication-defective retrovirus containing the lacZ gene. Retrovirus-mediated gene transduction was carried out as described previously (6). To estimate the percentage of cells expressing the newly introduced gene, we assayed duplicate cell culture plates for the presence of β-galactosidase by using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside as substrate. Cleavage of this substrate by β-galactosidase yields a blue precipitate, which results in diffuse staining of transfected cells.

Adenovirus-mediated Gene Transduction. The recombinant adenoviruses Adex1CAcMIL2 and Adex1CAcMIL7, which encode...
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Escherichia coli lacZ, murine IL-2 and IL-7, respectively, were constructed by homologous recombination between the expression cosmid cassette and the parental virus genome. The method of generating recombinant adenovirus was a modification of the method of Saito et al. (22) and the detailed procedure will be published elsewhere. Briefly, an expression cosmid cassette was constructed by inserting the expression unit (23), composed of the cytomegalovirus enhancer plus chicken β-actin promoter, a complementary DNA coding sequence, and the rabbit β-globin polyadenylate signal sequence, into the Sall site of pAdex1w which is a 42-kilobase cosmid containing a 31-kilobase adenovirus type 5 genome lacking E1A, E1B, and E3 genes. The expression cosmid cassette and adenovirus DNA-terminal protein complex were cotransfected into 293 cells (ATCC; CRL1573) by calcium phosphate precipitation. Incorporation of the expression cassette into the isolated recombinant virus was confirmed by digestion with appropriate restriction enzymes. The recombinant viruses were subsequently propagated with 293 cells and viral solution was stored at −80°C. The titers of viral stocks were determined by plaque assay on 293 cells. For in vitro infection of adenoviruses, medium was discarded from the cells seeded in 12-well culture plates, and 150 μl of viral stock were added to each well. After incubation for 1 h at 37°C, growth medium was added and cells were cultured for 2 to 3 days.

Lung Metastasis Model. B16F10, a metastatic subline of B16, was used for the lung metastasis model. Seven days after in vitro stimulation, the TIL were infected with the recombinant adenovirus at a multiplicity of infection of 500. Two days after the gene transduction, the TIL and 4 × 10⁵ B16F10 cells at an effector/target ratio of either 1 or 10 were injected i.v. After 16 days, mice were sacrificed and the lung metastatic tumor nodules in the lungs were counted on microscopic observation.

Intracerebral Tumor Model. BD2F1 mice received transplants into the right parietal lobe of the brain of 1 × 10⁶ B16 cells mixed with 3 × 10⁶ cells of the CD8+ TIL with or without gene transduction. The antitumor effect was assessed according to the survival of the mice.

**Results and Discussion**

CD8+ TIL were isolated from s.c. B16 melanoma tumor by using immunomagnetic beads. The cells were cultured with periodic in vitro stimulations with irradiated B16 tumor cells and mouse spleen cells. Flow cytometry revealed that the TIL consisted of Thy1-, CD3-, and CD8-positive, CD4- and NK-negative T lymphocytes (Fig. 1A). The CD8-positive TIL were IL-2 dependent as determined by [3H]thymidine uptake assay (Fig. 1B, a). Half-maximal growth stimulation was obtained at 4 lU/ml of IL-2. By microscopic observation, the B16 tumor monolayer was substantially damaged by the TIL after 1 day and completely disappeared after 2 days of cocultivation. Cytotoxicity assay using the β-galactosidase enzyme test confirmed this microscopic observation (Fig. 1B, b). Murine YAC-1 lymphoma cells, which are susceptible to natural killer activity, were not damaged by the CD8+ TIL (Fig. 1B, c). P815 mastocytoma cells, which are often used as target cells of CTL, were killed by the CD8+ TIL only when they were cocultured in the presence of anti-CD3 antibody (Fig. 1B, c). Taken together, these results indicate that the CD8+ TIL were CTL with specific cytotoxicity against B16 melanoma cells.

To develop an animal model for adoptive immunogene therapy, we attempted to genetically modify the CD8+ TIL. Retrovirus-mediated gene transduction is widely used in human gene therapy protocols (5). Using a reporter recombinant retrovirus MFGlacZ (Fig. 2a), we attained highly efficient gene transfer into murine fibroblasts (Fig. 2b), as well as B16 melanoma cells (data not shown). In contrast, the gene transduction efficiency of the murine CD8+ TIL was very low, resulting in less than 1% lacZ-positive CD8+ TIL (Fig. 2c). Cocultivation of the CD8+ TIL with the retrovirus producer pCRIP/MFGlacZ cells was tried, also resulting in less than 1% gene transduction. Thus, the efficiency of retrovirus-mediated gene transfer into murine
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We next tried an adenoviral vector derived from human adenovirus type 5 (Fig. 2d). With a reporter adenovirus Adex1CAlacZ, lacZ gene expression was observed in nearly 100% of the CD8+ TIL (Fig. 2f). We also attained efficient gene transduction of murine CD4+ TIL and primary-cultured lymphocytes from lymph nodes, indicating that the feasibility of gene transduction by the recombinant adenovirus is not limited to the CD8+ TIL (data not shown).

Since IL-2 and IL-7 are known as potent stimulators for CTL, recombinant adenoviruses encoding murine IL-2 and IL-7 were generated for in vivo studies. By infection of these viruses, we obtained gene-modified TIL that secrete more than 3000 IU/ml/10^6 cells/24 h of IL-2 and 2 ng/ml/10^6 cells/24 h of IL-7, respectively, while the nontransduced TIL produced undetectable level of these cytokines. Treatment of mice with the TIL genetically modified to produce IL-2 resulted in further reduction in the number of metastatic tumor nodules than the nontransduced TIL, while IL-7 gene transduction had no effect (Table 1). In the i.c. B16 tumor model, mice treated with the...
Gene transduction into other effector cells such as CD4+ CTL and natural killer cells could also enhance their antitumor activities. Genetic modification of antigen-presenting cells such as dendritic cells and macrophages may provide an insight into the recognition mechanisms of tumor antigens.

Our animal studies showed that IL-2 gene transduction made CTL more potent in their in vivo antitumor activity, suggesting the feasibility to apply the method for the treatment of clinical tumors. Micrometastases or minimal residual tumor cells after surgical resection of primary tumors are candidate targets for the adoptive immunogene therapy. Further investigations are necessary concerning the method of gene transduction, the choice of effector cells and genes to be introduced, as well as the appropriate combination with vaccine therapy. Our animal studies will provide basic data for the development of gene therapy of human cancers.

**References**

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