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In Vivo Antitumor Effect of Cytotoxic T Lymphocytes Engineered to Produce Interferon-γ by Adenovirus-Mediated Genetic Transduction

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Immunotherapy with adoptive transfer of genetically-modified cytotoxic T lymphocytes (CTL) is a promising approach for cancer gene therapy. We developed an adoptive therapy model with murine tumor-specific CTL, to which very efficient (up to 100%) gene introduction was achieved by using recombinant adenoviral vectors. Through a comparative study on the antitumor effects of CTL genetically modified with cytokine genes, transduction with interferon-γ gene resulted in a prominent increase in therapeutic efficacy of CTL in both metastatic and subcutaneous tumor models. Further additive effect was obtained by the adoptive cellular therapy in combination with vaccination of cytokine gene-modified tumor cells. Our findings provide a hopeful strategy of adoptive immunotherapy for human cancers.

Along with vaccination using tumor cells as antigens, adoptive cellular therapy is a major strategy of cancer immunotherapy (1–3). In the adoptive immunotherapy, autologous (or syngeneic) immunocompetent cells are expanded in vitro, and transferred to the tumor-bearing host (1,2). During the period of ex vivo cell culture, antitumor immune response is selectively augmented, leading to circumvention of immunosuppressive conditions which are often present in tumor-bearing host (reviewed in ref. 4). A marking study demonstrated, however, that adoptively transferred tumor-infiltrating lymphocytes (TIL) were rapidly cleared from the circulation and inefficiently localized to tumor sites, exerting an only insufficient therapeutic efficacy (5). In the face of the unsuccessful results of clinical studies, it is essential to develop novel modalities to improve therapeutic efficacies of the adoptive therapy (6,7).

Genetic transduction of the effector cells with cytokine genes has been proposed as a promising approach to improve antitumor activity, although investigations involving genetic modification of effector T cells have been hampered by the technical difficulties (6). In our previous report, we achieved highly efficient gene transfer into murine TIL using recombinant adenoviral vectors, and demonstrated that the production of IL-2 in the TIL enhanced the efficacy of adoptive therapy (7). In this report, we attempted to determine the cytokine which was beneficial for adoptive cellular therapy when expressed in cytotoxic T lymphocytes (CTL). Treatment of mice with the CTL producing interferon-γ resulted in an efficient suppression of tumor growth.

MATERIALS AND METHODS

Tumor cell lines and animals. B16F10, a metastatic subline of murine melanoma B16 originally developed by I. Fidler (8), was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 2 mM glutamine. Female C57BL/6 mice, purchased from Charles River Japan, Atsugi, were used at the age of 6 to 8 weeks.

Preparation of TIL. Preparation and characterization of TIL were described previously (7).

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Abbreviations: CTL, cytotoxic T lymphocytes; TIL, tumor-infiltrating lymphocytes; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; APC, antigen-presenting cells; E/T, effector to target ratio.

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Virus-mediated gene transduction. Retrovirus-mediated granulocyte-macrophage colony-stimulating factor (GM-CSF) gene transduction was carried out using the amphotrophic packaging cell line ψCRIP-MFGmGMCSF as described (9). We obtained GM-CSF-secreting B16 cells at 16.6 ng/m1/10 ^{6} cells/48 hr, while nontransduced B16 produced undetectable level of GM-CSF. The recombinant adenoviruses were constructed by homologous recombination between the expression cosmid cassette and the parental virus genome, propagated with 293 cells (ATCC, CRL1573), and titrated by plaque assay on 293 cells as described (7,10).

Treatment model against lung metastasis. For adoptive therapy, TIL were infected with recombinant adenovirus as described (7) at a multiplicity of infection (m.o.i.) of 500 and used 24 hr after the infection. Two days after the challenge with intravenous injection of tumor cells, genetically modified TIL at various E/T ratios were injected intravenously. For vaccination with tumor cells, irradiated (10,000 rads) genetically modified tumor cells (5×10^5) were prepared as described (9) and injected subcutaneously in the left flank of mice 2 days after the challenge. Sixteen days after the B16 challenge, mice were sacrificed and metastatic tumor nodules in the lung were counted under microscopic observation. Animal experiments were repeated at least twice. Statistical analysis was performed by the Mann-Whittney's U test.

