Combination Nonviral Interleukin 2 Gene Therapy and External-Beam Radiation Therapy for Head and Neck Cancer

David Bray, MD; Shu-Zhen Yu, MD; Hilary Koprowski II, MA; Juong Rhee, PhD; Sanjeev Kumar, PhD; Federica Pericle, PhD; Mohan Suntharalingam, MD; David A. Van Echo, MD; Daqing Li, MD; Bert W. O’Malley, Jr, MD

Objectives: To demonstrate that the combination of nonviral murine interleukin 2 (mIL-2) 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.

Methods: Randomized, controlled studies in the murine orthotopic model of HNSCC. Squamous cell carcinoma VII cells were injected into the floor of the mouth to establish tumors in immunocompetent mice. The intervention groups were treated with mIL-2, radiation therapy, empty plasmid, no treatment, combination mIL-2/XRT, and combination empty plasmid/XRT. Nonviral mIL-2 gene transfer was performed on days 5 and 9. The XRT was administered to the assigned groups 24 hours after first mIL-2 delivery. The mice were killed on day 13. Tumors and local lymph nodes were harvested and evaluated. Primary and secondary cytokine expression, cytotoxic T-lymphocyte activity, and apoptosis were assayed.

Results: The combination mIL-2/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, and this effect was preserved when mIL-2 treatment was combined with XRT. Combination therapy significantly increased apoptosis compared with monotherapy.

Conclusions: The present study demonstrates that combination mIL-2/XRT generates potent antitumor immune responses and significantly increases apoptosis in an orthotopic murine model of HNSCC. Further optimization of this strategy is warranted as well as consideration for human clinical trials.


Despite improvements in surgery, chemotherapy, and radiation therapy, the overall survival for head and neck cancer has not improved in 30 years. The overall 5-year survival rate remains at 50%, with only a 30% 2-year survival rate for cancer stages III and IV. Given these dismal figures, advances in gene therapy give new hope in the battle against head and neck cancer.

Multiple gene therapy approaches have been explored, including replacement gene therapy, suicide gene therapy, and immunotherapy. Despite these many approaches, gene therapy has encountered significant limitations. Current vector technology does not allow enough tumor cells to be transfected to eliminate tumors via replacement or suicide gene therapy. Immunotherapy is limited by the transient nature of gene expression, gross tumor burden, and toxic effects.

Steered by these limitations, current gene therapy efforts have moved toward combinations with traditional treatment modalities. Replacement therapy with p53 has been combined with chemotherapy and radiation therapy as a treatment for cancer. Adenovirus-mediated p53 gene therapy has shown enhanced efficacy when combined with paclitaxel. Pirollo et al have shown a synergistic effect when combining p53 replacement therapy with radiation therapy. Studies combining systemic interleukin 2 (IL-2) immunotherapy and external-beam radiation therapy (XRT) showed promising results in the treatment of renal carcinoma. Animal studies have shown that local tumor irradiation enhances the effect of IL-2 in a Renca murine renal adenocarcinoma model. Radiation therapy combined with IL-2 gene therapy in a head and neck cancer model has not yet been investigated.

In the present study, we investigated the efficacy and mechanism of com-
formulation nonviral murine IL-2 (mIL-2) gene therapy and XRT in our immunocompetent murine model for head and neck cancer. Our previous studies have demonstrated augmented immune activity and a significant antitumor benefit of nonviral mIL-2 gene therapy alone in this head and neck cancer model. Given the encouraging preclinical data in renal carcinoma, we hypothesized that mIL-2 gene therapy will enhance local expression of cytokine proteins, induce local regional immune responses, and augment the standard antitumor effect of radiation therapy. In our present study, we assessed the local expression of mIL-2 and murine interferon γ (mIFN-γ) and analyzed cytotoxic T-lymphocyte (CTL) activity and apoptosis as possible mechanisms for the antitumor effect.

**METHODS**

Two plasmids were used in this study: pVC1157 and pIL0555. All of the plasmids contained the pUC origin of replication and kanamycin-resistant gene. Plasmid pIL0555 contained the expression cassette for mIL-2 under transcriptional control by the cytomegalovirus. Plasmid pVC1157 contained no coding sequences and was used as a control. The plasmids were propagated in *Escherichia coli* strain DH5α, purified using alkaline lysis and column chromatography, and tested for endotoxin contamination using a Limulus assay (Bio-Whittaker, Walkersville, Md).

**FORMULATIONS**

The mIL-2 plasmid (pIL0555) and the 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 were prepared by 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 formulation was formulated at 0.25 mg plasmid DNA/mL at a DNA–lipid charge ratio of 1 (−/+).

**ANIMAL MODEL**

The animal model used was a syngeneic orthotopic murine model for squamous cell carcinoma of the head and neck. The care and use of the animals was in accordance with the guidelines of the Animal Welfare Committee of the University of Maryland School of Medicine, Baltimore. To establish a tumor model of the floor of the mouth, 48 mice had 1 × 10⁶ squamous cell carcinoma VII (SCCVII) cells injected into the floor of the mouth through the neck skin using sterile technique at day 0. At day 3, the tumors were surgically exposed and measured in 3 dimensions. The 48 mice were divided into 6 groups, with 8 mice in each group. On days 5 and 9, the tumors of 4 groups were directly injected with either 12.5 μg of formulated mIL-2 or 12.5 μg of formulated pVC1157. Three of the 6 groups were irradiated with 200 rad (2 Gy) on day 6, 24 hours after the initial injection of gene therapy. The 6 groups therefore received no treatment, mIL-2 therapy, empty plasmid therapy, XRT, combination mIL-2/XRT, or combination empty plasmid/XRT. On day 13, the mice were killed, the tumors measured, and local lymph nodes and tumor samples saved for designated studies.

