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

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Objective: To determine the feasibility and efficacy of combination nonviral lipid-formulated murine interleukin 2 (mIL-2) and polymer-formulated murine interleukin 12 (mIL-12) gene therapy for head and neck squamous cell carcinoma (HNSCC) in a murine model.

Methods: Randomized, controlled studies in a murine HNSCC model. Tumors were established in the floor of mouth in C3H/HeJ immunocompetent mice. Established tumors were directly injected with lipid-formulated mIL-2 and polymer-formulated mIL-12 alone and in combination. Antitumor responses, cytokine expression, and natural killer cell and cytolytic T-lymphocyte activity were assayed.

Results: The use of combined mIL-2 and mIL-12 gene therapy resulted in significant antitumor effects, compared with each of the single-cytokine and no-treatment (control) groups (P=.01 to P=.02). Tumors treated with the formulated cytokine genes showed an increased level of the corresponding proteins and decreased level of transforming growth factor β (TGF-β) expression. Combined mIL-2 and mIL-12 treatment consistently produced the greater activation of cytolytic T-lymphocyte and natural killer cells than did single-cytokine treatment or other controls at all concentrations tested. Augmented immune responses correlated with clinical antitumor effects.

Conclusions: The nonviral gene delivery system was well tolerated, and combined mIL-2 and mIL-12 gene transfer generated potent antitumor immune responses against HNSCC in our murine model. Combined nonviral IL-2 and IL-12 gene therapy may have great potential as a primary or adjuvant treatment for HNSCC in humans.


Head and neck cancer affects 50,000 new patients each year in the United States and more than 500,000 worldwide. During the past 30 years, the 3- to 5-year survival rate of patients with advanced T3 and T4 squamous cell carcinoma of the head and neck (HNSCC) has remained poor (20%-30%), despite considerable advances in surgical technique and radiation delivery and improvement in chemotherapeutic strategies. This reality has stimulated the development of novel therapeutic strategies for primary or adjuvant treatment of this disease. Immunotherapy represents a particularly promising treatment strategy for patients with HNSCC.

Interleukin 2 (IL-2) is naturally produced by T cells and serves as an important growth and activation factor for cytolytic T lymphocytes (CTLs), macrophages, natural killer (NK) cells, and B lymphocytes. Treatment with IL-2 has produced definite tumor regression in patients with advanced cancer such as renal cell carcinoma, melanoma, and colorectal cancer. In addition, evidence exists of local and systemic activation of immune cells by peritumoral injections of IL-2 in patients with HNSCC. In addition to IL-2, interleukin 12 (IL-12) has been shown to have potent antitumor efficacy. Produced by macrophages and dendritic cells, IL-12 enhances the cytolytic function of NK cells and CTL, and it activates NK and T cells to secrete interferon γ (IFN-γ), a potent activator of macrophages. It has been demonstrated that IL-12 gene therapy prevents the establishment of SCC, inhibits tumor growth, and elicits long-term antitumor immunity.

It has been well documented that the host immune system plays a major role in recognition and destruction of tumor cells, and locoregional immunosuppression allows the advancement of tumors. This immunosuppression is characterized by depressed mitogen responsiveness and re-
MATERIALS AND METHODS

PLASMIDS

The following 3 plasmids were used in this study: pIL0555, pLN0961, and pVC1157. All of the plasmids contained the kanamycin resistance gene. The expression cassette for murine IL-2 (mIL-2) was contained in pIL0555 with the cytomegalovirus promoter. Two complete and separate transcription units, 1 for each of the subunits p35 and p40 that combined to form the biologically active murine IL-12 (mIL-12) p70 molecule, were found in pLN0961. The transcription unit for each subunit contained the cytomegalovirus promoter. We used pVC1157 as a control, containing no coding sequences. The plasmids were propagated in Escherichia coli strain DH5α, purified using alkaline lysis and column chromatography, and tested for endotoxin contamination using an amebocyte assay (Limulus; BioWhittaker, Walkersville, Md).