Treatment model against subcutaneous tumor. Mice were challenged subcutaneously in the right flank with 2×10^5 B16F10 cells. Two days after the challenge, mice were treated with intravenous injection of TIL with or without genetic transduction. Tumor growth was monitored by measuring the longest diameter and the perpendicular diameter of the mass, and scored by using the formula $(0.4)(a \times b^2)$ where *a* being the longer diameter and *b* being the shorter diameter (11). Mice were sacrificed when challenge tumors exceeded 2 cm (longer diameter) or severe ulceration or bleeding developed as described previously (9). Animal experiments were repeated at least twice.

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

Flow cytometric analysis. Flow cytometric analysis of cells was performed using FACScan (Becton-Dickinson) as described (7). The R-phycoerythrin (R-PE)-labelled anti-H-2K^b (AF6-88.5) and FITC-labelled anti-H-2D^b (KH95) monoclonal antibodies were purchased from Pharmingen (San Diego, CA).

Quantitative measurement of murine interferon-γ. This was done by using an in vitro enzyme-linked immunosorbent assay kit obtained from Endogen, Cambridge MA.

RESULTS

Cytokine gene transduction of CD8+CTL. CD8+CTL with specific antitumor activities were prepared from tumor-infiltrating lymphocytes (TIL) of murine B16 melanoma as described by Nakamura et al. (7), and designated TIL/B16K (subclone K derived from TIL/B16) (7). CTL were cultured with periodical in vitro stimulations with irradiated tumor cells and mouse spleen cells. The phenotypic characters of the CTL used in this study were essentially the same as reported previously (7) (i.e., CD8+ immunophenotype, IL-2-dependent growth, specific cytotoxicity againts the tumor).

To study the effect of interferon-γ expressed in CD8+CTL, we generated a recombinant adenoviruses derived from human adenovirus type 5 with expression cassettes (10,12) containing murine interferon-γ cDNA essentially as described previously (7). High titer viral stock solutions with more than 10^9 plaque-forming unit (pfu)/ml were obtained, which enabled us to perform highly efficient (nearly 100%) genetic transductions of murine CTL as confirmed by X-gal staining of the lymphocytes transduced with a reporter lacZ adenovirus (7). While the interferon-γ secretion in vitro from nontransduced CTL was undetectable, that from the interferon-γ gene-modified CTL was as high as 110 ng per 10^5 cells per 48 hr.

Treatment with gene-modified CTL. TIL/B16K demonstrated only an insufficient antitumor effect when they were transferred to mice bearing B16 metastatic tumors in the lung (7). Interferon-γ gene transduction of CTL induced marked reduction of metastatic nodule formation (Fig. 1a). In an experimental condition where the effect of nontransduced CTL was 10–20% reduction, the CTL transduced with interferon-γ exerted 60–75% reduction of lung metastases ($p < 0.01$ compared with nontransduced CTL). Experiments were repeated for five times and the interferon-γ gene transduction was reproducibly more effective than the IL-2 gene transduction ($p < 0.05$).

Next we examined the therapeutic effect of cytokine gene transduction of CTL on their activities against established subcutaneous B16 tumor. As shown in Fig. 1b, TIL/B16K without genetic transduction (E/T 30) revealed only a limited inhibitory effect against established solid tumor. The IL-2 gene transduction gave a significant increase in antitumor activity of CTL ($p < 0.05$ compared with nontransduced CTL on day 16). The most prominent antitumor effect was obtained by the

