Combination Surgery and Nonviral Interleukin 2 Gene Therapy for Head and Neck Cancer

Daqing Li, Wen Jiang, Jeffery S. Bishop, Robert Ralston, and Bert W. O’Malley, Jr.1
Johns Hopkins University, Baltimore, Maryland 21287 [D. L., W. J., B. W. O.] and GeneMedicine, Inc., The Woodlands, Texas 77381 [J. S. B., R. R.]

ABSTRACT

We have developed a novel nonviral interleukin 2 (IL-2) gene therapy that demonstrates significant treatment-specific, antitumor efficacy in combination with subtotal surgical resection in a head and neck cancer murine model. Treatment of established head and neck tumors in immunocompetent mice was performed via direct injection with a cationic liposome composed of DOTMA and cholesterol formulation carrying DNA plasmid for human IL-2 (hIL-2) gene expression. ELISA assays of tumor extracts 24 h after treatment of hIL-2 gene therapy revealed increased local hIL-2 production as well as a formulation-specific secondary induction of murine IFN-γ and IL-12. We hypothesize that the paracrine production of multiple cytokines after IL-2 single gene transfer is important for generating a therapeutic effect, and that this strategy will be well tolerated and effective in combination with surgery for head and neck cancer. In animal experiments where surgery was performed in conjunction with an operative site injection of hIL-2 plasmid formulation, no pre-, intra-, or postoperative toxicity or compromise to wound healing was identified. In murine experiments combining partial surgical resection with the nonviral gene therapy, significant antitumor efficacy was demonstrated in the hIL-2 plasmid formulation group compared with empty plasmid formulation and lactose-injected controls. In a separate experiment using smaller tumor sizes, we also demonstrated that treatment outcomes were dependent on the technical aspect of the actual treatment injection as well as visualization with surgical access. The hIL-2 plasmid formulation gene therapy induces local expression of multiple cytokines, results in treatment-specific antitumor effects, and circumvents many of the concerns and toxicity encountered with viral gene transfer. These data support the need for continued preclinical investigation and the consideration of human clinical trials for combination nonviral hIL-2 gene therapy and surgery for head and neck cancer.

INTRODUCTION

Head and neck cancer afflicts 50,000 new patients each year in the United States and >500,000 worldwide. The 3–5-year survival rate of patients suffering from advanced T₃, T₄ squamous cell carcinoma of the head and neck has remained poor, ranging from 20 to 30% (1). This historically poor survival remains despite innovative surgery and improvements in radiation and chemotherapeutic strategies over the past 30 years.

It has been postulated for many years that the host immune system plays a major role in the recognition and destruction of tumor cells. The absence of an effective in vivo immune response to a tumor has been postulated to be largely due to a failure in activation of T lymphocytes and local production of lymphokines that generate an antitumor response (2). IL-2 is naturally produced by T cells and serves as an important growth and activation factor for CTLs, macrophages, natural killer cells, and B lymphocytes (3). Treatment with IL-2 and IL-2-activated peripheral blood lymphoid cells, or even with IL-2 alone, has produced definite tumor regression in patients with advanced cancer such as renal cell carcinoma, melanoma, and colorectal cancer (4, 5). However, systemic administration of lymphokines at pharmacological doses produces high concentrations of lymphokines in the vasculature and often suboptimal levels at the local tumor site. This large differential in IL-2 concentration results in limited antitumor effects but moderate to severe toxicity such as fever, chills, headaches, and capillary leak syndrome (6). Strategies that increase IL-2 expression in the local tumor environment while decreasing systemic exposure might enhance antitumor responses and eliminate toxicity. Using gene transfer technology is one such strategy that provides sustained local release of cytokines that stimulate antitumor immune response without significant systemic toxicity (2, 7). Although the pharmacokinetics of different cytokines vary tremendously, it is rare to detect greater than 1 ng/ml cytokine in the serum of mice, even after injection of 1 × 10⁷ transduced cells secreting high amounts of cytokine (2, 8). This hypothesis has been validated by several groups working in a variety of murine tumor models including melanoma, metastatic colon carcinoma, and head and neck squamous cell cancer (9).

