Effects of Combined Therapy With Interleukin 2 and Interleukin 12 Gene–Transfected Tumor Vaccine for Head and Neck Carcinoma

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**Background:** The biological effects of cytokines are coming to be understood. The therapeutic effects of interleukin (IL) 2, IL-12, and interferon γ (IFN-γ) in cancer treatment have been reported, but there are problems when these cytokines are systemically used as therapeutic agents.

**Objective:** To examine the efficacy of IL-2 and IL-12 gene–transfected tumor cell vaccines for head and neck squamous cell carcinoma (SCC).

**Methods:** Homozygous mice with the autosomal recessive nude gene (BALB/c nu/nu mice) were inoculated subcutaneously in the right flank with cells from a human oral floor SCC cell line (KB cells). The mice were then injected with IL-2 and IL-12 gene–transfected KB cells (KB/IL-2 and KB/IL-12 cells, respectively) irradiated with 2000 rad (20 Gy).

**Results:** No mice died soon after the injection of the gene immunotherapy. The treatment with either KB/human IL-2 (hIL-2) or KB/murine IL-12 (mIL-12) was not very effective. However, the treatment with both KB/hIL-2 and KB/mIL-12 cells significantly and safely inhibited the growth of established tumors ($P = .04$). There was no significant difference in antitumor effect between once-weekly and twice-weekly injections of both KB/hIL-2 and KB/mIL-12 cells.

**Conclusion:** Double gene immunotherapy is safe and effective treatment for SCC in mice.

ates CD4+ lymphocytes. It is made up of 2 disulfide-linked subunits designated p35 and p40, both of which are required for biological activities. Interleukin 12 is most noted for its ability to regulate the balance between type 1 and type 2 T helper cells and appears to be necessary for generation of optimal type 1 helper T-cell response in many experimental settings. It also appears to play a pivotal role in promoting cell-mediated immunity against predominantly intracellular microbial pathogens.

Another important property of IL-12 is its ability to induce the production of large amounts of interferon γ (IFN-γ) from resting and activated T and NK cells. Through direct action and through its ability to induce IFN-γ secretion from T and NK cells, IL-12 plays a central role in innate and adaptive immunity important to host defense against predominantly intracellular pathogens. Additionally, IL-12 has been shown to induce an antitumor immune response against several murine tumors, and clinical trials of this effect have commenced.

Thus, IL-2 and IL-12 have very strong antitumor effects. But it is difficult to use them systemically as drugs because of their adverse effects and short half-lives. However, there are some reports indicating that combination nonviral IL-2 and IL-12 gene therapy is effective in head and neck SCC and that the nonviral gene delivery system is without severe toxic effects. Indeed, viral vector has dangerous aspects and the dose of vector must be limited because of its toxic effects, but it has been reported that the nonviral delivery system is not as effective as the viral delivery system.

Rashleigh et al reported that the combination of IL-2 and IL-12 increased NK cell activity against head and neck SCC without increasing the toxic effects attendant with increasing doses of IL-2. There are many reports about the antitumor effects of IL-2 and IL-12. In those articles, the systemic administration of IL-2 and/or IL-12 was noted to be very toxic. To use these cytokines as systemic agents in safety, the dose must be limited, resulting in limited antitumor effects. So strategies have been explored to use viral delivery systems to transduce the genes of antitumor cytokines.

In our study, we used IL-2 and IL-12 double gene therapy with irradiated tumor vaccine made in vitro by a small quantity of viral vector without severe toxic effects. We describe herein the potential of a transfected IL-2 gene delivery system with amplicon-based HSV vector (HSV/human IL-2 [hIL-2]) and an IL-12 gene delivery system with retrovirus vector for the treatment of human head and neck SCC.

### METHODS

#### MICE, CELL LINE AND CULTURE, AND VERO CELLS

Female homozygous mice with the autosomal recessive nude gene (BALB/c nu/nu mice) were purchased from Japan Oriental Koubo (Tokyo, Japan) at age 6 weeks and were housed 5 per cage with a 12-hour light-dark cycle in a temperature-controlled environment. The KB cells were grown in RPMI-1640 medium (Life Technologies Inc, Gaithersburg, Md) supplemented with 10% fetal calf serum, 2mM glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin (complete medium) at 37°C in a 5% carbon dioxide atmosphere. African green monkey kidney (vero) cells transfected with promoter-based human cytomegalovirus/hIL-2, producing HSV/hIL-2 vector, were cultured in Dulbecco modified Eagle medium (Gibco BRL, Grand Island, NY) containing 10% heat-inactivated fetal calf serum under the above conditions.

