The Anti-Human Tumor Effect and Generation of Human Cytotoxic T Cells in SCID Mice Given Human Peripheral Blood Lymphocytes by the in Vivo Transfer of the Interleukin-6 Gene Using Adenovirus Vector

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ABSTRACT

Interleukin-6 (IL-6) was found to function as a late-acting killer helper factor in the differentiation of CTLs. In the model of tumor-bearing mice, the systemic administration of recombinant IL-6 was found to mediate the antitumor effect on the immunogenic murine tumors via the in vivo induction of murine CTLs but not on the poorly immunogenic murine tumors in our previous study. However, an in vivo experimental model capable of analyzing the antihuman tumor effect via the in vivo induction of human CTLs has not yet been established. Therefore, in the present study, severe combined immunodeficient mice were given human peripheral blood lymphocytes (SCID-PBL/hu), and thereafter human tumor cells were administered i.p. into these SCID-PBL/hu mice as a model of human patients with cancer. When these SCID-PBL/hu mice bearing allogeneic human CESS B blastoid tumor cells were treated in vivo with recombinant adenovirus vector expressing IL-6 cDNA, both the induction of CD8+ human CTLs against CESS cells in the spleen cells and peritoneal exudate cells and a prolongation in the survival of these mice were observed. Furthermore, SCID-PBL/hu mice were given peripheral blood lymphocytes from patients with cancer (gastric or rectal cancers) and autologous human tumor cells. The in vivo administration of recombinant adenovirus vector expressing IL-6 cDNA induced CD8+ human CTLs specific for autologous human tumor cells from human precursor T cells. The in vivo injection of the IL-6 gene also inhibited growth and metastasis in autologous human cancers. Based on the above findings, the experimental model using SCID-PBL/hu mice and the IL-6 gene delivered in vivo by an adenovirus vector might therefore provide a new strategy capable of analyzing an antihuman tumor effect and the in vivo induction of human CTLs by cytokine gene therapy without using the human body.

INTRODUCTION

Cytotoxic T cells play an important role in the immune responses against tumor cells and also induce an antitumor effect in vivo (1–3). Cytokines from helper T cells are required for the induction of cytotoxic T cells in both the murine and human systems (1, 4–9). IL-6 was found to function as a late-acting killer helper factor in the differentiation of CTLs (7). Furthermore, the systemic administration of rIL-6 was found to induce the in vivo generation of CD8+ CTLs against syngeneic murine tumors, FBL-3 erythro-leukemia, or fibrosarcoma while it induces an antitumor effect (a prolongation of survival time and an inhibition of micrometastasis; Refs. 1, 10, and 11). However, the systemic administration of rIL-6 did not mediate an antitumor effect on such poorly immunogenic murine tumors as RL98 and RL32T lymphoma (12).

In contrast, murine tumor cells transfected with IL-6 cDNA using a retrovirus vector induced a strong antitumor effect even on these poorly immunogenic murine tumors via the activation of CD4+ T cells and CD8+ T cells (12). Porgador et al. (13) reported that IL-6 gene-transfected murine lung cancer cells could exert an antitumor effect by the induction of tumor-specific CTLs and the activation of macrophages. The IL-6 gene-transfected murine fibrosarcoma also could mediate an antitumor effect by T cells or macrophages (14, 15).

Recently, murine tumor cells transfected with cDNA for several cytokines (such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-12, tumor necrosis factor α, granulocyte CSF, GM-CSF, and IFN-γ) were found to exert an antitumor activity based on different immunological mechanisms. These methods all strongly induce local antitumor immune responses by the activation of CTLs, CD4+ T cells, macrophages, eosinophils, granulocytes, or NK cells while causing a minimal degree of systemic toxicity (13–25). Fearon et al. (16) demonstrated that poorly immunogenic murine colon tumor cells transfected with IL-2 cDNA could induce the generation of CD8+ CTLs and elicit a strong antitumor immune response. Tepper et al. (21) reported that murine plasmacytomatas and mammary adenocarcinomas transfected with IL-4 cDNA could mediate an antitumor effect by an inflammatory infiltrate composed of eosinophils and macrophages. In contrast, Golumbek et al. (22) demonstrated that gene therapy using IL-4 cured renal cancer-bearing mice by the generation of CD8+ CTLs.

Dranoff et al. (23) compared the ability of different cytokines (GM-CSF, IL-6, IL-4, IFN, stem cell factor, granulocyte CSF, IL-2, IL-7, and so forth) and other molecules (such as adhesion molecules) to enhance the immunogenicity of tumor cells. Irradiated B16 melanoma cells expressing murine GM-CSF produced the most potent, long-lasting, and specific antitumor immunity, requiring both CD4+ and CD8+ T cells. However, Saito et al. (24) argued that GM-CSF gene-transfected murine bladder cancer vaccines are consistently less effective than IL-2 gene-transfected vaccines. Therefore, the antitumor effect of cytokine gene therapy may vary depending on the assay systems used and the type of tumors.

In human cancer patients, the antitumor effect of cytokine gene therapy might be different from mice with murine tumors depending on the tumors and cytokines. Therefore, by using the SCID-PBL/hu mice, we analyzed the in vivo anti-human tumor effect of cytokines. SCID-PBL/hu mice were established by the administration of human PBLs i.p. into CB-17-SCID mice. CD3+ and CD8+ human T cells were observed in vivo in spleen cells and PECs in these mice. The administration of rIL-6 augmented the generation of human CTLs in the SCID-PBL/hu mice stimulated with human B cell tumor CESS cells (12, 26).