FORMULATIONS

The mIL-2 plasmid (pIL0555) and control plasmid (pVC1157) were formulated in the cationic lipid N-(1-[2,3-dioleoyloxy] propyl)-N,N,N-trimethylammonium chloride (DOTMA; Avanti Polar Lipids, Alabaster, Ala), with cholesterol 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 1:0.5 (−/+). The mIL-12 (pLN0961) and control (pVC1157) plasmids were formulated in 5% polyvinylpyrrolidone. The plasmid DNA was mixed in a 1:17 mass ratio with polyvinylpyrrolidone. The plasmid DNA was mixed in a 1:17 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. 20 The care and use of all animals was in accordance with the guidelines of the animal welfare committee of University of Maryland School of Medicine, Baltimore. To establish a tumor model animal welfare committee of University of Maryland of all animals was in accordance with the guidelines of the

MEASUREMENT OF CYTOKINE EXPRESSION

IN TUMOR EXPLANTS

The measurement of cytokine expression in tumor explants has been published previously. 21 In brief, the harvested and finely minced tumors were cultured in 1 mL of Dulbecco Modified Eagle Medium (Sigma-Aldrich Corp, St Louis, Mo) 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 assays (ELISAs) for mIL-2, mIL-12 (p70), murine IFN-γ (R & D Systems, Minneapolis, Minn), and TGF-β1 (Promega, Madison, Wis).

NK-CELL ASSAY FROM SPLENOCYTES

Spleens were harvested and crushed to obtain splenocytes. Cells were washed in Hank balanced salt solution, centrifuged, and resuspended in the solution. Splenocytes were separated on Ficoll-Hypaque and again centrifuged before resuspension in CTL medium. Yac-1 target cells were labeled with sodium chromate Cr 51 (51Cr) and coincubated with 4 dilutions of effector cells to yield 4 different effector-target cell ratios (100:1, 33:1, 10:1, and 3:1). The resulting supernatant was extracted and measured on a γ-radiation counter 12 hours after coincubating in 96-well plates.

CTL ASSAY FROM REGIONAL LYMPH NODE

Regional neck lymph nodes were microscopically dissected from the animals and prepared as previously described. 21 With the use of CTL media, lymphocytes were washed twice, then plated into a 24-well plate at a concentration of 4 × 10⁶ cells/well. Mitomycin-treated SCC VII cells were used as stimulator cells and plated into each of the wells at a concentration of 1 × 10⁵ 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 with 51Cr, followed by 3 washes in culture medium. The 51Cr-labeled SCC VII cells were seeded into 96-well plates at a density of 3 × 10⁴ cells per well containing 100 µL of medium. Effector lymphocytes were labeled with sodium chromate Cr 51 (51Cr) and coincubated with 4 different effector-target cell ratios (3:1, 10:1, 33:1, and 100:1, in a final volume of 200 µL/well. Anti-CD4 or -CD8 blocking antibody (Pharmingen) experiments were performed to assess 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 for 51Cr release using a γ-radiation counter. The percentage of specific lysis was determined using the following formula:

(Sample Release−Spontaneous Release)/ (Maximum Release−Spontaneous Release)×100.

STATISTICAL ANALYSIS

The significance of differences between treatment groups was determined by Mann-Whitney analysis.
duced cytolytic activity, and by decreased cytokine production of tumor-infiltrating lymphocytes relative to regional lymph node and peripheral blood lymphocytes. Transforming growth factor beta (TGF-β) produced by tumor cells is one of the most potent immunosuppressive factors characterized to date. It has been shown to inhibit immunoregulatory cytokine production and to suppress the proliferative response of T cells to IL-2. Recently, Matthews et al reported the restoration of immunogenicity in prostate cancer cells by down-regulation of TGF-β production.

The objective of this study was to demonstrate the efficiency of nonviral gene transfer and to use this system to establish the superiority of combined IL-2 and IL-12 gene therapy combined with single-cytokine therapy in the treatment of HNSCC. Independently, IL-2 and IL-12 have been shown to have significant antitumor effects, and evidence exists that IL-2 and IL-12 may be more effective at inducing tumor rejection when given together.