#### TRANSFECTION OF hIL-2 GENE INTO KB CELLS

The KB cells were seeded in 24-well plates (Falcon, Becton Dickinson, San Jose, Calif) at 1×10⁶ cells/well and infected with HSV/IL-2 at an MOI (multiplicities of infection) of 50 in 1.0 mL of complete medium. After exposure to vector stocks for 2 hours, the plates were washed 3 times with phosphate-buffered saline. The transfection efficiency of the vector was about 60% to 70% at an MOI of 50. The determination of MOI was made previously.

#### RECOMBINANT RETROVIRAL VECTORS

The genes (p35 and p40) that code for the subunits of murine IL-12 (mIL-12) have been subcloned into the mammalian expression vector TFG (transduced fibroblast gene) mIL-12, as previously described. The TFG–mIL-12 was transfected into the CRIP packaging cell line, and CRIP-TFG–mIL-12 clones were selected with 0.75 mg/mL of geneticin (G418 sulfate) (Gibco BRL). Retroviral supernatant was generated from CRIP-TFG–mIL-12 clones at a concentration of 5×10⁵ to 5×10⁶ colony-forming units/mL. A second retroviral vector containing only the neomycin phosphotransferase gene (Neo) served as a negative control in all of our experiments (provided by Genetic Therapy Inc, Gaithersburg, Md).

#### TRANSFECTION OF mIL-12 GENE INTO KB CELLS

The KB cells were transfected with mIL-12 virus (KB/mIL-12) by incubating 5×10⁶ cells in a 25-cm² area in a sterile flask 24 hours before infection. After aspiration of the medium, 2 mL of CRIP-TFG–mIL-12 or neomycin viral supernatant (5×10⁵–5×10⁶ colony-forming units/mL) was added to each flask with gentle rocking. Two microliters of Polybrene (Gibco BRL, 8 mg/mL) was added to each flask followed by incubation at 37°C for 2 hours. Flasks were rocked every 15 minutes throughout the 2-hour incubation. Eight milliliters of complete medium was added to the flask, which were then incubated overnight at 37°C in 5% carbon dioxide. Fresh medium was added to the flask the following day, and cells were split at a 1:5 dilution in complete medium with 0.75 mg/mL of G-418 when confluent. Infection with the Neo-retrovirus for generation of the KB/Neo cell line was carried out in the same manner as described for KB/mIL-12. The KB/mIL-12 and KB/Neo transfectants were selected in 0.75 mg/mL of G418 sulfate medium for more than 2 weeks, with replacement of the medium every 3 to 4 days.

#### IRRADIATION METHOD, SURVIVAL CURVE OF IRRADIATED KB/mIL-12 CELLS, AND CYTOKINE ASSAY

The KB/mIL-12 cells and KB/mIL-12 cells were irradiated at room temperature with cobalt 60 at a dose rate of 84 rad (0.84 Gy)/min for 24 minutes (total dose, approximately 2000 rad [20 Gy]). After being irradiated, the KB/IL-12 cells were diluted with RPMI-1640 medium; supplemented with 10% fetal calf serum, 2mM glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin (complete medium) to seeding density (1×10⁶ cells/mL); and placed into 96-well plates (Sumitomo Bakelite,
Tokyo, Japan) (100 µL/well). The cells were then incubated at 37°C in a 5% carbon dioxide atmosphere for 72 hours. The cells were treated with trypsin and counted by microscope observation, using trypan blue to determine if the cells survived. The mIL-12 level in the supernatant of KB/mIL-12 cells was examined using a commercially available enzyme-linked immunosorbent assay kit (R & D Systems, Minneapolis, Minn).

TREATMENT WITH hIL-2 AND mIL-12 GENE–TRANSFECTED KB CELLS ON ESTABLISHED TUMORS

Mice were inoculated subcutaneously in the right flank with 5 × 10⁶ KB cells. Seven days after tumor cell inoculation, as a preliminary study, mice were injected peritumorally once a week for 4 weeks (total 4 times) with 5 × 10⁶ KB/hIL-2 and KB/mIL-12 cells or KB/mIL-12 cells irradiated with 2000 rad (20 Gy) or with phosphate-buffered saline as a control (5 mice were used in each group). No KB/β-galactosidase (lacZ) or KB/Neo was experimented with as a control. The location of the injection was a point of subcutaneous tissue bordering the established tumor. Tumor growth was calculated in cubic centimeters using the formula (a × b²) ÷ 0.4, where a is the larger and b the smaller of the dimensions. Tumors were measured every week.