Furthermore, cytokine genes introduced into human cancer cells...
In the present study, the administration of Adex IL-6 into SCID-PBL/hu mice bearing the human allogeneic B thyroid tumor CESS was found to induce the in vivo generation of human CD3⁺, CD8⁺ CTLs against CESS cells and thus prolong their survival. Furthermore, Adex IL-6 was injected into the SCID-PBL/hu mice bearing either autologous gastric cancer cells or rectal cancer cells. SCID-PBL/hu mice were given PBLs from a patient with gastric cancer or rectal cancer, injected with autologous cancer cells, and then treated with Adex IL-6. Human CTLs against autologous human cancer cells were generated in vivo by the treatment of Adex IL-6 from naive human T cells. An in vivo anti-human tumor effect also was observed in these SCID-PBL/hu mice after they were treated with Adex IL-6. Therefore, the experimental model using SCID-PBL/hu mice and cytokine genes delivered by an adenovirus vector might provide a new strategy capable of analyzing the anti-human tumor effect by cytokine gene therapy without using an actual human subject.

MATERIALS AND METHODS

Mice. CB-17 scid/scid (SCID) mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Kanagawa, Japan) and maintained in filter-capped isolator cages in our specific pathogen-free animal center (Kyushu University). The SCID mice were fed autoclaved food and water. All mice received daily trimethoprim/sulfamethoxazole (Bakter; 40 mg of trimethoprim and 200 mg of sulfamethoxazole per 5 ml of suspension; 0.125 ml of suspension for every 4 ml of drinking water per mouse) supplied by the Shionogi Co. (Osaka, Japan) to prevent infection with Pneumocystis carinii.

Reagents and Antibodies. FCS (lot 1111958) was purchased from HyClone Laboratories, Inc. (Logan, UT). Monoclonal anti-human CD3 (lot T329) and anti-human CD8 (lot T821) antibodies were purchased from Ortho Diagnostic System, Inc. (Raritan, NJ). Baby rabbit complement (lot 3441) was purchased from Cedarlane Laboratories Ltd. (Hornby, Ontario, Canada).

Generation of SCID-PBL/hu Mice. PBLs were obtained from patients with cancer or healthy volunteers after Ficoll Hypaque density centrifugation as described previously (5, 8). PBLs (2.5 X 10⁶) suspended in 300 μl of HBSS were mixed with 100 μl of matrigel (Becton Dickinson Labware, Bedford, MA). Six- to 8-week-old female SCID mice received pretreatment with whole-body X-ray irradiation (2 Gy; 100 kV, and 3.5 mA for 5 min) by using an X-ray generator (model MBR-1505R, Hitachi Medical Co., Tokyo, Japan) 1 h before the i.p. injection of PBLs.

Tumor Cell Lines. CESS, an EBV-transformed human B-lymphoid cell line, and K562, a NK-sensitive leukemia cell line, were maintained in complete medium (5). MKN7, MKN45, MKN74, NUG-C3, and Kato-III human gastric adenocarcinoma cell lines and Colo205, Colo320DM, RC422, and WiDr colorectal adenocarcinoma cell lines expressing melanoma antigen gene (MAGE)-1, -2, -3, -4, BAGE, GAGE1-2, or GAGE1-6 genes were obtained from the Japanese Cancer Research Bank (Tokyo, Japan; Refs. 27 and 28). TE6, TE7, TE9, TE13, KY30, KY450, and KY510 human esophageal squamous cancer cell lines expressing the MAGE-1, -2, -3, -4, -9, or -12 gene were provided by the cell bank of Tohoku University (Sendai, Japan) and Dr. Y. Shimada (Kyoto University, Kyoto, Japan; Ref. 29). GC408 and GC814, both gastric cancer cell lines, were generated in our laboratory from a patient (a 68-year-old female) with moderately differentiated adenocarcinoma. RC422, a rectal cancer cell line, was established from a patient (a 26-year-old female) with moderately differentiated adenocarcinoma. GC408 and GC814, both gastric cancer cell lines, were established from a 61-year-old female with mucinous adenocarcinoma and an 84-year-old male with poorly differentiated adenocarcinoma, respectively. The tumor cells obtained by surgical treatment from these patients with cancer were injected s.c. into SCID mice. After the formation of tumor masses in these mice, the tumor cells were transfected into in vitro cultures (Linbro multiwell plate), and these tumor cell lines were maintained in vitro. Murakami, an EB virus transformed human B blastoid cell line, was a gift from Dr. Fujie (Kyushu University). The tumor cell lines were maintained in vitro in RPMI 1640 (Flow Laboratories, Inc., McLean, VA) and supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml 2-mercaptoethanol.

