Intensification of Antitumor Effect by T Helper 1-dominant Adoptive Immunogene Therapy for Advanced Orthotopic Colon Cancer

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ABSTRACT

Purpose: T helper (Th) 1/Th2 balance, controlled by Th1 or Th2 cells producing cytokines, plays important roles in antitumor immunity. Interleukin-18 (IL-18) and IL-12 in promoting the generation of IFN-γ producing Th1 cells. The goal of this study was to determine whether cytotoxic T lymphocyte (CTL) secreted in a murine IL-18-induced Th1-dominant state inhibited the development of primary tumors and synchronous liver metastases in orthotopic colon cancer model.

Experimental Design: Murine IL-18 gene was transduced into activated T lymphocytes by an adenovirus vector encoding IL-18 (AdIL-18) liposome complex method. Efficacy of adoptive immunogene therapy using AdIL-18 with or without IL-12 was tested in advanced orthotopic xenograft of murine colon cancer. To elucidate the mechanism responsible for the adoptive immunogene therapy, serum IL-4, IL-6, IFN-γ, and tumor necrosis factor α production in Th1/Th2 cytokine balance and quantification of tumor vascularization were investigated.

Results: By a modified method of adenoviral gene transduction, T lymphocytes achieved efficient IL-18 production without cell toxicity. Against orthotopic colon cancer, when combined with low dose of recombinant (r) IL-12 (AdIL-18-CTL/rIL-12), the therapeutic efficacy showed much smaller tumor growth compared with the group treated with AdIL-18-CTL alone or other group (P < 0.01). In addition, the median survival time of the group treated with AdIL-18-CTL was 53.7 ± 5.8 days and that of AdIL-18-CTL/rIL-12 was 78.4 ± 6.1 days, which was also a significant difference (P < 0.01). These antitumor mechanisms were involved with Th1-dominant response in serum Th1/Th2 cytokine balance and suppression of neovascularization at primary tumor site.

Conclusion: These data suggest that a strategy of Th1/Th2 balance-based adoptive immunogene therapy might be useful for advanced cancer patients.

INTRODUCTION

A variety of adoptive cellular strategies, aimed at boosting the immune system, have been tested in the management of malignant diseases. Despite the drawbacks associated with in vitro cell manipulation and upscaling, several such approaches have been assessed in the clinic. The use of LAK cells, CTLs, and tumor infiltrating lymphocytes has been well studied, and additional trials are ongoing (1). Thus far, these approaches have not consistently shown benefits when compared with standard immune-based treatment with biological response modifiers, notably, high-dose IL-2 (2). More recently, it has been shown in various animal models that the in vitro transfer of genes to cells of the immune system can have a dramatic impact on cancer immunotherapy. The application of gene transfer techniques to immunotherapy has animated the field of cell-based cancer therapy research. In vitro strategies include gene delivery into tumor cells and into cellular components of the immune system, including T lymphocytes (3), NK cells, macrophages, and dendritic cells (4).

Historically, it has been demonstrated that CTLs recirculate and preferentially localize at the tumor site several days after adoptive transfer (5). For this reason, CTLs may be used as cellular vesicles to deliver some antitumor molecules to the tumor site. The neomycin phosphotransferase (NEO) gene has been successfully introduced and expressed in the T cells, and these gene-modified T cells have been safely transferred to cancer patients (6). Moreover, it has been reported that human T cells have been retrovirally transduced with the gene for TNF-α in an attempt to deliver a high concentration of TNF-α to the tumor site (7). We also have succeeded previously in genetically modifying T lymphocytes with human IL-2 by using the retrovirally double chamber method (8).

