Combination Nonviral Murine Interleukin 2 and Interleukin 12 Gene Therapy and Radiotherapy for Head and Neck Squamous Cell Carcinoma

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Objectives: To demonstrate that the combination of nonviral murine interleukin (mIL) 2 and mIL-12 gene therapy and external beam radiation therapy (XRT) have an enhanced therapeutic effect for the treatment of head and neck squamous cell carcinoma (HNSCC) in an orthotopic murine model and to elucidate the mechanism of action.

Design: A randomized, controlled study in a murine HNSCC model.

Interventions: Tumors were established in the floor of the mouth in C3H/HeJ immunocompetent mice with the SCC VII cell line. These tumors were directly injected with single lipid–formulated mIL-2 or single polymer–formulated mIL-12 or a combination of them and with phosphate-buffered saline or vector without mIL-2 and mIL-12 gene as controls. Then the local tumor was radiated twice with a dose of 1 Gy the next day and injected again 4 days later. Antitumor responses, cytokine expression, and natural killer cell and cytolytic T-lymphocyte activity were assayed. Meanwhile, tumor sizes were measured before and after treatment and compared among the different treatment groups and the controls.

Results: The combination mIL-2 + mIL-12 + XRT demonstrated a significant increase in antitumor effects compared with single therapy or controls. Increased expression levels of primary and secondary cytokines were found in the group treated with mIL-2 + mIL-12, and this effect was preserved when mIL-2 and mIL-12 treatments were combined with XRT. Combination therapy significantly increased antitumor effects, T-lymphocyte infiltration of CD4+ and CD8+, and the numerous necroses compared with monotherapy.

Conclusions: Combination mIL-2 and mIL-12 gene therapy and XRT generates potent antitumor immune responses against HNSCC and significantly increases necrosis (apoptosis) in an orthotopic murine model of HNSCC. The nonviral mIL-2 and mIL-12 gene delivery system was well tolerated. Further optimization of treatment strategy for patients with HNSCC is warranted as well as consideration for human clinical trials.


More than 500,000 new patients each year worldwide contract head and neck cancers.1 During the past 30 years, the 3- to 5-year survival rate of patients with advanced T3 and T4 squamous cell carcinoma (SCC) of the head and neck (HNSCC) has remained poor (20%-30%) despite considerable advances in surgical techniques and irradiation delivery and improvement in chemotherapeutic strategies. Given these dismal figures, advances in gene therapy give new hope for primary or adjuvant treatment of HNSCC. Patients with HNSCC have been well documented to exhibit local immunosuppression with depressed T-cell–mediated responses as well as depressed natural killer (NK) cell and antibody-dependent cell cytotoxic effects.2 Gene therapy approaches include replacement gene therapy, suicide gene therapy, and immunotherapy. Despite these many approaches, gene therapy still has been limited significantly. Current vector technology does not allow enough tumor cells to be transfected to eliminate tumors. Immunotherapy is limited by the transient nature of gene expression, gross tumor burden, and toxic effects.3 Steered by these limitations, gene therapies combined with traditional treatment methods have evolved into current gene therapy efforts. Replacement gene therapy with antioncogene has been combined with chemotherapy and radiation therapy as a treatment for HNSCC. A study combining systemic interleukin (IL) 2 or IL-12 immunotherapy with external beam radiation therapy (XRT) has shown promising results in the treatment of lung and renal
An animal study has shown that local tumor XRT enhances the effect of IL-2 or IL-12 in a small-cell lung cancer model. Interleukin 2 and IL-12 gene therapy combined with XRT in a head and neck cancer model has not yet been investigated.

In our study, we investigated the feasibility, efficacy, and mechanism of combination nonviral mIL-2 and mIL-12 gene therapy and XRT in our immunocompetent murine model for HNSCC. O'Malley et al. have demonstrated augmented immune activity and significant antitumor benefit of nonviral mIL-2 gene therapy alone in this HNSCC model. Given the encouraging preclinical data in these carcinomas, we hypothesized that mIL-2 and mIL-12 gene therapy would enhance local expression of 2 cytokine proteins, induce local regional immune responses, and augment the standard antitumor effect of XRT. In the present study, we assessed the local expression of mIL-2 and mIL-12 and analyzed NK cell and cytolytic T-lymphocyte (CTL) activity and necrosis or apoptosis as possible mechanisms for the antitumor effect.