Fig. 1. Establishment of Adex1SRahIL-6 (Adex IL-6) and Adex1SRahW (Adex SW) and the production of IL-6 in SCID-PBL/hu mice. A, the recombinant adenovirus is based on adenovirus type 5 and lacks the E1A, E1B, and E3 regions. The expression unit of human IL-6 with SRα promoter and SV40 poly(A) signal sequences was inserted in the deleted E1 region of pAdex1C (pAdex1SRahIL-6). No expressing gene with SRα promoter and SV40 poly(A) signal sequences was inserted in the deleted E1 region of pAdex1C (pAdex1SRahW). B, the kinetics of human IL-6 activity in sera from SCID-PBL/hu mice treated with i.p. injection of Adex IL-6 was assessed using the chemiluminescence enzyme immunosay system as described in "Materials and Methods." Data points, mean of IL-6 activity in five mice; bars, SD.
Adenovirus Vector. The recombinant adenovirus vector was based on adenovirus type 5 and lacks the E1A, E1B, and E3 regions (Fig. 1A; Ref. 30). The recombinant human IL-6 adenovirus vector (AdexSRaIL-6; Adex IL-6) contained SRa promoter, human IL-6 cDNA, and the SV40 poly(A) signal sequence inserted into the E1-deleted region of a cosmid cosmid vector. Recombinant adenovirus (AdexSRaW; Adex SW) was used as a control contained the SRa promoter and the SV40 poly(A) signal. Viral stocks were generated from confluent monolayers of 293 cells. The supernatant that contained virus particles was stored at −80°C until use. After the viruses were concentrated, the virus stock (1.0 × 10^11 plaque-forming units/ml) was stored at −80°C (31, 32). The tumor cells were found to be transduced with the adenovirus in vivo by using an adenovirus vector containing the LacZ gene (Adex LacZ) and β-gal staining.

Treatment of Cells with Antibodies and Complements. Spleen cells (2 × 10^7/ml SCID-PBL/hu) and PECs were treated with an anti-human CD3 or CD8 monoclonal antibody (1:20 diluted) for 15 min at 4°C and then incubated with baby-rabbit serum complements (1:10 diluted) for 45 min at 37°C as described previously (1, 4, 5).

In Vivo Induction of Human Cytotoxic T Cells in SCID-PBL/hu Mice Against Allogeneic Human Tumors by the Injection of Adex IL-6. CESS was used as an allogeneic tumor cell line. PBLs (2.5 × 10^7) from the healthy donor were administered i.p. into SCID mice on day 0. These mice (eight animals in each group) were inoculated i.p. with 2 × 10^7 human tumor CESS cells on day 2. The SCID-PBL/hu mice bearing human tumor cells were then treated i.p. with Adex IL-6 at a.m.o.i. of 100 or with Adex SW on day 3 after the injection of PBLs. Twenty-four days after the injection of human PBLs, the mice were sacrificed. The spleen cells and PECs obtained from these SCID-PBL/hu mice were assessed for lytic activity against human tumor cells.

In Vivo Induction of Human Cytotoxic T Cells in SCID-PBL/hu Mice Against Autologous Human Tumors by the Injection of Adex IL-6. GC629 and GC408 gastric cancer cell lines and RC422, a rectal cancer cell line, were used as autologous tumor cells. PBLs (2.5 × 10^7) from patients with gastric cancer or a patient with rectal cancer were administered i.p. into SCID-PBL/hu mice on day 0. These mice (either eight or five animals in each group) were injected i.p. with 2 × 10^7 human tumor CESS cells and K562 cells.

In Vivo Generation of CD3+ CD8+ Human Cytotoxic T Cells against Allogeneic Human Tumor Cells in SCID-PBL/hu Mice. To investigate the in vivo activation of human T cells, PBLs from healthy volunteers were administered i.p. into CB-17 SCID mice and then these SCID-PBL/hu mice were in vivo stimulated with human CESS tumor cells. In the spleen cells, lymph node cells, and PECs obtained from SCID-PBL/hu mice on a day between day 21 and day 28 after the injection of human PBLs, 10—30% of CD3 + CD8 + human tumor cells and CD3 + CD4 + human tumor cells were observed (data not shown).

RESULTS

In Vivo Generation of CD3+ CD8+ Human Cytotoxic T Cells against Autologous Human Tumor Cells in SCID-PBL/hu Mice. In Vivo Generation of CD3+ CD8+ Human Cytotoxic T Cells against Allogeneic Human Tumor Cells in SCID-PBL/hu Mice. In Vivo Generation of CD3+ CD8+ Human Cytotoxic T Cells against Allogeneic Human Tumor Cells in SCID-PBL/hu Mice. To investigate the in vivo activation of human T cells, PBLs from healthy volunteers were administered i.p. into CB-17 SCID mice and then these SCID-PBL/hu mice were in vivo stimulated with human CESS tumor cells. In the spleen cells, lymph node cells, and PECs obtained from SCID-PBL/hu mice on a day between day 21 and day 28 after the injection of human PBLs, 10—30% of CD3 + CD8 + human tumor cells and CD3 + CD4 + human tumor cells were observed (data not shown).

As shown in Table 1 (Experiments I and II), the cytotoxic cells were generated in the in vivo spleen cells and PECs from these SCID mice on day 24 after the injection of PBLs. When SCID-PBL/hu mice were given 2.5 × 10^7 human PBLs and 2.0 × 10^7 CESS cells and treated with Adex IL-6, spleen cells and PECs from these SCID-PBL/hu mice at 24 days after the injection of human PBLs was assessed against several kinds of human tumor cells.

Assay for Cytolytic Activity. The percentage of specific cytolytic activity was measured by means of a microcytotoxicity assay using 51Cr-labeled human tumor cells as target cells (5, 8). Specific cytotoxicity (%) = [(experimental 51Cr release − control 51Cr release)/maximum 51Cr release − control 51Cr release)] × 100.

Assay for IL-6 Activity. Human IL-6 activity was assessed using the Chemiluminescence Enzyme Immuno Assay system (SRL Co., Tokyo, Japan).