The focus of these previous investigations has been on the use of gene transfer of cytokines to the local tumor environment using the replication defective retrovirus or adenovirus (7). Adenovirus-mediated gene therapy has recently become a major area of investigation and development in cancer research be-

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1 To whom requests for reprints should be addressed, at Otolaryngology-Head and Neck Surgery, The University of Maryland School of Medicine, 16 South Eutaw Street, Suite 500, Baltimore, MD 21201. Phone: (410) 328-5828; E-mail: bomalley@surgery1.umm.edu. B. W. O. is a consultant and holds equity interest in GeneMedicine, Inc.

2 The abbreviations used are: IL, interleukin; hIL, human IL; mIL, murine IL; CMV, cytomegalovirus.
cause of the inherent benefits over retroviral-based strategies (10–12). The recombinant adenovirus is much more efficient in transferring genes into most target tissues and does not require active cell division for gene uptake and expression. The major disadvantage of adenoviral vectors, however, is that they induce antiviral immune responses and may generate toxicity from systemic dissemination. Attempts at rechallenging an immunocompetent host with an adenoviral vector have resulted in diminished transgene expression (13). Given the limitations and potential pitfalls including immunogenicity, risks of genetic recombination, toxicity, and cytopathic effects of viral gene therapy in general, nonviral systems have been studied as an alternative method of gene delivery in vivo (14).

The major focus of nonviral gene therapy has been on the development of adequate vehicles to deliver therapeutic genes to target tissues in vivo. The most commonly investigated vehicle is the synthetic liposome, which is a positively charged lipid that complexes with DNA. These liposomal complexes facilitate fusion with the negatively charged cell membrane, resulting in transfer of DNA into cells (13). The major criticism and limiting factor of nonviral systems, however, has classically been the low efficiency of in vivo gene transfer compared with viral strategies. The objective of our study is to test the efficacy of a novel hIL-2 cationic lipid formulation gene therapy in combination with surgery for head and neck cancer in an immunocompetent murine model. This novel lipid formulation has been designed to induce the local secondary production of IFN-γ and IL-12 that augments the immune response generated by IL-2 transgene expression.

MATERIALS AND METHODS

hIL-2 Plasmid. The hIL-2 plasmid used in this study is derived from pUC19, in which the selectable marker for ampicillin resistance was replaced with a gene for kanamycin resistance. The expression cassette for hIL-2 used a HCMV MIE promoter, a 56 nucleotide synthetic 5′ untranslated region based on β-globin, and the cDNA for human IL-2. The plasmid for expression of human IL-2 (pIL0697) used the poly(A) signal and site from human growth hormone. An “empty” plasmid (pVC0612), identical to pIL0697 while only lacking the hIL-2 cDNA sequence, was used as a control for analysis of hIL-2 gene-specific effects. Plasmids were propagated in Esherichia coli strain DH 5α and purified using alkaline lysis and column chromatography. The resulting plasmid preparations were tested for contamination by endotoxin using a Limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD). Only plasmid preparations containing <50 IU/mg DNA were used for additional experiments.

Formulations. The formulation selected used DOTMA as the cationic lipid and cholesterol as the colipid to optimize the delivery of CMV promoter-driven chloramphenicol acetyltransferase expression plasmid for hIL-2. Small unilamellar vesicles (cationic liposomes) composed of the DOTMA and neutral lipid cholesterol in a 1:1 m ratio were prepared by microfluidization. The resulting cationic liposomes were mixed with purified plasmid at a DNA:lipid charge ratio of 1.0.5 –+/ (≈1:1 w/w) under controlled conditions in a solution containing 10% lactose as an isotonic agent. The DNA concentration of the final formulation was 0.6 mg/ml.

Animal Model. Animal experiments, including designations for survival outcomes, were approved by the Johns Hopkins University Animal Care and Use Committee. A syngeneic orthotopic murine model for squamous cell carcinoma of the head and neck that we previously developed and described was used for these experiments (15). Floor of the mouth tumors were established in C3H/HeJ mice by percutaneous injection of 5 × 105 SCC VII (squamous carcinoma) cells using sterile techniques under a laminar flow hood. The animals were maintained in standard housing conditions until an appropriate tumor size was observed by using neck exposure. Tumors were measured in three dimensions using calipers following surgical exposure. Smaller tumors (30–40 mm3) were achieved 5 days after initial implantation of SCC VII cells, and they were used in the experiment that demonstrated the importance of delivery techniques. Larger tumors (150–200 mm3 and 180–240 mm3) were achieved 7 days after implantation and were used for the initial assessment of tolerability and later partial surgical resection in combination with hIL-2 treatment. The designed treatments were then applied according to assigned experimental groups. Initial treatment were performed under direct visualization with surgical exposure and subsequent treatments when performed were via a percutaneous route.