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2 The abbreviations used are: LAK, lymphokine activated killer; CTL, cytokotic T lymphocyte; IL, interleukin; NK, natural killer; TNF, tumor necrosis factor; Th, T helper; AdIL-18, adenoviral vector encoding murine interleukin 18; MACS, magnetic-activated cell sorting; MFI, multiplicity of infection; r, recombinant; PE, phycoerythrin; HSE, hepatic sinusoidal endothelial.
To succeed in immunogene therapy for malignancies, it is important to choose a suitable kind of cytokine to transduce into target cells. IL-18 is an attractive cytokine, which was initially identified as a cytokine that facilitates the production of IFN-γ induced by endotoxin (9). It is an essential factor for IFN-γ production in response to microbial agents, and can act together with IL-12 in promoting the generation IFN-γ-producing Th1 cells (10). In IL-12-deficient mice, there are defects in the generation of Th1 response (11). These results indicate that IL-18 plays an essential role in inducing the Th1 response in vivo.

Furthermore, recent studies have demonstrated that the Th1/Th2 balance controlled by Th1 or Th2 cell producing cytokines plays important roles in various immune responses, including antitumor immunity (12). These include Th1 cells producing IFN-γ and IL-2 that are essential for the induction of cellular and tumor immunity, whereas Th2 cells producing IL-4, IL-6, and IL-10 are associated with suppression of cytolytic activity (13, 14). Several studies have shown that Th2 type cytokine production increased in the peripheral blood of tumor-bearing mice or cancer patients (15, 16). In particular, great changes in cytokine environments and subsequent immune dysfunction have been demonstrated in advanced malignant disease (17, 18), and progression of the malignant stage is related to an increase in serum levels of IL-6 and transforming growth factor β (19). Consistent with the notion that Th1 cells are essential to induce antitumor immunity, preliminary studies have been examined (20–23). Actually, administration of IL-18 with or without IL-12 has significant antitumor potency for murine s.c. tumors. However, combination therapy with rIL-18 and rIL-12, when administered i.p., induces an immune response with enhanced IFN-γ production and significant toxicity leading to death of the treated animals (23). In the development of therapeutic strategies to circumvent these problems, alternative gene transfer methods able to overcome the limitation of cytokine therapy must be identified.

Hence, if T lymphocytes can be genetically modified to produce IL-18, they may secrete sufficient amounts of IL-18 in the microenvironment of the tumor site to enhance the antitumor activity of resident immune-competent cells (helper function); this would thus reduce toxicity because of the amount of IL-18 administered systemically.

In the current study, to enhance the antitumor effects of IL-18 administration without such severe side effects, we examined adoptive immunogene therapy using AdIL-18 as an alternative strategy for malignancies. In addition, to translate murine models into a clinical setting, we have evaluated the antitumor effect using an orthotopic colon cancer model, which resembles human colon cancer patients.

**MATERIALS AND METHODS**

**Mice.** Female BALB/c (H-2b) mice (obtained from Crl SLC, Inc., Atsugi, Japan) were bred under specific pathogen-free conditions and used for experiments at the age of 7–10 weeks.

**Cell Lines and Reagents.** CT26 cells, a subline of the N-nitroso-N-methylurethane-induced BALB/c undifferentiated colon adenocarcinoma (C26), were kindly provided by Hoffmann-La Roche Co. Ltd. (Kamakura, Japan). The 293 cells (Ad5 E1-transformed human embryonic kidney cell line) and YAC-1 cells (NK cell-susceptible targets) were purchased from American Type Culture Collection (Rockville, MD). Cells were cultured in DMEM (Nissui Pharmaceutical, Co., Tokyo, Japan) supplemented with 10% heat-inactivated FCS and antibiotics at 37°C in a humidified 5% CO₂ atmosphere. Supplements were purchased from Life Technologies, Inc. Murine rIL-18 was purchased from MBL (Nagoya, Japan), and murine rIL-12 was done from PharMingen (San Diego, CA).

**Construction of Recombinant Adenoviral Vectors.** Murine IL-18 cDNA was obtained by reverse transcription-PCR. Briefly, total RNA was prepared from spleens of lipopolysaccharide-treated mice, and the full length of IL-18 cDNA was amplified with primers: 5’-primer: GGGAATTCACCAT-GGCTGCCATGTCAAGACTCTTGTC and 3’-primer: CCGAATTCTAATTTGATGTAAGTTAGTGAGAGT.