Statistics. Survival and tumor growth were observed every day after the injection of human tumor cells into SCID-PBL/hu mice for more than 100 days. Experimental data on survival in SCID-PBL/hu mice bearing human tumors treated with Adex IL-6 or Adex SW was statistically evaluated using the Kaplan-Meier test (33).

Table 1. The in vivo induction of human CD8+ positive cytotoxic T cells in the SCID-PBL/hu mice by administration of Adex IL-6

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Splenic effector cells from SCID mice treated with:</th>
<th>% Specific cytotoxicity against CESS (E:T ratio)</th>
<th></th>
<th>% Specific Cytotoxicity against K562</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>CESS cells</td>
<td>20:1</td>
<td>4:1</td>
<td>20:1</td>
</tr>
<tr>
<td>Human PBLs</td>
<td>2.5 × 10^7</td>
<td>2.0 × 10^7</td>
<td>85.5 ± 9.2</td>
<td>24.0 ± 2.2</td>
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<td></td>
<td>2.5 × 10^7</td>
<td>1.0 × 10^7</td>
<td>52.2 ± 4.4</td>
<td>20.2 ± 2.2</td>
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<td></td>
<td>1.25 × 10^7</td>
<td>1.0 × 10^7</td>
<td>23.0 ± 3.2</td>
<td>9.1 ± 2.1</td>
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<td></td>
<td>2.5 × 10^7</td>
<td>2.0 × 10^7</td>
<td>1.0 ± 0.1</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>2.5 × 10^7</td>
<td>2.0 × 10^7</td>
<td>2.5 ± 0.3</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>PEC</td>
<td>2.5 × 10^7</td>
<td>2.0 × 10^7</td>
<td>16.1 ± 1.1</td>
<td>4.1</td>
</tr>
<tr>
<td>2.5 × 10^7</td>
<td>2.0 × 10^7</td>
<td>88.9 ± 6.5</td>
<td>27.8 ± 3.1</td>
<td>5.2 ± 1.1</td>
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<tr>
<td></td>
<td>2.5 × 10^7</td>
<td>2.0 × 10^7</td>
<td>11.1 ± 1.1</td>
<td>6.1 ± 0.2</td>
</tr>
<tr>
<td>Effector splenic cells treated with antibody</td>
<td>Anti-human CD3 mAb</td>
<td>50.1</td>
<td>25.1</td>
<td>12.1</td>
</tr>
<tr>
<td></td>
<td>Anti-human CD8 mAb</td>
<td>21.9 ± 2.3</td>
<td>18.8 ± 1.1</td>
<td>17.7 ± 3.2</td>
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<tr>
<td></td>
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<td>0.0 ± 0.1</td>
<td>2.6 ± 0.2</td>
<td>0.4 ± 0.1</td>
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<tr>
<td></td>
<td></td>
<td>0.0 ± 0.0</td>
<td>2.5 ± 0.1</td>
<td>1.1 ± 0.2</td>
</tr>
</tbody>
</table>

Data represent the mean ± SD of cytotoxic activity in the triplicate cultures of spleen cells from eight mice in each group.

Two days after the administration of various numbers of human PBLs i.p. into the SCID mice, various numbers of tumor cells (CESS) were inoculated i.p. and then Adex IL-6, or Adex SW (m.o.i. = 100) was injected on day 3. These mice (eight mice in each group) were sacrificed on day 24. The cytotoxic activity in spleen cells was assessed against CESS cells and K562 cells.

SCID mice constructed with 2.5 × 10^7 human PBLs were injected with 2.0 × 10^7 CESS cells and then treated with Adex IL-6 or Adex SW. On day 24 after the injection of PBLs, the cytotoxic activity in the PECs was assessed against CESS cells and K562 cells.

Spleenic effector cells from SCID-PBL/hu mice injected with Adex IL-6 were treated with a monoclonal anti-human CD3 antibody or an anti-human CD8 antibody in the presence of complement as described in "Materials and Methods." After the treatment, effector cells were assessed for cytotoxic activity against CESS.

mAb, monoclonal antibody.
The survival of these mice is shown. A, 2.0 × 10^7 viable CESS human tumor cells were administered i.p. into the SCID-PBL/hu mice given 2.5 × 10^7 human PBLS (16 mice). On day 1 after the injection of tumor cells, these mice were treated with Adex IL-6 (8 mice; ●) or Adex SW (8 mice; ○). The survival of these mice is shown.

On day 1 after the injection of tumor cells, these mice were treated with Adex IL-6 were administered i.p. into SCID-PBL/hu mice given 2.5 × 10^7 human PBLS (10 mice).

Furthermore, the treatment of effector cells in spleen cells from SCID-PBL/hu mice with a monoclonal anti-human CD3 antibody or a monoclonal antihuman CD8 antibody in the presence of complements almost abolished the cytotoxic activity against CESS cells (Table 1, Experiment III). These results demonstrated that the cytotoxic cells generated in the SCID-PBL/hu mice immunized with CESS cells and treated with Adex IL-6 were CD3^+ CD8^+ human cytotoxic T cells capable of lysing CESS human allogeneic tumor cells specifically.

**In Vivo Generation of Human CTLs against Syngeneic (Autologous) Cancer Cells in SCID-PBL/hu Mice by the Injection of Adex IL-6.** To investigate the *in vivo* generation of cytotoxic activity against syngeneic (autologous) human tumors, SCID-PBL/hu mice given PBLS from cancer patients and autologous cancer cells were treated with Adex IL-6. Gastric cancer cell lines and rectal cancer cell lines were established from cancer patients with gastric and rectal cancer, respectively.