Seven days after tumor cell inoculation, the rest of the mice were injected twice a week for 4 weeks (total 8 times) peritumorally with 5 × 10⁶ KB/hIL-2 and/or KB/mIL-12 cells or KB/lacZ or KB/Neo cells irradiated with 2000 rad (20 Gy), or with phosphate-buffered saline as a control. Tumors were measured once a week as described above. The mice were killed 12 weeks after tumor cell inoculation for evaluation of the IFN-γ levels. Serum samples were obtained by cardiac puncture. All data were expressed as mean ±SD; the t test was used for analysis. The concentration of supernatant murine interleukin 12 (mIL-12) in mIL-12–transfected cells from a head and neck squamous cell carcinoma cell line. Error bars indicate SD calculated from 3 experiments.

The concentration of KB/mIL-12 cells in the culture supernatant gradually increased every day, which suggests that KB/mIL-12 cells keep producing mIL-12 (Figure 1). The concentration of the culture supernatant of KB/mIL-12 cells irradiated with 2000 rad (20 Gy) increased in the first 24 hours but then leveled off, which suggests that KB/mIL-12 cells irradiated with 2000 rad (20 Gy) produce mIL-12 for only the first 24 hours (Figure 2).

SURVIVAL CURVE OF IRRADIATED KB/mIL-12 CELLS

To examine the survival time of KB/mIL-12 cells irradiated with 2000 rad (20 Gy) and to see whether they had an ability to proliferate, we counted the number of cells every 12 hours. The number gradually decreased, and 72 hours after irradiation, almost all of the KB/mIL-12 cells were dead. Error bars indicate SD calculated from 3 experiments. The concentration of supernatant murine interleukin 12 (mIL-12) in mIL-12–transfected cells from a head and neck squamous cell carcinoma cell line. Error bars indicate SD calculated from 3 experiments.

INHIBITION OF TUMOR GROWTH BY IRRADIATED KB/hIL-2 AND/OR KB/mIL-12 CELLS IN VIVO

Tumors were measured in mice treated with KB/hIL-2 and KB/mIL-12 cells or KB/mIL-12 cells irradiated with 2000 rad (20 Gy). The KB tumor–bearing mice were treated with peritumor injections of those cells for 4 weeks (5 × 10⁶ cells, once a week). The injection of 5 × 10⁶ irradiated KB/hIL-2 cells and 5 × 10⁶ irradiated KB/mIL-12 cells once a week significantly inhibited the growth of established tumors (P = .05) (Figure 4). Based
on the survival curve of irradiated KB/mIL-12 cells (Figure 3), it was supposed that those injected cells lived for half a week, so we administered the treatment with peritumoral injections of those cells twice a week for 4 weeks (5 × 10⁶ cells). The tumors were measured in mice treated with KB/hIL-2 and/or KB/mIL-12 cells 2 weeks after tumor cell inoculation. The tumor volume was not significantly different between once-weekly and twice-weekly treatment with both KB/hIL-2 and KB/mIL-12 twelve weeks after tumor cell inoculation (data not shown).

The serum murine IFN-γ levels in each treatment group (irradiated KB/hIL-2 and/or irradiated KB/mIL-12, 5 × 10⁶ cells, twice-weekly injections, 12 weeks after tumor cell inoculation) were determined by enzyme-linked immunosorbent assay. The serum IFN-γ levels of the mice treated with both irradiated KB/hIL-2 and irradiated KB/mIL-12 cells were significantly higher than those of the other groups (P < .05) (Figure 6).

**COMMENT**

The therapeutic efficacy of IL-2 gene–transfected tumor vaccine for head and neck carcinoma has been reported. It is suggested particularly as treatment for advanced and recurrent cases. Indeed, IL-2 gene therapy is effective to some degree, but used alone, it cannot attain a clinically complete response in advanced cases. Interleukin 12 is clearly an important regulator of immune systems and has many biological functions related to antitumor immunity, antimetastasis, and antiangiogenesis. In the present study, IL-12 gene–transfected vaccine therapy was combined with IL-2 gene therapy. Our goals were to determine the efficacy of the IL-2 and IL-12 double gene therapy for head and neck carcinomas and to elucidate the mechanism of the antitumor response with particular interest in the role of IFN-γ.