**METHODS**

**PLASMIDS**

We used PIL0555, PIN0961, and pVC1157 plasmids for our study, all containing the kanamycin resistance gene. The expression cassette for mIL-2 is contained in PIL0555 with the cytomegalovirus promoter. Two complete and separate transcriptions unite for each of the subunits p35 and p40 that combine to form the biologically active mIL-12 p70 molecule, and these are found in PIN0961. The transcription unit for each subunit contains the cytomegalovirus promoter. mIL-12 plasmids contain no coding sequences and was used as control. The plasmids were propagated in Escherichia coli strain DH5α, purified using alkaline lyses and column chromatography, and tested for endotoxin contamination using an amebocyte assay.

**FORMULATIONS**

The mIL-2 (PIL0555) and control (pVC1157) plasmids were formulated in the cationic lipid N-trimethylammonium chloride—N-(1-[2, 3-dioleoyloxy]-propyl)-N—(DOTMA; Avanti Polar Lipids, Alabaster, Ala), with cholesterol used as a colipid to optimize plasmid delivery. Small unilamellar vesicles of a 1:1 molar ratio of DOTMA and cholesterol (Avanti Polar Lipids) were prepared by means of microfluidization. Plasmid lipid complexes were prepared by mixing purified plasmid with these liposomes under controlled conditions in a solution containing 10% lactose as an isotonic agent. The final plasmid-lipid mixture was formulated at a 0.25-mg/mL concentration of plasmid DNA at a DNA-lipid charge ratio of approximately 1:0.5. The mIL-12 (PIN0961) and control (pVC1157) plasmids were formulated in 5% polyvinylpyrrolidone. The plasmid DNA was mixed in a 1:1.7 mass ratio with polyvinylpyrrolidone at a final 1.92 mg/mL concentration of plasmid DNA.

**ANIMAL MODEL**

The animal model used for this study was a syngeneic orthotopic murine model for HNSCC. To establish a tumor model in the floor of the mouth, C3H/HeJ mice were infected with 2 × 10⁵ cells/mL of SCC VII cells using a 25-gauge needle. Injections were made into the floor of the mouth through the neck skin using a sterile technique at day 0. After day 6, established tumors were surgically exposed and measured in 3 dimensions using vernier calipers. Intratumoral multidirectional injections were then given of the respective gene transfer per assigned treatment group. The next treatment was given 1 day after the first gene delivery via 2 administrations of 1 Gy each of x-ray irradiation at 90 and 270 angle points. The injections were given again 4 days after irradiation. Animals were killed 8 days after the initial gene transfer treatment (on day 14 of the experiment), and tumor size was again measured in 3 dimensions using vernier calipers. Harvested tumors and spleens from each animal were used for designated studies.

**MEASUREMENT OF CYTOKINE EXPRESSION IN TUMOR EXPLANTS**

The measurement of cytokine expression in tumor explants has been published previously. In brief, the harvested and finely minced tumors were cultured in 1 mL of Dulbecco Modified Eagle Medium with 10% fetal bovine serum in 3.8-cm² wells. Medium was extracted after 24 hours, and cytokine assays were performed using commercially available monoclonal antibody enzyme-linked immunosorbent assay for mIL-2, mIL-12 (p70), murine interferon (INF)γ (R & D Systems, Minneapolis, Minn), and transforming growth factor β1, (Promega, Madison, Wis).