These enclosed the EcoRI restriction sites by the method of reverse transcription-PCR. 5’- and 3’- primers should amplify the published full-length cDNA (9). The mL-18 cDNA clone we used was sequenced to confirm normal coding potential. After then, the fragment encoding the leader peptide of murine IL-1ra was fused to the sequence encoding mL-18 cDNA, and this fragment was termed IL-1ra/mL-18 (24). Recombinant replication-defective adenoviral vectors harboring the IL-1ra/ mL-18 gene were prepared according to the COS-TPC method (Taraka Shuzo Co., Ltd., Kyoto, Japan). Briefly, an expression cosmid cassette was constructed by inserting the expression unit composed of a cytomegalovirus immediate early enhancer, a modified chicken β-actin promoter (25), a cDNA coding sequence and rabbit β-globin polyadenylacylic acid signal sequence into the SwaI site of pAxCAwt, which is a 42-kb cosmid containing the 31-kb adenovirus type 5 genome lacking E1A, E1B, and E3 genes. The expression cosmid cassette and adenoviral DNA-terminal protein complex were cotransfected into 293 cells by the calcium phosphate precipitation method (26).

The generated vector was designated AdIL-18. All of the recombinant viruses were propagated with 293 cells, and purified by two rounds of CsCl density centrifugation, dialyzed, and stored at −80°C as described previously (27). The titers of viral stocks (pfu/ml) were determined by plaque assay on the 293 cells. All of the viral stocks were checked for the presence of replication-competent adenoviral vector by PCR (28). In none of the stocks of adenoviral vectors used in this study was contamination of replication-competent viruses detectable.

**Preparation and Activation of T Lymphocytes.** Mice with s.c.-administered tumor cells served subsequently as donors of lymphocytes for adoptive transfer experiments. These mice were killed 14 days after tumor cell inoculation, followed by the preparation of single-cell suspensions by mincing freshly resected spleen and passing it through a steel sieve. Lymphocytes were separated by Ficoll-Hypaque gradient centrifugation (600 × g, 20 min). Subsets of CD8+ T cells were isolated by MACS (Miltenyl, Auburn, CA). Briefly, lymphocytes were labeled with paramagnetic anti-CD8+ Micro Beads and isolated with a Mini MACS separation unit, according to the manufacturer’s guidelines. The CD8+ T cells were suspended at 2 × 10⁶/ml in the CTL culture medium. The CTL culture medium consisted of RPMI 1640 with 10% heat-inactivated FCS, 2 mM...
l-glutamate, 5 × 10⁻⁵ M 2-mercaptoethanol, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.5 μg/ml amphotericin B, 10 mM 3-(N-morpholino)propanesulfonic acid, and murine rIL-2 (20 units/ml) plus rIL-12 (100 units/ml). All of the cultures were stimulated on day 1 with 2 × 10⁵ mitomycin C (at a concentration of 100 μg/ml)-treated tumor cells and 1 × 10⁶ mitomycin C-treated normal murine splenocytes per well. The in vitro sensitization was repeated every 7–14 days. Cultures were split when confluent and replated at 2 × 10⁶ cells/ml in fresh culture medium. Cultures received fresh medium every 2–3 days. Afterwards, cells were harvested and used sequentially for the following experiments in vitro and in vivo. Flow cytometry analysis was performed with a FACScan (Becton-Dickinson), using FITC-conjugated antimurine CD3 (145–2C11), PE- or FITC-conjugated antimurine CD8 (53–6.7), and PE-conjugated antimurine CD4 (RM4–5). For each staining, we used isotype matched FITC- or PE-conjugated antimurine monoclonal antibodies as negative controls. Monoclonal antibodies were purchased from PharMingen.