Measurement of Primary and Secondary Cytokines. Posttreatment residual tumor masses or local-regional lymph nodes were harvested and minced. A consistent 6 × 6 × 6 mm3 (0.216 ml) volume of tissue was placed in culture in 3.8-cm2 wells containing 1 ml of DMEM plus 10% FCS. After 24 h, conditioned medium was harvested from the explant cultures, and the presence of cytokines was measured using commercially available monoclonal antibody ELISAs (from Genzyme, hIL-2; from R&D Systems, mIL-2, mIFN-γ, and IL-12 p40).

PCR. Posttreatment local-regional lymph nodes were harvested, and DNA material was extracted from the tissue using standard methods. The hIL-2 plasmid-specific sequence was amplified by PCR using forward and reverse primers (CMV78/IL206 from GeneMedicine). A 10-μl reaction consisted of 1.5 μg of purified DNA, 1.5 mM dextonucleotide triphosphates, 0.5 μM of each primer, 1X PCR buffer, and 0.5 unit of Taq. When the OmniGene PCR machine was used, the amplification conditions were: 95°C for 3 min. (1 cycle); denaturation at 95°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 30 s (30 cycles); and 72°C for 10 min (1 cycle). The size of this PCR product is 265 bp.

Statistical Analysis. The significance of differences between treatment groups was determined by Mann-Whitney analysis.

RESULTS

Tolerance and Limitation of hIL-2 Plasmid Formulation. Forty C3H/HeJ mice with established floor of the mouth tumors ranging from 150 to 200 mm3 were assigned to two experimental groups, preoperative and intraoperative injection. The preoperative group of animals received the first hIL-2 plasmid formulation treatment 24 h before the surgical access procedure, whereas the intraoperative group received the first
treatment during the procedure. The two groups were subsequently divided into subgroups and received up to four sequential direct percutaneous injections of hIL-2 plasmid formulation into palpable tumors every 48 h. All of the animals were inspected daily with special attention to wound healing, assessing the presence of incisional ulcers, wound dehiscence, or frank infection with purulence or seroma. Photographs were taken for an objective evaluation of wound conditions. At 2 weeks post-treatment, animals were sacrificed, and level of wound healing was assessed by gross inspection, manual evaluation of tensile strength, and microscopic analysis as compared with untreated controls. Three-dimensional caliper measurements of the tumor mass were obtained. No clinical wound healing complications such as incisional ulcers, seroma, or frank infection were observed in any of the treatment groups. Manual tensile strength was normal in all groups. The surgical incisions were completely healed in 5–7 days, and repeated needle injections did not appear to affect normal wound healing. H&E staining of surrounding normal tissue (muscle, blood vessels, and salivary glands) demonstrated no necrosis or significant inflammatory infiltrate. Microscopic analysis showing no histological changes in distant organs such as lung, liver, bowel, and kidney (data not shown) established relative clinical tolerability of the hIL-2 plasmid formulation. Although all of the animals tolerated the gene therapy administration, there were no significant antitumor effects in the treatment groups versus a control group that received five PBS injections. Bars, SD.

**hIL-2 Gene Therapy in Smaller Tumors and the Importance of Delivery Technique.** On the basis of the previous lack of efficacy upon treatment of a very large tumor burden for this murine model, the following experiment was designed to determine whether gross tumor burden was a limiting factor. This experiment was also designed to evaluate the importance of delivery techniques of the nonviral gene therapy strategy on treatment outcome. A total of 15 C3H/HeJ mice with established FOM tumors ranging from 30 to 40 mm³ were divided into three experimental groups that received injections following a neck exposure operation: (a) hIL-2 standard injection with no leakage of formulation from the injection site; (b) hIL-2 injection with visible leakage ≥30% of total volume; and (c) standard injection using 10% lactose (the base solution for the plasmid formulations). Standard injection was significantly more effective (P = 0.01 to 0.009) in delaying tumor progression than either leaky or control. Bars, SD. B, diminished cytolytic T lymphocyte activity using leaky injection technique compared with standard injection of hIL-2 plasmid formulation. □, IL-2 standard injection; ○, IL-2 leaked injection.
delaying tumor progression than leaked injection (\( P = 0.01 \)) or control lactose injection (\( P = 0.009 \); Fig. 2A). When CTL activity was assessed using block antibody, it was observed that tumors treated with hIL-2 plasmid formulation using the leaked injection method had diminished CTL activity compared with the standard injection technique (Fig. 2B).