**NK CELL AND CTL ASSAY FROM SPLENOCYTES**

Lactate dehydrogenase assay was used to detect NK cell and CTL activity, Yac-1 target cells to detect NK cell activity, and SCC VII to detect CTL activity. Splenocytes were harvested and crushed to obtain splenocytes. Cells were washed in Hank balanced salt solution, centrifuged, and resuspended in the solution. Splenocytes were separated using the Ficoll-Hypaque technique and again centrifuged before resuspension in CTL medium. The effector and target cells were then mixed in 96-well plates at different ratios (10:1, 36:1, 50:1, and 100:1) and cultured at 37°C in 5% carbon dioxide overnight. We harvested a 100-µL suspension from each well and placed it into a flat-bottom 96-well plate, added 100 µL of fresh lactate dehydrogenase medium, coincubated at room temperature for 10 minutes, and then stopped the coincubation by adding 0.1 M citric acid to each well. The resulting supernatant of each well was extracted and measured on an enzyme-labeling analyzer at 570 nm (A value) after coincubating in 96-well plates.

With the use of CTL media, splenocytes were washed twice and then plated into a 24-well plate at a concentration of 4 × 10⁶ cells per well. Mitomycin-treated SCC VII cells were used as stimulator cells and plated into each of the wells at a concentration of 1 × 10⁷ cells/mL of media. Murine IL-2 (Pharmingen, San Diego, Calif) was then added to each well at a concentration of 1 ng per well, and the cells were incubated for 7 days. The SCC VII target cells were prepared by means of incubation for 1 hour followed by 3 washes in culture medium. The SCC VII cells were seeded into 96-well plates at a density of 3 × 10⁵ cells per well containing 100 µL of medium. Effector splenocytes then were added to give effector-target ratios of 10:1, 36:1, 50:1, and 100:1 in a final volume of 200 µL/well. Anti-CD4 or CD8 blocking antibody (Pharmingen) experiments were performed to assay the specificity of the tumor response. Antibodies were added at a concentration of 0.2 µg/10 µL for each tissue culture well, and blocking assays were performed in triplicate. Plates were incubated for 16 hours and centrifuged, and the supernatant was assayed on an enzyme-labeling analyzer. The percentage of specific lysis was determined using the following formula: (sample A value–spontaneous A value)/(maximum A value–spontaneous A value) × 100%.
The animal ethics committee of West China Hospital at Sichuan University approved the present study. Tumor-bearing mice were killed humanely, and the explants were excised. The immunoperoxidase staining was performed on paraffin sections of 4-μm thickness. Primary antibodies used were goat and anti-rabbit (CD4, 1:100 dilution; CD8, 1:100 dilution) (Santa Cruz Biotechnology Inc, Santa Cruz, Calif). Secondary antibodies were horseradish peroxidase–conjugated rabbit antimouse IgG (Santa Cruz Biotechnology Inc). Phosphate-buffered saline was substituted for primary antibody as the negative control, and the positive control was provided by assay kit. Simultaneously, traditional hematoxylin-eosin staining was performed for all of the paraffin sections.

STATISTICAL ANALYSIS

All values were calculated as mean ± SD. A paired t test and 1-way analysis of variance were used. The significance of difference between treatment groups was determined using SPSS software (version 11.5). All P values less than .05 were considered statistically significant.

RESULTS

EFFECT OF COMBINATION mIL-2 AND mIL-12 GENE THERAPY AND XRT ON TUMOR GROWTH

We divided C3H/HeJ mice with established floor of the mouth tumors into the following 10 experimental treatment groups: lipid-formulated mIL-2 only; lipid-formulated mIL-2 + XRT; polymer-formulated mIL-12 only; polymer-formulated mIL-12 + XRT; lipid-formulated mIL-2 + polymer-formulated mIL-12; lipid-formulated mIL-2 + polymer-formulated mIL-12 + XRT; XRT only; vacant vector of mIL-2; vacant vector of mIL-12; and no treatment (phosphate-buffered saline). Lipid-formulated mIL-2 was given at a dose of 12.5 μg, whereas polymer-formulated mIL-12 was used at a dose of 150 μg; XRT was used at an absorbed dose of 1 Gy every time; vacant vector and phosphate-buffered saline were used at a dose of 50 μL.