Adenoviral-mediated Gene Transfer into T Lymphocytes. Activated T lymphocytes (5 × 10⁵) were suspended in 0.5 ml of serum-free RPMI 1640, and adenovirus stock was added at various MOIs for 1 h at 37°C in a humidified atmosphere 5% CO₂ in air. After incubation, 0.5 ml of RPMI 1640 containing the same cytokines used in in vitro sensitization and 10% heat-inactivated FCS was added. To additionally increase the efficiency of transduction, we added LipofectAMINE (Life Technologies, Inc., Grand Island, NY) together with adenoviral vector. LipofectAMINE is a cationic lipid that should increase the uptake of viral particles into the cells by promoting their binding to the cell surface. Briefly, AdIL-18-LipofectAMINE complex was formed by mixing 15 μl of LipofectAMINE (30 μg) with AdIL-18 at MOI range from 100 to 1000 in 0.5 ml of serum-free RPMI 1640. The mixture was incubated at room temperature for 30 min and then added to target cells. After incubation at 37°C in a 5% CO₂ incubator for 2 h, the cells and virus mixture was washed twice in PBS. After 48 h of culture, cells were harvested and used for the following experiments.

Evaluation of Viable Cells and Murine IL-18 Production in Vitro. Secretion of IL-18 in the culture medium from the IL-18 gene-transduced T lymphocyte was determined using ELISA kit (MBL, Nagoya, Japan). Briefly, the cells (5 × 10⁵/well) were infected in vitro with adenoviral vectors at MOI range from 100 to 1000. Viable cell counting was performed by trypan blue dye exclusion test. The supernatants were collected at 48 h after plating 5 × 10⁵ cells in a six-well dish containing 2 ml of medium and a quantitated amount of IL-18.

CTLs Assay. To measure the T lymphocyte-mediated cytotoxicity, the in vitro sensitization T lymphocytes or AdIL-18-CTL were used as effector cells, whereas ⁵¹Cr-labeled CT26 cells were used as target cells described previously (29). Briefly, 1 × 10⁶ parental CT26 cells were labeled with 100 μCi of Na₂¹⁵CrO₄ for 1 h at 37°C. The cells were then washed five times, and 1 × 10⁵ of labeled CT26 cells were added to each well of a 96-well round-bottomed plate. Effector cells were then added to triplicate wells at various E:T cell ratios in a final volume of 200 μl/well. The plates were incubated at 37°C for 4 h. Supernatant (100 μl) was collected from each well and counted in a gamma counter. The percentage of specific cytotoxicity was calculated as follows: % cytotoxicity = (experimental counts – spontaneous release)/(maximum release – spontaneous release) × 100. The spontaneous release did not exceed 10% of the maximum release obtained by adding 1 M HCl.

Experimental Design of in Vivo Tumor Therapy for Orthotopic Colon Cancer Models. To mimic the clinical scenario more closely, we evaluated whether the orthotopically transplanted tumor could be suppressed after adoptive transfer of T lymphocytes genetically modified to express mIL-18. Fresh surgical colon cancer specimens were obtained from resected s.c. tumors of CT26 tumor-bearing mice. Tumor specimens were aseptically removed, and after necrotic tissue and noncancerous tissue of the specimens were carefully cut away with scissors, the remaining cancerous tissue was divided into small pieces ~2 mm in diameter. The tumor species were surgically transplanted orthotopically to the cecum serosa in syngeneic BALB/c mice according to the method reported previously (30). Briefly, mice were anesthetized with a 2.5% solution of a tert-amylalcohol (1:1), and a small midline incision was made and the ileocecal part of the intestine was exteriorized. A tumor piece was then fixed on each injured site of the cecal surface with 6–0 Prolene sutures (Ethicon, Somerville, NJ). The intestine was then returned to the peritoneal cavity, and the abdominal wall and skin were closed.