SCID mice were given PBLS from a patient with gastric cancer and autologous gastric cancer cell line GC629 cells. Those SCID-PBL/hu mice were then treated with Adex IL-6 *in vivo*. Cytotoxic activity against autologous GC629 cancer cells was induced in the spleen cells and PECs of these mice by treatment with Adex IL-6 but not with Adex SW. Cytotoxic cells against GC629 cells were not generated when SCID mice were given GC629 cells in the absence of PBLS from a patient with gastric cancer even after treatment with Adex IL-6 (Table 2, Experiment I). On the other hand, cytotoxic cells specific against autologous RC422 cancer cells were generated in the SCID-PBL/hu mice when SCID mice were given PBLS from a patient with rectal cancer, and the autologous rectal cancer cell line RC422 cells were then treated with Adex IL-6 *in vivo* (Fig. 4B and Table 2, Experiment II).

The cytotoxic activity of these effector cells was almost completely abolished by the treatment of the effector cells in the spleen cells and PECs from SCID-PBL/hu mice with an anti-human CD3 or CD8 antibody and complements. These results demonstrate that the *in vivo* administration of Adex IL-6 into the SCID-PBL/hu mice bearing autologous human cancer cells induced CD3^+*, CD8^+ human cytotoxic T cells against autologous cancer cells.

**Specificity of in Vivo CTLs Generated in the SCID-PBL/hu by the Administration of Adex IL-6.** To investigate the specificity of human CTLs generated *in vivo* in the SCID-PBL/hu mice, cytotoxic activity of these CTLs was assessed using a panel of many kinds of allogeneic human gastric, colorectal, and esophageal cancer cells. The cytotoxic activity induced by the administration of Adex IL-6 in SCID-PBL/hu mice given PBLS from a patient with gastric cancer (GC629) and autologous cancer cells (GC629) was not exhibited against other cancer cell lines such as GC408, GC814, NUG-C3, MKN7, MKN45, MKN74 (gastric adenocarcinoma cells), RC422, Colo205, Colo320DM, RCm1, WiDr (colorectal adenocarcinoma cells), KY30, KY450, KY510, TE5, TE7, TE9, TE13 (esophageal squamous cell carcinoma) cells, CESS, Murakami K562 cells, and autologous PBLS (Table 2, Experiment I; Fig. 4A). In contrast, the cytotoxic activity induced by the administration of Adex IL-6 in SCID-PBL/hu mice given PBLS from a patient with rectal cancer (RC422) and autologous cancer cells (RC422) was specifically exerted against autologous RC422 tumor cells (Fig. 4B; Table 2, Experiment II). As shown in Fig. 4C, the cytotoxic activity induced by the administration of Adex IL-6 in the SCID-PBL/hu mice given PBLS from a patient with rectal cancer (RC422) and autologous cancer cells (RC422) was specifically exerted against autologous RC422 tumor cells (Fig. 4B; Table 2, Experiment II). As shown in Fig. 4C, the cytotoxic activity induced by the administration of Adex IL-6 in the SCID-PBL/hu mice given PBLS from a patient with rectal cancer (RC422) and autologous cancer cells (RC422) was specifically exerted against autologous RC422 tumor cells (Fig. 4B; Table 2, Experiment II). As shown in Fig. 4C, the cytotoxic activity induced by the administration of Adex IL-6 in the SCID-PBL/hu mice given PBLS from a patient with rectal cancer (RC422) and autologous cancer cells (RC422) was specifically exerted against autologous RC422 tumor cells (Fig. 4B; Table 2, Experiment II). As shown in Fig. 4C, the cytotoxic activity induced by the administration of Adex IL-6 in the SCID-PBL/hu mice given PBLS from a patient with rectal cancer (RC422) and autologous cancer cells (RC422) was specifically exerted against autologous RC422 tumor cells (Fig. 4B; Table 2, Experiment II). As shown in Fig. 4C, the cytotoxic activity induced by the administration of Adex IL-6 in the SCID-PBL/hu mice given PBLS from a patient with rectal cancer (RC422) and autologous cancer cells (RC422) was specifically exerted against autologous RC422 tumor cells (Fig. 4B; Table 2, Experiment II). As shown in Fig. 4C, the cytotoxic activity induced by the administration of Adex IL-6 in the SCID-PBL/hu mice given PBLS from a patient with rectal cancer (RC422) and autologous cancer cells (RC422) was specifically exerted against autologous RC422 tumor cells (Fig. 4B; Table 2, Experiment II). As shown in Fig. 4C, the cytotoxic activity induced by the administration of Adex IL-6 in the SCID-PBL/hu mice given PBLS from a patient with rectal cancer (RC422) and autologous cancer cells (RC422) was specifically exerted against autologous RC422 tumor cells (Fig. 4B; Table 2, Experiment II).
PECs from SCID-PBL/hu mice bearing autologous human cancer cells by treatment with Adex IL-6, can recognize the antigen specific to the autologous human cancer cells.