All the animals received 2 injections and were irradiated twice. The first injection was performed after surgical exploration of the initial tumor, and the same injection was done again in 4 days. The animals were irradiated locally twice simultaneously at different angle points after the first gene delivery. All the animals were observed carefully with attention to change in tumor size, and then the animals were humanely killed on day 8 after the first injection and irradiation. The tumor size in each animal was determined using a dimensional vernier caliper measurement. As summarized in Table 1, the mIL-2 only, mIL-12 only, XRT only, mIL-2 + XRT, mIL-12 + XRT, mIL-2 + mIL-12, and mIL-2 + mIL-12 + XRT treatment groups showed significantly more delay in tumor progression than the no-treatment control (PCB) group (P < .001 vs P = .01). In the 10 experimental treatment groups, the greatest antitumor efficacy was observed in the groups of combination mIL-2 + mIL-12 + XRT (Table 1) (P < .001 vs P = .003). The combination group mIL-2 + mIL-12 showed greater effect than the mIL-2 only, mIL-12 only, XRT only, mIL-2 + XRT, and mIL-12 + XRT groups (P < .001 vs P = .01). Combined XRT + mIL-2 or XRT + mIL-12 showed improved antitumor efficacy over the mIL-2 only and mIL-12 only groups (P < .01 vs P = .03). The XRT only group compared with the mIL-2 only and mIL-12 only groups showed no significance difference (P > .05).

CYTOKINE EXPRESSION

Increased expression levels of mIL-2 were found in the mIL-2 only, mIL-2 + XRT, mIL-2 + mIL-12, mIL-2 + mIL-12 + XRT groups, which showed statistical significance when compared with mIL-12 alone, mIL-12 + XRT, and no-treatment control groups (P < .05) (Figure 1A). Similarly, the greatest mIL-12 expression was seen in the mIL-12 alone, mIL-12 + XRT, mIL-2 + mIL-12, and mIL-2 + mIL-12 + XRT treatment groups. The greatest mIL-2 expression was seen in the mIL-2 only and mIL-2 + XRT groups compared with the no-treatment control group (Figure 1B). Overall, assays of cytokine expression demonstrated that formulated cytokine gene transfer sufficiently induced the intended transgene expression and stimulated a desirable secondary cytokine expression.

NK CELL ACTIVITY

We harvested NK cells from splenocytes to evaluate their ability to lyse tumor cells after the various treatment regimens. The greatest NK cell activity occurred in the mIL-2 + mIL-12 treatment group followed by the mIL-2 + mIL-12 + XRT group. At an effector-target cell ratio of 50:1, the mIL-2 + mIL-12 and mIL-2 + mIL-12 + XRT treatments resulted in 72.4% and 49.4% target cell lysis, respectively. At an effector-target cell ratio of 100:1, that
percentage increased to 96.7% and 80.4%, respectively. At the same concentrations, mIL-2 only and mIL-12 only resulted in 41.4% and 48.3% target cell lysis (50:1) and 67.0% and 71.7% (100:1) target cell lysis, respectively. The XRT and control groups showed limited NK cell activity of about 20% to 30% (50:1) and about 31.2% to 38% (100:1), respectively. An augmented antitumor NK cell activity found in the mIL-2 + mIL-12 treatment group correlated well with the observation of inhibition of tumor growth (Table 2).

CTL ACTIVITY

To evaluate immune responses in the animal model with HNSCC, splenocytes were obtained from spleens and tested for their ability to lyse tumor cells in vivo. As summarized in Table 2, the greatest CTL activity occurred in the combined mIL-2 + mIL-12 treatment group. At an effector-target cell ratio of 100:1, the mIL-2 + mIL-12 group showed more than 60% target cell lysis. The mIL-2 only and mIL-12 only groups showed 35.6% and 44.3% CTL activity, respectively. Low-level CTL activity was found in the XRT, lipid-formulated, polymer-formulated, and no-treatment control groups (17.4%, 24.7%, 23.3%, and 21.0% cell lysis, respectively). The results of CTL activity correlated well with the data for inhibition of tumor growth and NK cell activity. To determine whether the antitumor immunity was associated with the presence of tumor-specific CD8+ CTLs and CD4+ helper T lymphocytes, monoclonal antibodies against CD8+ or CD4+ were incorporated as blocking reagents in each CTL assay. As summarized in Table 3, the monoclonal antibody against CD4+ was ineffective in blocking cell lysis. This result may indicate that the antitumor response observed was primarily mediated by CD8+ CTLs.