**hIL-2 Plasmid Formulation Therapy in Combination with Partial Surgical Resection.** In the preceding experiments, we demonstrated a lack of antitumor efficacy in large tumors (150–200 mm\(^3\)) and a significant antitumor benefit in smaller tumors (30–40 mm\(^3\)). This suggests that a threshold of tumor size may limit efficacy of this nonviral hIL-2 gene therapy. Thus, surgical resection of large tumors is likely to increase the efficacy of the gene therapy by reducing tumor burden while also providing direct visualization and access to the tumor itself. The following experiment was designed to test the hypothesis that hIL-2 plasmid formulation can be effectively combined with partial surgery for large tumors.

Established floor of the mouth tumors were allowed to grow to sizes ranging from 180 to 240 mm\(^3\), and then partial surgical resections were performed, leaving residual tumors in the mylohyoid muscle measuring ~18–24 mm\(^3\). Exposed residual tumors were then injected with 50 \( \mu l \) of one of the following solutions: hIL-2 plasmid formulation, control plasmid formulation, 10% lactose, or PBS. Repeat percutaneous injection was performed 4 days after neck exposure operation, and animals were sacrificed 8 days after first injection. Statistical significance was achieved for hIL-2-treated tumors compared with all remaining groups (\( P = 0.01–0.002 \)). Control plasmid formulation was significantly more effective than lactose or PBS controls (\( P = 0.02–0.004 \)). Bars, SD.

**Fig. 3** Antitumor effect of the hIL-2 plasmid formulation in combination with surgery. Exposed residual tumors in 44 animals were injected with 50 \( \mu l \) of one of the following solutions: hIL-2 plasmid formulation, control plasmid formulation, 10% lactose, or PBS. Repeat percutaneous injection was performed 4 days after neck exposure operation, and animals were sacrificed 8 days after first injection. Statistical significance was achieved for hIL-2-treated tumors compared with all remaining groups (\( P = 0.01–0.002 \)). Control plasmid formulation was significantly more effective than lactose or PBS controls (\( P = 0.02–0.004 \)). Bars, SD.

formulation was also effective in delaying tumor progression versus either 10% lactose or PBS controls (\( P = 0.02 \) and 0.004, respectively).

**Induction of Secondary Cytokines after hIL-2 Plasmid Formulation Treatment.** In this experiment, established FOM tumors were treated with 10% lactose solution, empty control plasmid formulation, or hIL-2 plasmid formulation injections. Twenty-four h after treatment injections, the tumors were harvested, minced, and placed in fresh media in tissue culture. After 24 h in tissue culture, the presence of various cytokines in the culture media was analyzed by ELISA using species-specific monoclonal antibodies. The endotoxin levels of each preparation were measured and matched to rule out the dependence of these effects on LPS. Only tumors treated with hIL-2 plasmid formulation expressed a significant amount of hIL-2 protein compared with empty plasmid or 10% lactose-treated tumors. None of the treatments induced expression of endogenous murine IFN-\( \gamma \) and mIL-12; and treatment with hIL-2 plasmid formulation achieved an even higher level of expression for both cytokines (\( P = 0.05–0.009 \); Fig. 4). Similar
secondary induction of murine IFN-\(\gamma\) and mIL-12 was also observed in local-regional cervical draining lymph nodes after initial intratumoral injections of either hIL-2 plasmid or control plasmid formulations (Fig. 5), and the presence of the hIL-2 plasmid DNA in the lymph nodes after intratumoral injections of hIL-2 plasmid formulation was verified by PCR (Fig. 6).