**RESULTS**

**EFFECT OF COMBINATION mIL-2 GENE THERAPY/XRT ON TUMOR GROWTH**

The greatest antitumor effect occurred when mIL-2 gene therapy was combined with XRT (Figure 1). The combination mIL-2/XRT had statistically significant smaller tumors compared with the following controls: no treatment (P < .01), empty plasmid (P < .02), mIL-2 alone.
Cytokine expression

Murine IL-2 gene therapy showed a statistically significant increase in mIL-2 expression when compared with control groups (Figure 2). The addition of radiation therapy, however, did not further enhance mIL-2 expression.

Secondary cytokine expression

Murine IL-2 is known to induce secondary production of mIFN-γ. We evaluated whether the addition of XRT would affect production. The groups that underwent mIL-2 gene therapy when compared with the control groups showed a statistically significant increase in mIFN-γ expression (Figure 3). However, the expression of mIFN-γ did not increase with the addition of XRT.

CTL activity

All treatment groups that underwent mIL-2 gene therapy showed a significant increase in CTL activity (Figure 4A).

There was no difference between combination mIL-2/XRT and mIL-2 gene therapy alone, indicating that the addition of XRT does not influence CTL activity. This was further demonstrated by comparable CTL activity in the XRT alone group and the no treatment control group.

Monoclonal antibodies against CD8+ or CD4+ were incorporated as blocking reagents in each CTL assay to determine whether the antitumor response was associated with the presence of tumor-specific CD8+ CTLs or CD4+ helper T lymphocytes. CD8+ blockade resulted in the greatest drop in CTL activity, suggesting a primary CD8+-mediated antitumor response (Figure 4B).
Apoptosis

After immunohistochemical analysis, the number of apoptotic cells in various treatment groups was measured at ×200 magnification. Both XRT alone and mIL-2 alone increased apoptotic staining cells compared with control groups. The combination mIL-2/XRT demonstrated significant increases in apoptotic-stained cells when compared with either treatment alone (Figure 5A). Multiple sections of tumors were analyzed. Cells staining positive for apoptosis were counted using the IPLab software, and data were graphed. As shown in Figure 5B, XRT alone and mIL-2 therapy alone showed statistically significant increases in apoptosis (P<.05). The mIL-2/XRT combination demonstrated statistical superiority over either treatment alone and appeared to have a more than additive effect on the number of apoptotic cells (P<.05).

Comment

Despite significant progress in gene therapy, limitations have been encountered. As a result, a trend is evolving whereby existing gene therapy strategies are being combined with traditional chemoradiation treatment modalities. The present study focuses on the combination of nonviral mIL-2 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.9 Our data confirm the results of these studies; however, we found that the addition of XRT did not enhance immune activity as represented by CTL activity or local cytokine expression. These findings suggest that the antitumor mechanism of XRT is separate from and does not influence the antitumor immune response induced by IL-2.

Radiation therapy kills cells by direct and indirect DNA damage, resulting in mitotic cell death or apoptosis.11,12 Interleukin 2 therapy induces apoptosis through a 2-step process. First, local IL-2 expression stimulates production and activation of CTLs and natural killer cells.9 Second, the activated CTLs and natural killer cells secrete cytotoxic granules containing perforin and granzymes that induce apoptosis.13,14 Interleukin 2–activated T cells also express the Fas ligand, which causes apoptosis via a cell-surface ligand-receptor interaction.13 Herein we demonstrated an increase in the number of apoptotic cells in the IL-2 and XRT groups individually. When the 2 therapies were combined, there was a significant enhancement in tumor apoptosis.

Previous studies have shown that XRT enhances the therapeutic effect of systemically administered IL-2 on pulmonary metastases from renal adenocarcinoma.7,8 These studies report that macrophages, mobilized by radiation, cause an enhanced local and systemic CTL immune response.9 In contrast, our results do not suggest an enhanced immune response with the addition of XRT; however, we did not look directly at macrophage activation. There are no preclinical tumor studies using mIL-2 gene therapy and XRT to compare with our study. From our data, it appears that the antitumor effects of combination mIL-2/XRT occur through parallel mechanisms. As is well known for XRT, apoptosis and mitotic cell death result from DNA damage in tumor cells. In the case of IL-2 gene therapy, the antitumor response results from a stimulated immune response and direct tumor cell killing by CD8+ cells as well as a secondary apoptotic mechanism.

The significant antitumor effect seen when mIL-2 gene therapy and XRT were combined supports further investigation and development of this therapeutic strategy. Future studies should include dose variation and fractionated treatment of XRT combined with the mIL-2 gene therapy. Further mechanism studies could include evaluation of macrophage activity. In addition, mIL-2 gene therapy and XRT could be combined with surgery or chemotherapy in a classic adjuvant strategy.

Regarding the potential of this novel combination therapy for human application, irradiation is a common therapy for head and neck cancer, and the safety of nonviral IL-2 gene therapy has been demonstrated in phase 1 and phase 2 human trials for head and neck cancer (B.W.O. and Shelly McQuone, MD, unpublished data, 2003).
While more preclinical animal work will allow optimization and assessment of survival, this novel strategy warrants consideration in human clinical trials for head and neck cancer.

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Corresponding authors and reprints: Bert W. O'Malley, Jr, MD, or Daqing Li, MD, Otolaryngology–Head and Neck Surgery, University of Maryland School of Medicine, 16 S Eutaw St, Suite 500, Baltimore, MD 21201 (e-mail: bomalley@smail.umaryland.edu or dli@smail.umaryland.edu).

REFERENCES


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