For adoptive therapy, T lymphocytes were infected with AdIL-18 at a MOI of 500 and used 24 h after the infection. Seven days after the orthotopic transplantation of CT26 tumor tissues, the mice received i.v. administration of gene-modified CTL (at number of 5 × 10⁶) with or without i.p. administration.
of murine rIL-12 at a dose of 50 ng/day for 5 consecutive days. The mice were divided into four groups (n = 5 in each group) as follows: group A, PBS; group B, CTL; group C, AdIL-18-CTL, and group D, AdIL-18-CTL followed by rIL-12. Thirty-five days after the orthotopic transplantation, the mice were euthanized and examined to ascertain whether they had developed peritoneal dissemination and tumors in the cecum. Tumors on the mesentery and cecum were evaluated as to tumor weight. Their livers were then removed and fixed in 10% formalin neutral-buffered solution (pH 7.4). The numbers of metastatic foci on the liver surface were measured. To evaluate whether the therapeutic effects of the reduction of primary and metastatic tumors were reflected by a prolongation of the animal survival, we prepared and treated an additional set of mice by the protocol described above (n = 8 in each group) and evaluated their survival.

Cytokine Production Assay in Vivo. To measure systemic cytokine production, serum was collected when the mice were euthanized. Serum cytokine levels (IL-4, IL-6, IFN-γ, and TNF-α) were measured by murine ELISA kits (Endogen, Woburn, MA).

Quantification of Tumor Vascularity. For histological studies, primary tumors were harvested, fixed in 10% formalin, embedded in paraffin, sectioned, and stained with H&E. Slides were then examined under a light microscope, and the number of blood vessels per ×200 field was counted. Blood vessel counts/field were then averaged. For immunohistochemical staining, formalin-fixed, paraffin-embedded blocks were sectioned at 2 μm and stained with a rabbit antihuman von Willibrand factor (Dako Corp., Carpinteria, CA) antibody as described previously (31).

Statistical Analysis. Quantitative results were expressed as mean ± SD of the mean. Statistical analysis was performed by ANOVA and Fisher’s PLSD test using Statview 5.0 software (SAS Institute, Inc., Cary, NC). A P < 0.01 was considered significant.

RESULTS

Viability and Kinetics of IL-18 Production of Transduced Cells. As shown in Fig. 1, the highest cell viability and IL-18 production were obtained with an MOI of 500 (95.5 ± 1.2% viable cells and 12.5 ng/5 × 10^6/48 h). At higher MOI (=500), we obtained a negligible increase in IL-18 production but an increase in toxicity (74.3% viable cells). On the other hand, with lower MOI, the IL-18 gene transduced T lymphocytes (representing 2.63% viable cells) were sectioned at 2 μm and stained with a rabbit antihuman von Willibrand factor (Dako Corp., Carpinteria, CA) antibody as described previously (31).

Therapeutic Efficacy of Genetically Modified T Lymphocytes in Orthotopic Colon Cancer Models. To evaluate whether in vivo antitumor effect of genetically modified CTLs would be reflected on a more stringent and clinically relevant
examined whether they developed peritoneal dissemination and tumors days after the orthotopic transplantation, the mice were euthanized and AdIL-18-CTL; and 50 ng/day for 5 consecutive days. A, the range of administration dose of IL-12 was 1.0 administration of recombinant IL-12 (32, 33). In these reports, model, orthotopic colon cancer models were used. It has well described above (n = 8 in each group) and evaluated their survival.

Table 1 Responses of gene-modified CTLs for CT26 orthotopic cancer model

<table>
<thead>
<tr>
<th>Group/treatment</th>
<th>Primary tumor weight (g)</th>
<th>Number of hepatic metastases</th>
<th>Survival time (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: PBS</td>
<td>4.23 ± 0.93</td>
<td>14.0 ± 2.0</td>
<td>38.9 ± 3.2</td>
</tr>
<tr>
<td>B: CTL</td>
<td>3.04 ± 0.67</td>
<td>9.6 ± 1.8</td>
<td>44.7 ± 3.5</td>
</tr>
<tr>
<td>C: AdIL-18-CTL</td>
<td>0.89 ± 0.17</td>
<td>4.0 ± 1.5</td>
<td>53.7 ± 5.8</td>
</tr>
<tr>
<td>D: AdIL-18-CTL/rIL-12</td>
<td>0.16 ± 0.04×,b</td>
<td>0.1 ± 0.2×,b</td>
<td>78.4 ± 9.6×,b</td>
</tr>
</tbody>
</table>

× Significant difference was observed compared with PBS or CTL group (P < 0.05).
×,b Significant difference was observed compared with other groups (P < 0.01).