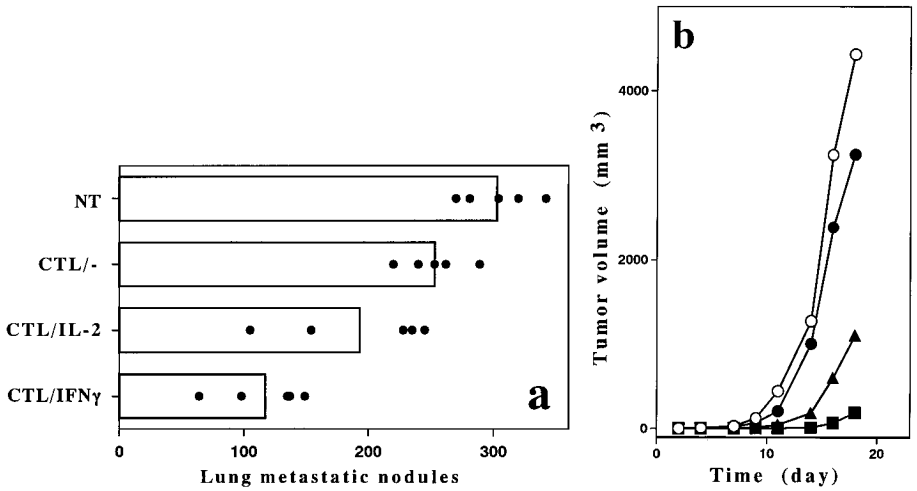

FIG. 1. Therapeutic effect of CTL transduced with IL-2 or interferon-γ. **a,** Treatment against B16 lung metastasis. Mice were intravenously injected with 3×10^5 B16 cells, followed 2 days later by intravenous injection of 4.5×10^6 of TIL/B16K (E/T 15) without gene transduction (CTL/−), with IL-2 gene transduction (CTL/IL-2), or with interferon-γ gene transduction (CTL/IFNγ). Control mice were injected with the same volume of saline (nontreated, NT). The points represent the number of metastatic nodules of individual mouse, and the bar shows the mean of five determinations. **b,** Treatment against B16 subcutaneous tumor. 2×10^5 B16 cells were transplanted subcutaneously into mice. On day 2, mice were confirmed of the presence of visible subcutaneous tumors, randomized into groups each consisting of five, and treated with 6×10^6 TIL/B16K (E/T 30) with or without genetic transduction. The data represent the mean tumor volume of each group treated with control saline (&hb9;), with nontransduced TIL/B16K (&sb9;), with TIL/B16K transduced with either IL-2 (▲) or interferon-γ (■) gene.

CTL transduced with interferon-γ gene. Almost complete suppression of tumor growth was observed during the first two weeks after the administration of interferon-γ-producing CTL ($p < 0.01$ compared with the groups treated with IL-2 gene-modified CTL or nontransduced CTL on day 16)(Fig. 1b).

Serum concentrations of interferon-γ from mice treated with CTL with or without the cytokine gene transduction were monitored. The interferon-γ concentration was the highest during the initial 2 to 3 days after the injection of CTL transduced with interferon-γ; ∼8–10 ng/ml was observed by intravenous injection of 6×10^6 of the interferon-γ-producing CTL (Fig. 2). In contrast, mean serum concentration of interferon-γ from control mice injected with 6×10^6 of nontransduced CTL was 230 ± 230 pg/ml, which was comparable to the level with that of normal mice (120 ± 130 pg/ml), indicating that the rise in serum concentration of interferon-γ was due to the effect of the genetic modification of CTL.

In vitro effect of interferon-γ on B16 cells. Flow cytometry analysis showed that the B16 melanoma line used in this study was MHC class I negative (< 1% positive). However, both H-$2K^b$ and H-$2D^b$ were strongly induced in the B16 cells cultured in vitro for 24 hr in the presence of 10 ng/ml of interferon-γ (99.9% positive)(Fig. 3). The induction of the MHC class I molecules is one of the possible action mechanisms of interferon-γ leading to the in vivo therapeutic efficacy.

The in vitro growth of the B16 cells was only marginally suppressed in the presence of 10 ng/ml interferon-γ (data not shown). The systemic concentration of interferon-γ was, at the most, ∼10 ng/ml (Fig.2), which is unlikely to be high enough for the direct inhibition of the in vivo growth of B16 tumor. However, it remains possible that the interferon-γ-producing CTL could accumulate in the tumor lesions, resulting in much higher local concentration of interferon-γ which directly suppress the growth of B16 tumor.

Adoptive transfer of CTL in combination with gene-modified tumor vaccination. In the previous

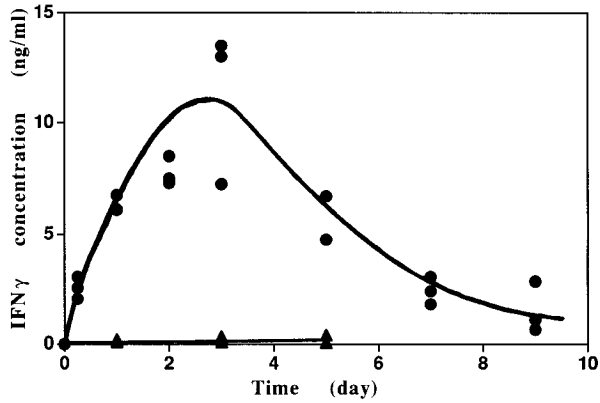

FIG. 2. Serum concentration of interferon-γ in mice injected with CTL. Mice were injected intravenously with 6×10^6 TIL/B16K (E/T 30) with or without interferon-γ gene transduction. Each point demonstrates the serum concentration of interferon-γ of an individual mouse administered with nontransduced TIL/B16K (▲), or TIL/B16K transduced with interferon-γ gene (&sb9;). Experiments were repeated twice and similar results were obtained.