Interleukin 2 plays an important role in the amplification of immune responses. It stimulates the proliferation of CTLs, T helper cells, NK cells, lymphokine-activated killer cells, and macrophages. Our group has reported the therapeutic efficacy of IL-2 gene–transduced tumor vaccine for head and neck carcinoma and suggested that IFN-γ was secreted by NK cells and acti-

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**Figure 4.** Inhibition on tumor growth by treatment with murine interleukin 12 (mIL-12)–transfected cells from a head and neck squamous cell carcinoma cell line (KB cells) and human interleukin 2 (hIL-2)–transfected KB cells in vivo. Mice were implanted subcutaneously with 5 × 10⁶ KB cells per mouse. After implantation, irradiated KB/hIL-2 cells and/or KB/mIL-12 cells were injected peritumorially once a week for 4 weeks. The treatment with both KB/hIL-2 and KB/mIL-12 significantly inhibited the growth of established tumors. lacZ indicates β-galactosidase; Neo, neomycin phosphotransferase gene; PBS, phosphate-buffered saline; asterisk, P < .05; dagger, P < .01; and double dagger, not significant. Error bars indicate SD calculated from 5 experiments.

**Figure 5.** Inhibition on tumor growth by treatment with murine interleukin 12 (mIL-12)–transfected cells from a head and neck squamous cell carcinoma cell line (KB cells) and human interleukin 2 (hIL-2)–transfected KB cells in vivo. Mice were implanted subcutaneously with 5 × 10⁶ KB cells per mouse. After implantation, irradiated KB/hIL-2 cells and/or KB/mIL-12 cells were injected peritumorially twice a week for 4 weeks. The treatment with both KB/hIL-2 and KB/mIL-12 significantly inhibited the growth of established tumors. PBS indicates phosphate-buffered saline; asterisk, P < .05; dagger, P < .01; and double dagger, not significant. Error bars indicate SD calculated from 5 experiments.

**Figure 6.** The concentration of serum murine interferon γ (mIFN-γ) in mice treated with only human interleukin 2 (hIL-2)–transfected cells from a head and neck squamous cell carcinoma cell line (KB cells) was higher than that of control mice. The concentration of serum mIFN-γ in mice treated with only murine interleukin 12 (mIL-12)–transfected KB cells was also higher than that of control mice. The concentration of serum mIFN-γ in mice treated with both KB/hIL-2 and KB/mIL-12 was higher than in those treated with only KB/hIL-2 or only KB/mIL-12. Moreover, the mIFN-γ concentration in mice treated with both KB/hIL-2 and KB/mIL-12 was higher than that of control mice. lacZ indicates β-galactosidase; Neo, neomycin phosphotransferase gene; asterisk, P < .01; dagger, not significant. Error bars indicate SD calculated from 3 experiments.
vated by IL-2 and that IFN-γ promoted the activation of CTLs and amplified the tumoricidal activity of NK cells. It has also been reported that IL-12 administration in vivo was associated with dramatically increased serum levels of IFN-γ and played a role of immunologic effects.

In the present study, the IL-2 and IL-12 double gene therapy was much more effective than IL-2 gene therapy alone. Twelve weeks after tumor cell inoculation, the murine serum IFN-γ level of the group treated with both IL-2 and IL-12 gene–transfected tumor vaccine was much higher than that of either the IL-2 or IL-12 gene therapy group alone. So serum IFN-γ level seems to be concerned with antitumor activities. And the IL-12 gene–transfected KB cells irradiated with 2000 rad (20 Gy) could not proliferate and survived only about 3 days. Therefore, irradiated KB/IL-12 cells might be clinically safe as an agent to inject. However, there was not a significant difference of antitumor effect between once-weekly and twice-weekly injection of both KB/hIL-2 and KB/mIL-12 cells. It seems that the antitumor effect of the double gene therapy was not merely based on each antitumor effect of IL-2 and IL-12, but on synergistic functions, including the antigenicity of the vaccine.

In conclusion, a new combined therapy using both IL-2 and IL-12 gene–transfected tumor vaccine has a much stronger antitumor effect than either IL-2 or IL-12 single gene therapy alone, and part of the antitumor effect seems to be related to activated CTLs, NK cells, and macrophages promoted by high-level secretion of IFN-γ. Furthermore, in terms of inhibition on tumor growth, antiangiogenic effects of IL-12 might have important roles by down-regulating the expression of vascular endothelial growth factor and basic fibroblast growth factor.