PATHOLOGIC AND IMMUNOHISTOCHEMICAL ANALYSIS OF EXPLANTS IN TUMOR-BEARING MICE

Pathologic Analysis

Paraffin sections and hematoxylin-eosin stain were used to analyze the excised explants. Considerable cellular infiltration of macrophages, neutrophilic granulocytes, and lymph leukocytes was found in the excised tumor tissue. These findings were mostly shown in the mIL-2 + mIL-12 and mIL-2 + mIL-12 + XRT groups vs the noncytokine and control groups (Figure 2A).

Immunohistochemical Analysis

We immunohistochemically evaluated the explants of tumor-bearing mice for the presence of CD4+ or CD8+ T cells under the condition of streptavidin peroxidase immunization with both mIL-2 and mIL-12 (Figure 2B and C). The infiltration of CD4+ and CD8+ T lymphocytes in combined treatment groups was considerably enhanced compared with that observed in the control and noncytokine groups (Table 3).

While the study of gene therapy has shown significant progress, limitations have been encountered. As a result, a new gene therapy trend is evolving, by which existing gene therapy strategies are being combined with traditional chemoradiation treatment methods. The present study focuses on the combination of nonviral mIL-2 and mIL-12 gene therapy with XRT.

It has been shown that nonviral IL-2 gene therapy stimulates primary and secondary cytokine expression and activates tumor-specific CD8+ CTLs, while nonviral IL-12 gene therapy stimulates primary and secondary cytokine expression and activates tumor-specific CD4+ CTLs. Our data confirm the results of these studies; however, we found that the addition of XRT did not enhance or decrease immune activity as represented by CTL activity or local cytokine expression. These findings sug-
gest that the antitumor mechanism of XRT is separate from1 and does not influence the antitumor immune response induced by IL-2 and IL-12. Radiation therapy kills cells by direct and indirect DNA damage, resulting in mitotic cell death or apoptosis.8,9 Interleukin 2 and IL-12 are known to have antitumor properties, and used in combination these cytokines will afford greater effects than are known to have antitumor properties, and used in combination these cytokines will afford greater effects than either alone.10

Some studies have reported the therapeutic efficacy of IL-2 and IL-12 nonviral gene–transfected tumor for head and neck carcinoma. Interleukin 12 is clearly an important regulator of immune systems and has many biological functions related to antitumor immunity, anti-metastasis, and antiangiogenesis. Interleukin 2 gene therapy is effective to some degree, but used alone, it cannot attain a clinically complete response in advanced cases. Kimura et al12 have suggested that INF-γ is secreted by NK cells and activated by IL-2 and that INF-γ promotes the activation of CTLs and amplifies the tumorcidal activity of NK cells. It has also been reported that IL-12 is associated in vivo with dramatically increased serum levels of INF-γ and plays a role in immunologic effects.13,14

Interleukin 12 produced by macrophages is a potentially powerful antitumor cytokine. By enhancing the cytokolytic activity of both NK cells and CTLs, inducing the production of IFN-γ, and favoring the response of type 1 T-helper (Th1) cells over Th2 cells, IL-12 is thought to be a key participant in vivo NK T-cell activation and in the generation of Th1-like T cells.15,16 The superior antitumor activity of mIL-2 and mIL-12 when used together may be explained by augmented NK cell and CTL responses.