studies on cytokine gene-modified tumor vaccination, a remarkable antitumor response was induced by tumor cells transduced with GM-CSF gene (9.13) In this study, we examined the effect of interferon-γ gene-transduced CTL in combination with GM-CSF-producing B16 tumor vaccine in the lung metastatic model. Only a slight suppression (∼15%) of metastasis was observed when mice were treated with nontransduced CTL (Fig. 4). Vaccination with the GM-CSF-producing tumor vaccine demonstrated a significant therapeutic effect; ∼50% reduction of lung metastatic nodules was achieved. The combined use of the GM-CSF-producing tumor vaccine with the CTL significantly enhanced the antitumor activity. The interferon-γ gene-transduced CTL in combination with GM-CSF gene-modified tumor vaccine resulted in the most efficient suppression of metastatic nodule formation (∼85% reduction in the number of metastatic nodules; $p < 0.01$ compared with the group treated singly with the interferon-γ-transduced CTL; $p < 0.01$ compared with the group treated singly with the GM-CSF-producing tumor vaccine)(Fig.4).

DISCUSSION

In this report, the transduction of tumor-specific CTL with interferon-γ gene demonstrated a prominent augmentation of antitumor immunity in the adoptive cellular therapy in B16 melanoma model (Fig. 1). Although the IL-2 gene transduction also showed increases in antitumor activity of CTL (Fig. 1; ref. 7), the antitumor effect by the CTL augmented by the interferon-γ gene transduction was superior to that by the IL-2 gene (Fig. 1).

Interferon-γ is a pleiotropic cytokine with a number of actions on many cell types (for reviews, see ref. 14,15). Recombinant interferon-γ has been reported to demonstrate antitumor activity when administered systemically to tumor-bearing hosts (14,15). Previous reports showed an increase in tumor suppression by a glioma-specific murine CTL line producing interferon-γ (16,17). Several mechanisms are attributable to the efficacy of interferon-γ gene transduction of CTL in our adoptive therapy model: 1) interferon-γ could activate the adoptively transferred CTL in an autocrine or a paracrine manner; 2) interferon-γ could regulate specific effector mechanisms by direct actions on host helper T cells, NK cells or cytotoxic T cells; 3) amplified expression of MHC class I molecules on the tumor cells could enhance the host antitumor response as well as the tumor susceptibility to CTL; 4) interferon-γ would upregulate the expression of MHC class II or costimulator molecules (e.g., B7-1)(18) in the host antigen-presenting cells (APC; i.e., monocytes/macrophages, dendritic cells) and/or the nonprofessional APC (i.e., epithelial, endothelial, and connective tissue cells), leading to effective tumor-antigen presentation to T lymphocytes; 5)