**In Vivo Antitumor Effect on Autologous Human Cancer Cells by the Administration of the IL-6 Gene Using Adenovirus Vector.** An in vivo anti-human tumor effect was observed in the SCID-PBL/hu mice bearing autologous human gastric cancer (GC629 cells), as well as CTL activity against autologous cancer cells in those mice by the administration of Adex IL-6 (Fig. 5A). Neither a tumor mass nor metastasis was shown in the peritoneal cavity and organs (liver, spleen, kidney, pancreas, lung, and brain) in these SCID-PBL/hu mice treated with Adex IL-6. On the other hand, tumor masses were observed in the peritoneal cavity of those SCID-PBL/hu mice bearing GC629 cancer cells treated with Adex SW (Fig. 5B). No antitumor effect was observed in the SCID mice bearing autologous human GC629 tumor without the injection of PBLs from a GC629 cancer patient even when treated with Adex IL-6 (data not shown). The in vivo administration of Adex IL-6 7 days after the injection of GC629 cancer cells also mediated the in vivo antitumor effect (tumor regression and prolongation of survival) in the SCID-PBL/hu mice (data not shown), suggesting that Adex IL-6 is effective even against established human tumors.

These results indicate that the direct injection of the IL-6 gene in vivo using adenovirus vector could mediate anti-human tumor effect on the autologous cancer and that the experimental model of SCID-PBL/hu provides a useful strategy for analyzing the in vivo immunoregulatory mechanisms against anti-human tumors by the activation of human T cells.

**DISCUSSION**

In the present study, CD8+ human CTLs specific against autologous human cancer cells, as well as CD8+ human CTLs specific against allogeneic human cancer cells, were generated in the in vivo
The in vivo induction of human cytotoxic T cells against autologous human cancer cells in the SCID-PBL/hu mice by treatment with Adex IL-6

<table>
<thead>
<tr>
<th>Experiment I* Treated with:</th>
<th>Spleen cells a specific cytotoxicity, E:T ratio</th>
<th>PECs b % specific cytotoxicity, E:T ratio</th>
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<tr>
<td>PBL</td>
<td>GC629</td>
<td>Adex IL-6</td>
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<tr>
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<th>Spleen cells a E:T ratio</th>
<th>PECs E:T ratio</th>
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<td>GC629</td>
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<th>Spleen cells E:T ratio</th>
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<td>Anti-human CD3 mAb + C</td>
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<td>11.5 ± 1.5</td>
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<td>Anti-human CD8 mAb + C</td>
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<tr>
<td>C</td>
<td>18.5 ± 1.3</td>
<td>13.6 ± 1.4</td>
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| a 2.5 x 10^7 PBLs from a patient with gastric cancer (GC629) were administered i.p. into the SCID mice. On day 2 after the injection of PBLs or without the injection of PBLs, GC629 cells (obtained from GC629 cell line) were injected i.p. and then treated with Adeex IL-6 or Adeex SW. On day 24, the cytotoxic activity in spleen cells and PECs was assessed. |
| b Results were expressed as mean % cytotoxicity ± SD in triplicate cultures of spleen cells or PECs in each group (eight mice per group). The cytotoxic activity against GC629 gastric cancer cells, K562, autologous PBL, RC422 rectal cancer cells, gastric cancer cell lines (NUG-C3, MK7N, MKN45, MKN74, or GC408), colorectal cancer cell lines (Colo205, Colo205DM, RCM1, or WiDr), or esophageal cancer cell lines (KY450, KY450, TE5, TE7, TE9, or TE13) was assessed. |
| c Autologous PBLs were stimulated with 0.2% phytohemagglutinin-P in the presence of 1000 units/ml of rIL-2 for 2 days in vitro, and blastoid PBLs were used as target cells. |