We hypothesized that the underlying mechanism of the observed augmented NK cell and CTL responses was related to an increase in the expression levels of mIL-2 and mIL-12. It has been shown that intratumoral delivery of mIL-12 results in increased infiltration of NK cells and CD4+ and CD8+ T cells. It would be advantageous if IL-2 and IL-12 could be used in combination because increased cytokine expression and decreased levels of transforming growth factor β, which correlates well with tumor growth inhibition expression, may contribute to the superior antitumor effects of combined IL-2 + IL-12 therapy. The observed inhibition in tumor growth seen in our study probably results from overcoming the immunosuppression and reactivating suppressed tumor-specific CTL in our mouse model with HNSCC.

Herein, we have demonstrated an increase in the number of necrosis cells in the IL-2, IL-12, and XRT groups individually. When the 3 therapies were combined, there was a significant enhancement in tumor necrosis. Dezso et al17 and Younes et al18 reported that XRT enhances the therapeutic effect of systemically administered IL-2 on pulmonary metastases from renal adenocarcinoma. Macrophages play an important role in enhanced local and systemic CTL immune response. In contrast, our results do not suggest an enhanced immune response with decreased levels of INF-γ...
The addition of XRT; however, we did not look directly at macrophage activity. As is well known for XRT, apoptosis and mitotic cell death result from DNA in tumor cells. From our data, it appears that the antitumor effects of combination mIL-2 + mIL-12 + XRT occur through parallel mechanisms. In our case of IL-2 only and IL-12 only gene therapy, the antitumor response resulted from a stimulated immune response and direct tumor cell killing by CD4+ and CD8+ cells.

The significant antitumor effect was seen when mIL-2 gene therapy and XRT were combined or mIL-12 gene therapy and XRT were combined. The much more significant antitumor effect was seen when the 3 therapies were combined. These data support further investigation and development of this therapeutic strategy. Dose variation and fractionated treatment of XRT combined with mIL-2 and mIL-12 gene therapy should be included in further studies. Evaluation of macrophage activity could be an area of investigation as well. In addition, mIL-2, mIL-12, and XRT could be combined with surgery or chemotherapy in a classic adjuvant strategy.

Although nonviral systems have shown low efficacy for in vivo gene transfer compared with viral strategies, the lower toxicity profile, greater availability, and persistent clinical effects make them worthy of further investigation. In our study, nonviral systems were used to deliver IL-2 and IL-12 genes and proved their efficacy in vivo. We have not only demonstrated the antitumor activity of IL-2 and IL-12 gene transfer but also observed greater antitumor activity when the two were used in combination with each other and with XRT. This nonviral delivery system to the local tumor mass has proven efficient in its gene transfer and, in addition, circumvents the severe toxic effects of systemic therapy and the limitations of viral-based gene therapy. The safety of nonviral IL-2 and IL-12 gene therapy has been demonstrated in phase 1 and phase 2 human trials for head and neck cancer.

This novel combination therapy has great potential for human application. Irradiation is a common therapy for head and neck cancer, and more preclinical animal work will allow optimization and assessment of survival of this combination therapy. This novel strategy warrants consideration in human clinical trials for head and neck cancer.

In conclusion, the present study provides evidence that the nonviral gene delivery system is well tolerated and that IL-2 and IL-12 gene therapy combined with XRT generate a much more potent antitumor immune response and greater inhibition against HNSCC in our murine model than any 1 of the 3 therapies alone. It further demonstrates that IL-2 and IL-12 gene transfer each activate target cells and antitumor immune responses. Combining IL-2 with IL-12 gene therapy improves the effect of local antitumor and systematic immune reactions and enhances radiotherapeutic efficacy. Furthermore, XRT greatly augmented the antitumor efficacy of IL-2 and IL-12 in our murine model. The results indicate that part of the antitumor effect is related to activated CTLs, NK cells, and macrophages promoted by high-level secretion of IFN-γ, and combined nonviral IL-2 and IL-12 gene therapy with XRT may have great potential as a primary or adjuvant treatment for HNSCC. The XRT materially augmented the antitumor effect after combination of IL-2 and IL-12. The nonviral gene delivery system is a good tool that warrants further laboratory investigation.

Submitted for Publication: November 29, 2004; final revision received May 5, 2005; accepted June 21, 2005.
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Financial Disclosure: None.

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