AdIL-18-CTL and rIL-12 Induced a Th1-dominant State in Orthotopic CT26 Tumor-bearing Mice. To elucidate the characteristics of the orthotopic CT26 tumor-bearing mice, we first estimated the ability of the tumors to induce abnormalities associated with serum cytokine level. After 35 days of orthotopic tumor implantation, the PBS-treated mice induced significant elevations of serum Th2 type cytokines such as IL-4 and IL-6, and suppressed serum IFN-γ. However, treatment with AdIL-18-CTL and rIL-12 showed a significantly high level of IFN-γ and suppression of Th2-type cytokine production (Table 2). In addition, these mice did not die of IFN-γ-related toxicity. In this experimental design, a significant difference of serum TNF-α production was not observed (data not shown).

Suppression of Tumor Neovascularization. We finally explored the possibility that AdIL-18-CTL initiates antitumor responses by a mechanism independent of direct cytotoxic cellular immunity. After euthanization of mice, the primary tumor was resected and stained with H&E. As shown in Fig. 4, A and B, AdIL-18-CTL-treated tumors clearly demonstrate a decrease in the number of intratumoral blood vessels compared with the PBS-treated control. In addition, to evaluate precisely the inhibitory effect of IL-18 on tumor angiogenesis, tumor tissue sections treated with AdIL-18-CTL were immunohistochemically stained with an endothelial-specific antibody against von Willebrand factor (Fig. 4, C and D), and microvessels in tumor tissues were randomly counted. Fig. 4E represents an average of vessel density from six random fields in tumors from mice of each group. A dramatically decreased microvessel density in tumor tissues was revealed in the AdIL-18-CTL-treated mice.

Fig. 3 Therapeutic effect of the IL-18 gene-transduced CTL for orthotopic colon cancer. Seven days after the orthotopic transplantation of CT26 tumor tissues, mice received i.v. administration of geno-modified CTL (at number of 5 × 10^7) with or without murine rIL-12 at a dose of 50 ng/day for 5 consecutive days. A, PBS as control; B, CTL alone; C, AdIL-18-CTL; and D, AdIL-18-CTL followed by rIL-12. Thirty-five days after the orthotopic transplantation, the mice were euthanized and examined whether they developed peritoneal dissemination and tumors in the cecum.
Adoptive Immunogene Therapy Inducing Th1-dominant State

Table 2 Cytokine production after treatment of IL-18 secreting CTL and rIL-12

A CT26 tumor specimen was implanted orthotopically into the cecal wall of syngeneic BALB/c mice. After 7 days of implantation, CT26 or AdIL-18-CTLs (at number of 5 × 10⁴) were administered i.t. into the tail vein. rIL-12 was administered i.p. for 5 consecutive days. After 28 days of intravenous administration, all of the mice were euthanized and the serum collected. Then the serum cytokine production was measured by ELISA.

<table>
<thead>
<tr>
<th>Group</th>
<th>IL-4 (pg/ml)</th>
<th>IL-6 (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>65.3 ± 10.3</td>
<td>500 ± 24.6</td>
<td>12.4 ± 5.64</td>
</tr>
<tr>
<td>CTL</td>
<td>49.2 ± 6.32</td>
<td>356 ± 45.3</td>
<td>27.2 ± 4.26</td>
</tr>
<tr>
<td>AdIL-18-CTL</td>
<td>31.3 ± 6.36ᵃ</td>
<td>231 ± 52.1ᵃᵇ</td>
<td>39.3 ± 6.27ᵃᵇ</td>
</tr>
<tr>
<td>AdIL-18-CTL+rIL-12</td>
<td>7.4 ± 3.89ᵃᵇ</td>
<td>104 ± 24.5ᵃᵇ</td>
<td>223.4 ± 12.2ᵃᵇ</td>
</tr>
</tbody>
</table>

ᵃ Significant difference was observed compared with the CTL group (P < 0.01).
ᵇ Significant difference was observed compared with the other groups (P < 0.01).

compared with that of those treated with PBS and CTL. Moreover, combination treatment of AdIL-18-CTL and rIL-12 had a stronger suppression compared with the other groups (P < 0.01).