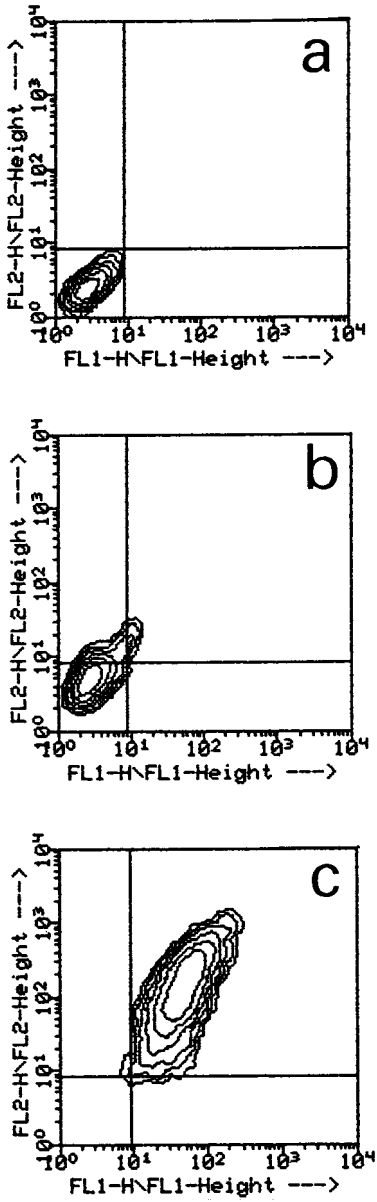

FIG. 3. Induction of MHC class I by interferon-γ. The data demonstrate the flow cytometric analysis of B16 cells cultured in the presence of 10 ng/ml murine interferon-γ for 0 hr, **a;** 4 hr, **b;** and **c,** 24 hr. Ordinate, fluorescence intensity stained with R-phycoerythrin (R-PE)-labelled anti-H-$2K^b$; abscissa, fluorescence intensity stained with FITC-labelled anti-H-$2D^b$.

interferon-γ could induce the expression of specific tumor-associated antigens on tumor cells which are recognized by host immunocompetent cells. In addition to the effects similar to those of the systemic administration of recombinant cytokines, more advantageous therapeutic effects could be anticipated in the adoptive transfer of the gene-modified CTL as a cytokine delivery system. Tumor-specific CTL transduced with cytokine gene(s) could possibly localize in the tumor lesions and/or regional lymph organs, leading to a high-dose local cytokine delivery to tumor cells as well as host immunocompetent cells. Since systemic concentration of the cytokine could remain rela-

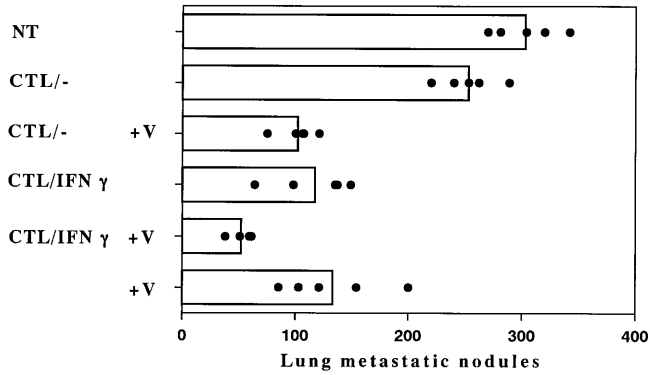

FIG. 4. Therapeutic effect of adoptive therapy in combination with GM-CSF-producing tumor vaccine in the B16 lung metastasis. Mice were first intravenously injected with 3×10^5 B16 cells. Two days after the challenge they were intravenously injected with 4.5×10^6 of TIL/B16K (E/T 15) without gene transduction (CTL/−), or with interferon-γ gene transduction (CTL/IFNγ). Control mice were injected with the same volume of saline (nontreated, NT). For vaccination with tumor cells, irradiated (10,000 rads) GM-CSF-producing tumor cells (5×10^5) were inoculated subcutaneously in the left flank of mice 2 days after the challenge, either alone (V) or in combination with adoptive cellular therapies (CTL/− + V or CTL/IFNγ + V).

tively low in the face of a very high local concentration, adverse effects encountered in the high-dose systemic administration (19–22) could be controlled to minimal levels without losing the therapeutic efficacies.

A potent therapeutic effect was obtained by the combination of the interferon-γ-producing CTL and the GM-CSF gene-transduced tumor vaccine (Fig. 4). Administration of the irradiated gene-modified tumor cells could work as a specific immunomodulator, keeping the CTL in an activated state with specificity against tumor antigens. GM-CSF is supposed to stimulate the professional APC of the host (9,13), leading to a tumor-specific activation of CD4+ helper T cells, which could eventually stimulate the tumoricidal activities of the CD8+CTL. The findings could have implications to the clinical application of specific immunotherapy for cancers. Since the mechanisms for tumor rejection could involve a highly regulated host immune system, therapeutic advantages may not necessarily be attained by simply increasing the dosage of a single therapeutic advantages may not necessarily be attained by simply increasing the dosage of a single therapeutic modality (i.e., adoptively transferred CTL, or gene-modified tumor cell vaccines). It would be desirable to perform the adoptive therapy accompanied with a tumor-specific immunization (i.e., gene-modified tumor cell vaccination or tumor antigen-based vaccination)(6), which would support the specific antitumor activity of the adoptively transferred T lymphocytes.

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