IL-6 GENE THERAPY FOR HUMAN CANCERS IN SCID-PBL/hu MICE

SCID-PBL/hu mice by the administration of the IL-6 gene using an adenovirus vector (Adex IL-6). Furthermore, the in vivo antitumor effect (proliferation of survival, tumor regression, and inhibition of metastasis) on these SCID-PBL/hu mice bearing human cancer cells (autologous gastric cancer, autologous rectal cancer, or B blastoid tumor CESS) was demonstrated by the analysis of IL-6 expression. Several lines of evidence support these conclusions. (a) When adenovirus vector without the insertion of the IL-6 gene (Adex SW) instead of IL-6 gene was administered into SCID-PBL/hu mice bearing human tumor cells, no cytotoxic cells specific against human immunized tumor cells were generated. Therefore, these SCID-PBL/hu mice died because of tumor development. (b) No cytotoxic cells were induced in vivo in the SCID mice bearing these human tumor cells even when Adex IL-6 and human tumor cells were given to the SCID mice without the administration of human PBLs. (c) The cytotoxic activity of effector cells generated in the SCID-PBL/hu mice by the injection of Adex IL-6 was almost completely abolished by the treatment of either anti-human CD8 antibody or anti-human CD3 antibody in the presence of complements. Human T cells enriched in E rosette sheep RBC-positive fraction exerted cytotoxic activity, whereas PBL cells in E-rosette-SRBC-negative fraction did not exert such activity (data not shown). (d) Based on the analysis of surface antigens on the cells using monoclonal antibodies against human T cells antigens and fluorescence-activated cell sorting, 15−30% CD3+ human T cells, 5−15% human CD4+ T cells, and 10−25% CD8+ human T cells were observed in the spleen cells from SCID-PBL/hu mice. Thirty to 60% CD3+ or CD8+ human T cells were also shown in PECs from those mice. (e) CD8+ human CTL cell lines capable of exerting the cytolytic activity against human tumor cells (CESS) and living for more than 10 weeks were established from the spleen cells of the SCID-PBL/hu mice stimulated with CESS cells and Adex IL-6 by in vitro re-stimulation with CESS cells (data not shown). (f) Walker and Gallagher (34) described the antibodies against human ovarian cancer cells that play an important role in the successful active immunotherapy against those human tumors in human PBL-reconstituted SCID mice. However, antibodies against...
Fig. 4. The specific cytolytic activity of human cytotoxic cells in vivo generated in SCID-PBL/hu mice against autologous human cancer cells. 2.5 × 10^7 PBLs from patients with gastric cancer or a patient with rectal cancer were administered i.p. into the SCID mice on day 0. These mice were injected with autologous human cancer cells on day 2 and then were treated with Adex IL-6 on day 3. The cytolytic activity in the spleen cells and PECs from these SCID-PBL/hu mice (5 mice in each group) 24 days after the injection of human PBLs was assessed against several kinds of human cancer cells. Columns, mean of cytotoxic activity in each group; bars, SD. A, SCID-PBL/hu mice were given PBLs from a patient with gastric cancer (GC629), and autologous GC629 cancer cells obtained from this cell line were treated with Adex IL-6 in vivo. The cytotoxic activity in the spleen cells (■) or in the PECs (▲) was assessed against GC629, RC422 human rectal cancer cells, GC408 human gastric cancer cells, K562, Murakami B blastoid cells, and K510 human esophageal cancer cells. The cytolytic activity in the spleen cells is shown at an E:T ratio of 40:1 and in the PECs at an E:T ratio of 5:1. B, SCID-PBL/hu mice given PBLs from a patient with rectal cancer (RC422) and autologous RC422 cancer cells obtained from the RC422 in vitro cell line were treated with Adex IL-6 in vivo. The cytotoxic activity in the spleen cells (■) or in the PECs (▲) was assessed against RC422, GC629, GC408, Kato-III human gastric cancer, CESS, and K562 cells. The cytolytic activity in the spleen cells is shown at an E:T ratio of 12:1 and in the PECs at an E:T ratio of 2:1. C, SCID-PBL/hu mice given PBLs from a patient with gastric cancer (GC408) and autologous GC408 cancer cells obtained from the GC408 in vitro cell line were treated with Adex IL-6 in vivo. The cytotoxic activity in the spleen cells (■) at an E:T ratio of 20:1 and in the PECs (▲) at an E:T ratio of 20:1 was assessed against GC408, RC422, and K562 cells.

Fig. 5. The inhibition of engraftment of autologous human cancer cells by the in vivo treatment with Adex IL-6 in SCID-PBL/hu mice. 2.5 × 10^7 PBLs from a patient with gastric cancer (GC629) were administered into the SCID mice (10 mice). On day 2 after the injection of PBLs, GC629 autologous human gastric cancer cells obtained from the in vitro cell line were inoculated i.p. into the SCID-PBL/hu mice. These mice were treated with Adex IL-6 (5 mice; A) or Adex SW (5 mice; B). On day 24, these mice were sacrificed and the engraftment of human gastric cancer GC629 was investigated.
SCID-PBL/hu in the present study mice model. CTLs generated by
treatment with Adex IL-6 in the SCID-PBL/hu mice given PBLs from
a patient with GC629 gastric cancer (expressing the MAGE-3 gene)
showed little cytotoxic activity against many kinds of gastric and
decorctal adenocarcinoma cells or several kinds of esophageal squa-
mous cancer cells, thus suggesting that these CTLs might not recog-
nize shared antigens such as the MAGE-3 antigen. To clearly prove
the tumor specificity of CTLs, SCID-PBL/hu mice should be given
PBLs from a patient with double cancers and injected with one of
these cancer cells, and then cytotoxic activity against other cancer
cells should be assessed. We have had no opportunity to study such
a patient with double cancer. Therefore, these SCID-PBL/hu mice
might provide a useful tool for the establishment of cytokine gene
therapy against cancer in humans.

SCID-PBL/hu mice given syngeneic PBLs from a lung cancer
patient were injected i.p. with lung cancer cells transfected with IL-6
gene.\(^5\) As a result, in vivo cytotoxic activity was generated. In addi-
tion, SCID-PBL/hu mice treated with PBLs from a cancer patient
and IL-6 gene-transferred cancer cells showed a prolonged survival time.\(^6\)
Recently, IL-6 transgenic SCID mice have been established by Y.
Osugi.\(^6\) IL-6 transgenic SCID-PBL/hu mice treated with PBLs from a
lung cancer patient and syngeneic lung cancer cells both exhibited a
strong cytotoxic activity (12). Therefore, IL-6 transgenic SCID mice
may provide a useful model for the rapid analysis of anti-human
tumor immunity without using gene-transfected tumor cells.\(^5\)

However, the findings regarding the engraftment of human PBLs
into SCID mice have varied depending on the investigators; some
report more success than others (41). This may be due to the human
PBL graft rejection mediated by NK cells of the SCID mouse (41, 43).
In such situations, human growth hormone promotes the engraftment
of murine or human T cells in SCID mice (44). It has been reported
that the activation of human PBLs with anti-CD3 antibody and the
subsequent administration of human rIL-2 after cell transfer provides
a means for optimizing human T cell engraftment in SCID mice (41).
Barry et al. (43) have shown that the irradiation of SCID mice before
the engraftment or treatment of SCID mice with anti-asialo GM-1
antiserum throughout the engraftment improved the survival of human
thalamic grafts by the abrogation of NK cell activity and/or macrophage
activity.