DISCUSSION

We demonstrated previously that inoculation of mice with fibroblasts engineered to express IL-2 resulted in a failure of an otherwise inevitable tumor establishment, usually a consequence of a strongly induced CT26 tumor-specific T-cell response for the orthotopic CT26 colon cancer model (30). In the study reported here, we have additionally examined the impact of IL-18-mediated adoptive immunogene therapy. The goal of the study was to determine whether an IL-18 secreting CTL inhibited the development of primary tumors and synchronous liver metastases in orthotopic colon cancer.

Firstly, in this study, we used an adenoviral vector to transduce the IL-18 gene into T lymphocytes. An adenoviral vector is a highly efficient and reproducible method of gene transfer. Indeed, several studies have shown that successful adenoviral gene transfer into T lymphocytes resulted in inducing an immunological response against tumors (3). Ad vectors bind to coxsackievirus and adenovirus receptor, which is a high-affinity receptor for the adenovirus fiber protein, and transduce the gene into cells. However, T lymphocytes have a small amount of surface expression of coxsackievirus and adenovirus receptor, and, therefore, high doses of adenoviral vectors are required for high transduction efficiency, resulting in low viability of transduced T lymphocytes. To overcome this problem, we used a combined method of cytokine and liposome for adenoviral gene transfer into T lymphocytes as in previous reports (34, 35). As a result, transgenic expression in T lymphocytes showed a remarkable high efficiency (~50%) keeping high viability at a MOI of 500, which was a fairly lower dose than that in previous studies. It is suggested that adenoviral gene transduction by a combined method of adequate cytokine and liposome is markedly efficient, and makes it possible for more
and thereafter, the mice became severely cachexic with peritoneal dissemination. For these reasons, we evaluated liver metastases on day 35 in this orthotopic model. This study demonstrated that control mice and mice treated with CTL alone had not only a large primary tumor accompanied with colon obstruction, but also multiple liver metastases, which is similar to highly advanced colon cancer. Surprisingly, however, in the mice treated with AdIL-18-CTL, the growth of the primary tumor was suppressed, and moreover, the mice treated with AdIL-18-CTL in combination with rIL-12 had remarkably suppressed liver metastases, peritoneal dissemination, and ascites for a long time. In addition, these mice lived significantly longer than other mice. These observations implicate the possibility for clinical application. Of course, we need to investigate additionally the timing of AdIL-18-CTL and rIL-12 administration, how many CTLs are needed for one injection, and how many times CTLs should be injected. In addition, CTL preparation used in this study contained some NK and LAK activity. We also need to confirm whether these populations potentiate specific activities by depletion of NK1.1 + NK cells or some other method.

Several studies have shown that Th1-type cytokines enhance the therapeutic efficacy of antitumor responses and that Th1-dominant immunity is superior to Th2-dominant immunity in the induction of antitumor immunity (12, 38); therefore, to confirm these phenomena, serum Th1 and Th2 cytokine were measured. As a result, we propose the following possibilities in addressing the mechanism underlying our successful therapeutic model of mice with advanced colon cancer. Initially, in this model, adoptive transferred CTL were exposed to a Th2-biasing environment, and tumor-derived suppressors such as IL-4 and IL-6. Subsequently, these factors suppressed the function of CTL and the induction of immunoregulatory cells, whereas in mice treated with AdIL-18-CTL and rIL-12, many kinds of immune cells such as T lymphocytes, NK cell, NKT cells, and macrophages were enhanced in the Th1-biasing environment around the tumor site. These Th1-dominant environments at the tumor site may differentiate many kinds of immune cells that induce systemic antitumor immunity.