**RESULTS**

**DOSE RESPONSE OF FORMULATED mIL-2 AND mIL-12 GENE TRANSFER**

We divided C3H/HeJ mice with established floor of the mouth tumors into the following 3 experimental treatment groups: lipid-formulated mIL-2, polymer-formulated mIL-12, and no treatment. The mIL-2 and mIL-12 treatment groups were further divided into 3 subgroups and each animal received variable concentration of treatment. Lipid-formulated mIL-2 was given at 3 different doses of 6.25, 12.5, and 25 μg, whereas polymer-formulated mIL-12 was used at 3 doses of 48, 96, and 150 μg. All the animals received 2 injections, the first during surgical exploration of the initial tumor and a subsequent injection on day 4 after first gene delivery. All the animals were observed carefully with attention to change in tumor size, and animals were humanely killed on day 8 after the first injection. The tumor size in each animal was determined using 3-dimensional caliper measurements. As shown in Figure 1, the mIL-2 and mIL-12 treatment groups were significantly more effective in delaying tumor progression than the no-treatment control group (P<.03 to P=.04). The antitumor efficacy of mIL-2 and mIL-12 improved with increasing concentration of injected dose. The optimal concentrations for mIL-2 and mIL-12 were determined to be 12.5 μg and 96 μg, respectively (Figure 1), and any higher concentration provided minimal improvement in efficacy with no statistical significance (P>.80). For further experiments in assessing the efficacy of combined mIL-2 and mIL-12, the concentration was used as the optimal dosing for mIL-2 and mIL-12.

**INHIBITION OF TUMOR GROWTH**

The greatest antitumor effect was observed with combination therapy consisting of mIL-2 and mIL-12 (Figure 2). Mann-Whitney analysis revealed statistical significance between combined mIL-2 and mIL-12 therapy and mIL-2 or mIL-12 therapy alone (P=.01 to P=.02). Combined mIL-2 and mIL-12 therapy and each single-cytokine therapy showed statistically significant antitumor response when compared with the lipid- and polymer-formulated plasmid group and with the no-treatment control groups (P<.01 with combined therapy; P=.009 with single-cytokine therapy). There was no statistical significance between the formulated...
control plasmid and no-treatment control groups ($P > .50$). There was also no statistical significance between single-cytokine and combined mIL-2 and mIL-12 therapies ($P > .50$).

**CYTOKINE EXPRESSION**

Increased levels of mIL-2 were found in the mIL-2 and combined mIL-2 and mIL-12 treatment groups, which showed statistical significance when compared with mIL-12 alone and combined formulated plasmid and no-treatment control groups ($P < .05$) (Figure 3A). Similarly, the greatest mIL-12 expression was seen in the single mIL-12 and combined mIL-2 and mIL-12 groups ($P < .05$) (Figure 3B). Secondary cytokine expression of murine IFN-γ was demonstrated in the combined mIL-2 and mIL-12 treatment group and in the mIL-2 and mIL-12 treatment groups, with statistical significance between these groups and the combined formulated plasmid and no-treatment control groups ($P < .05$) (Figure 3C). Decreased levels of TGF-β, a marker for tumor activity, were found in the combined mIL-2 and mIL-12 group and in the single-cytokine treatment groups, with significance when compared with combined formulated plasmid and no-treatment control groups ($P < .05$) (Figure 3D). There was no significant difference in decreased TGF-β expression between the combined- and single-cytokine therapies. 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 combined mIL-2 and mIL-12 treatment group. At an effector-target cell ratio of 100:1, combined mIL-2 and mIL-12 treatment resulted in 38% target cell lysis. Single-cytokine therapy with mIL-2 or mIL-12 resulted in 31% and 29% target cell lysis, respectively. The lipid- and polymer-formulated and the no-treatment control groups resulted in limited NK-cell activity of 14% and 6%, respectively (Figure 4). An augmented antitumor NK-cell activity found in the combined mIL-2 and mIL-12 treatment group correlated well with the observation of inhibition of tumor growth.
To evaluate regional cell-mediated immune responses in the animal model with HNSCC, lymphocytes were obtained from the local lymph nodes and tested for their ability to lyse tumor cells in vivo. As shown in Figure 5A, the greatest CTL activity occurred in the combined mIL-2 and mIL-12 treatment group. At an effector-target cell ratio of 100:1, the combination group resulted in 40% target cell lysis. Single-cytokine therapy with mIL-2 and mIL-12 resulted in 35% and 34% CTL activity, respectively. Low levels of CTL activity were found in the lipid- and polymer-formulated and the no-treatment control groups (15% and 10% 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/or CD4+ helper T lymphocytes, monoclonal antibodies against CD8 or CD4 were incorporated as blocking reagents in each CTL assay. As seen in Figure 5B, 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.