In the present SCID-PBL/hu mouse model study, spleen cells and
PECs contained monocytes/macrophages that were detectable by anti-
human monocyte monoclonal antibodies, which thus might contribute
to the capability of the in vivo induction of human CTLs. Irradiation
of SCID mice and the use of matrigel may promote the engraftment of
human macrophages from human PBLs into spleen cells and PECs.
Low-dosage irradiation played an important role in the engraftment of
human spleen cells in the hu-Spl-SCID mice (SCID mice prepared
human spleen cells) as reported by Alegre et al. (45). In their SCID
model, human T cells retained their proliferative responses to mito-
gens and to alloantigens when tested 3 weeks after engraftment into
SCID mice. Mice engrafted with unprimed human splenic T cells
rejected human allografts. Sandhu et al. (46) also described a SCID-
PBL/hu model in which recipient mice were treated with irradiation
and anti-asialo GM-1 antiserum. In the model, high levels of human
leukocyte engraftment were observed and primary human antibody
responses to antigens were obtained. Because the degree of human
CD4\(^+\) proliferation in the spleen cells from these SCID-PBL/hu mice
increases along with human KLH-specific antibody titers, it was
suggested that human antigen presenting cells, B cells, and helper T
cells are functional in the SCID mouse environment.

In contrast, it has been reported that human mature T cells in
SCID-PBL/hu mice were anergic in vitro. Tary-Lehman et al. (47)
discussed that freshly isolated cells from SCID-PBL/hu mice lacked
any in vitro proliferative response to anti-CD3 antibody. The differ-
ence between these results and our findings might be due to these
components of our study: (a) the use of matrigel, (b) the stimulation
of IL-6, (c) the period of sacrifice after the administration of human
PBLs into SCID mice, (d) the pretreatment with irradiation before the
administration of PBLs, and (e) the in vivo stimulation of human
tumor cells after the administration of PBLs. Tary-Lehman did not
inject antigens in vivo into SCID mice after the injection of human
PBLs.

The production of IL-6 in SCID mice by the injection of Adex IL-6
may also promote the engraftment of human T cells and the in vivo
generation of CTLs, as reported in IL-3 transgenic SCID mice. In the
IL-3 transgenic SCID mice, the engraftment of bone marrow cells was
augmented (48). In fact, using IL-6 transgenic SCID mice, an increase
in the number of CD3\(^+\) human T cells and CD8\(^+\) human T cells in
spleen cells and PECs from IL-6 transgenic SCID-PBL/hu mice was
observed (data not shown).

Furthermore, the in vivo injection of the IL-2 gene using an ade-
novirus vector could induce the regression of tumors (49). Therefore,
using the administration of the IL-6 and IL-2 genes, significant anti-
tumor immune responses were induced as shown using rIL-2 and
rIL-6 (1). The problem with using adenovirus vector is that repetitive
injections induce the generation of neutralizing antibody against the
protein of the adenovirus vector. A strategy for overcoming this
problem has been reported recently (50). When IFN-y or rIL-12 was
administered with the recombinant adenovirus vector, the formation
of neutralizing antibody to adenovirus was blocked. The infiltration
of inflammatory cells was observed slightly in the liver from SCID-
PBL/hu mice treated with Adex IL-6 by a microscopic histological
examination. On the other hand, few inflammatory cells were detected
in the liver of SCID-PBL/hu mice treated with Adex SW (data not
shown). However, no histological changes were observed in other
organs in the peritoneal cavity, such as the kidneys and intestine in the
SCID-PBL/hu mice treated with Adex IL-6.

In the present study, the in vivo administration of the IL-6 gene using
the adenovirus vector into SCID-PBL/hu mice mediated an anti-human
tumor effect via the induction and differentiation of human CTLs from
precursors of human T cells. By using an adenovirus vector, many
cytokine genes can thus be directly delivered in vivo without the need
to manipulate the genes\(^6\) in vivo. Therefore, the experimental model using
SCID-PBL/hu mice and cytokine genes delivered by an adenovirus
vector might be a useful tool for elucidating the anti-human tumor effect
of gene therapy using various kinds of cytokine genes.

ACKNOWLEDGMENTS

We thank Hiroko Tomonaga, Junko Takano, and Ryoko Nyuta for their
excellent technical assistance. We are grateful to Prof. Izumu Saito and Dr.
Shizuko Harada (Laboratory of Molecular Genetics, University of Tokyo) for
helpful discussions and for the generous gift of adenovirus vectors. We are
grateful to Assoc Prof. Yasuji Yoshikawa (Department of Pathology,
Medical Institute of Bioregulation, Kyushu University) for help with the
microscopic pathological examination of SCID mice treated with Adex IL-6
and human gastric and rectal cancer cells in the tumors and metastatic lesions
in the SCID mice. We thank Prof. Shizuo Akira (Department of Immunology,
Hyogo College of Medicine) for helpful discussions. Finally, we thank
Yo-shikazu Watanabe (Kyushu University) for his technical support in maintain-
the animals used in this study.

\(^5\) M. Okada, Y. Osugi, K. Nakahara, M. Kitahara, S. Akira, T. Nomura, T. Kishimoto,
and T. Suzuki. The in vivo anti-human tumor effect using lung cancer cells transfected
with IL-6 cDNA and IL-6 transgenic SCID mice, manuscript in preparation.

\(^6\) Personal communication.
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The Anti-Human Tumor Effect and Generation of Human Cytotoxic T Cells in SCID Mice Given Human Peripheral Blood Lymphocytes by the in Vivo Transfer of the Interleukin-6 Gene Using Adenovirus Vector

Fumiaki Tanaka, Masako Abe, Tsuyoshi Akiyoshi, et al.


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