Another possibility is antiangiogenesis involved in an antitumor mechanism, which nonspecifically impairs tumor growth by limiting access by vital nutrients and metabolites to rapidly growing tumors such as advanced colon cancer. Among various cytokines, the antiangiogenic effects of IL-12 have been well documented (39, 40). In addition, it has been demonstrated recently that the antitumor action of IL-18 is also because of its inhibitory effect on angiogenesis (31). In vitro, IL-18 inhibits the proliferation of capillary endothelial cells by fibroblast growth factor. Exogenous IL-18 also suppresses corneal neovascularization in vivo. Administration of IL-18 leads to inhibition of fibrosarcoma growth accompanied by hypovascularization. Combination therapy using IL-12 and IL-18-expressing tumor cells shows more efficacies in tumor regression-mediated antiangiogenic effects (41). Therefore, IL-18, particularly in combination with IL-12, may become a more potent antiangiogenic agent. In this study, we have shown the effect of antiangiogenesis in combination with AdIL-18-CTL and rIL-12 without IFN-γ toxicity.

The functions of IL-18 in vivo are very heterogeneous and complicated. Because IL-18 is constitutively expressed in ovarian or intestinal epithelial cells (42, 43), carcinoma derived from epithelial cells seems to be relatively benign for the host; however, IL-18-positive colon cancer cell lines do not contain its processing enzyme caspase-1, resulting in their acquisition of malignant potency (44). Moreover, it has been suggested that soluble products from B16 melanoma cells stimulate HSE cells to release TNF-α, IL-1β, and IL-18, which up-regulates the expression of vascular cell adhesion molecules on HSE cells, promoting cancer cell adhesion on HSE cells and metastasis to the liver (45). Furthermore, B16B10 melanoma cells, which express both IL-18 and IL-18R, increase FasL expression and intracellular reactive oxygen intermediate levels in an autocrine manner. Thus, endogenous IL-18 in melanoma cells may help them to evade the host immune system by regulating the expression of FasL as well as reactive oxygen intermediate levels (46). In a CT26 tumor-bearing model, a significant difference of serum IL-18 production was not observed (data not shown). For these reasons, a careful manipulation of IL-18 may be required for practical immunotherapy.

Finally, the ability to introduce foreign genes into T lymphocytes has opened exciting opportunities for enhancing the adoptive immunotherapy of cancer. For adoptively transferred lymphocytes to be most effective, they must survive and proliferate in vivo, recognize tumor cells, and respond with an adequate effector mechanism. Attempts are under way to improve on each of these steps through gene modification (47). In this study, the introduction of the gene for IL-18 may enhance the T-lymphocyte effector function by enabling lymphocytes to deliver reasonable concentrations of IL-18 to tumor sites, without causing systemic IFN-γ toxicity. For these approaches to succeed, we need to additionally confirm whether genetically engineered T lymphocytes migrate to and target tumor microenvironments by the methods such as 51Cr-labeled activated T lymphocytes or tetramethylrhodamine B isothiocyanate-labeled T lymphocytes (48), and also, we need the development of vectors (49), resulting in enhanced transduction efficiencies and gene expression in primary T lymphocytes. Ultimately, vectors of which the expression can be regulated in vivo may be a great clinical utility.

In conclusion, our preliminary studies showed that IL-18 gene transduction made T lymphocytes more potent in their in vivo antitumor activity, and their efficacy enhanced in combination with rIL-12. These results have suggested the feasibility of this strategy for the treatment of clinical tumors.

REFERENCES

Adoptive Immunogene Therapy Inducing Th1-dominant State in Patients with Metastatic Melanoma.


Intensification of Antitumor Effect by T Helper 1-dominant Adoptive Immunogene Therapy for Advanced Orthotopic Colon Cancer

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