**COMMENT**

Interleukin 2 and IL-12 are known to have antitumor properties, and evidence exists that, given in combination, these cytokines will afford greater effects. We observed the greatest antitumor effect with combination mIL-2 and mIL-12 therapy, and these results support the proposed additive strength of mIL-2 and mIL-12.

The superior antitumor activity of mIL-2 and mIL-12 when given together may be explained by augmented NK-cell and CTL responses. The underlying mechanism of the observed augmented NK-cell and CTL responses is thought to be related to an increase in the expression levels of mIL-2, mIL-12, and secondary cytokine IFN-γ, resulting in activation of local and systemic immune cells. It has been known that IL-2 is a major T-cell growth and activation factor and a potent growth and activation factor for NK cells. Numerous studies have shown IL-2–activated tumor inhibition in vivo. Interleukin 12 produced by macrophages and dendritic cells enhances the cytolytic function of NK cells and CTLs. It has been shown that intratumoral delivery of IL-12 results in increased infiltration of NK cells and CD4+ and CD8+ T cells and up-regulation of major histocompatibility complex class I molecules in tumors and lymph nodes. Induction of secondary cytokines such as IFN-γ is also believed to augment the antitumor immune response and incite a cascade in which activated lymphocytes add to the stimulus by increasing their cytokine pro-
duction. It would therefore be advantageous if IL-2 and IL-12 could be used in combination.

In addition to increased cytokine expression, decreased TGF-β expression may contribute to the superior antitumor effects of combined IL-2 and IL-12 therapy. Transforming growth factor β is a potent immunosuppressive cytokine. Recent study has shown that down-regulation of TGF-β production restores immunogenicity in prostate cancer cells. Results of the present study have demonstrated the greatest reduction in TGF-β expression with mIL-2 and mIL-12 combination therapy, which correlates well with tumor growth inhibition. The observed inhibition in tumor growth probably results from overcoming the immunosuppression and reactivating suppressed tumor-specific CTL in our mouse model with HNSCC.

With the role of cytokines in tumor regression established, an effective protocol for local and sustained delivery to the tumor is needed for clinical application. Systemic administration of lymphokines at pharmacological doses produces high concentrations of lymphokines in the vasculature and suboptimal levels at the local tumor site, resulting in limited antitumor effects but moderate to severe toxic effects such as fever, chills, headaches, and capillary leak syndrome. As a result of these limitations, strategies have been explored to use viral vector systems for cytokine gene delivery. Although adenoviral vectors have proven efficient in transferring genes into target tissues and do not require active cell division for uptake and expression, they induce antiviral immune responses and may generate toxic effects from systemic dissemination. The use of retroviral vector systems is limited by the danger of cotransferring contaminating infectious and potential transforming viruses. Given these limitations, nonviral systems have been studied as an alternative method of gene delivery in vivo.

The major criticism and limiting factor of nonviral systems has been the low efficiency of in vivo gene transfer compared with viral strategies. One objective of this study was to demonstrate the efficiency of nonviral gene transfer using a unique plasmid-based therapy specifically designed to facilitate diffusion of the plasmid as well as gene uptake and expression. Our results demonstrate significant cytokine expression and antitumor effect after this nonviral therapy using lipid-formulated mIL-2 and polymer-formulated mIL-12. This nonviral delivery system to the local tumor mass has proven to be efficient in its gene transfer and, in addition, to circumvent the severe toxic effects of systemic therapy and the limitations of viral-based gene therapy.

CONCLUSIONS

The present study provides evidence that the nonviral gene delivery system is well tolerated and further demonstrates that combined mIL-2 and mIL-12 gene transfer generates potent antitumor immune responses against HNSCC in our murine model. The results indicate that combined nonviral IL-2 and IL-12 gene therapy may have great potential as a primary or adjuvant treatment for HNSCC. The nonviral gene delivery system is a promising new tool that warrants further laboratory investigation.

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Accepted for publication June 12, 2001.

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