**DISCUSSION**

Although gene therapy has great potential as a new treatment strategy for head and neck cancers and other solid malignancies, it may find its most promising present application in combination with surgical procedures. Our murine model experiments demonstrated that hIL-2 single gene delivery via a specifically designed cationic lipid formulation could be well tolerated with head and neck surgical procedures. With respect to the surgical site, there were no clinical wound healing complications in either treatment or control groups. Furthermore, there were no identifiable gross or microscope adverse changes in the incision site, the surrounding normal tissue, or distant organs. The lack of serious clinical morbidity associated with hIL-2 plasmid formulation in animal experiments is encouraging; however, more complete systemic safety and biodistribution analyses are required prior to considering human clinical trials.

We demonstrated a lack of antitumor efficacy in large tumors treated with hIL-2 plasmid formulation alone. This is partially due to the historically lower efficiency of gene transfer seen in nonviral delivery systems. Also, the limited diffusion capacity of the nonviral formulation after direct *in vivo* injection might explain the decreased therapeutic efficacy in treating large tumors. We hypothesize that this limitation may be overcome or lessened when the gene therapy is combined with surgery. Surgical resection will not only reduce tumor burden but will provide direct access to the tumor site. Surgical access ensures that the formulation is delivered to the specified tumor site and maximizes the formulated plasmid to tumor cell ratio. After the initial intraoperative treatment, subsequent percutaneous injections appear to be the only clinically feasible alternative at this time. The need to control leakage of formulation and issues of inaccurate delivery emphasize the general limitations of current gene transfer strategies. There is clearly a need for the development of improved delivery techniques such as image-guided direct delivery in the postsurgical setting or more importantly, the development of novel systemically deliverable gene therapies.

Despite the fact that the nonviral strategy of gene delivery is a low efficiency system, we achieved significant antitumor efficacy with the hIL-2 plasmid formulation. This can be explained by the paracrine production of endogenous murine IFN-\(\gamma\) and mIL-12 elicited by both hIL-2 gene product and the plasmid formulation itself. The production of secondary cytokines after delivery of the control plasmid formulation demon-

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**Fig. 5** hIL-2 plasmid formulation treatment effect on secondary cytokine production (murine IFN-\(\gamma\) and mIL-12) in local-regional cervical lymph nodes. Draining lymph nodes from 40 animals were harvested 24 h after direct injection of established floor of mouth tumors. Nodal extracts were assayed for secondary cytokine production. A, lymph node production of murine IFN-\(\gamma\) was significantly increased in the hIL-2-treated tumors versus controls. B, lymph node production of mIL-12 was equally significant for both the control (empty) plasmid and hIL-2 formulation. Bars, SD.

**Fig. 6** Presence of hIL-2 plasmid in local-regional cervical draining lymph nodes after initial intratumoral injection of hIL-2 plasmid formulation as verified by PCR amplification of plasmid containing the hIL-2 cDNA sequence. Lane M, marker; Lane C, positive control for hIL-2 plasmid; Lanes 1–7, PCR products from draining cervical lymph nodes of seven different animals, all positive for the plasmid sequence containing hIL-2 cDNA; Lane W, water; Lane B, buffer.
strated that both cationic lipid and bacterial plasmids play a role in this cytokine induction process. Our results are consistent with previous nontumor studies that show mIFN-γ and mIL-12 production by normal mouse lung in response to intratracheal administration of plasmid DNA formulated in cationic lipid (16). This secondary cytokine response was not observed after administration of formulated methylated plasmid DNA or formulated eukaryotic DNA. Neither cationic lipid alone nor plasmid formulation in saline in our tumor model produced comparable levels of mIFN-γ and mIL-12 (data not shown). DNA of bacterial origin has been shown to elicit the production of several Th-1 cytokines (IFN-γ, IL-12, and IL-6) and increased natural killer cell activity in vitro (17, 18). Induction of these cytokines also can be elicited by oligonucleotides containing a central unmethylated Cpg dinucleotide (17, 18), suggesting that this is an innate immune response to a molecular structure characteristic of bacteria. Moreover, combining the oligonucleotide with Lipofectin can further enhance the production of IFN-γ and mIL-12 by mouse splenocytes treated with such oligonucleotides in vitro. In a separate experiment, we also demonstrated secondary induction of murine IFN-γ and mIL-12 in local-regional cervical draining lymph nodes. This may play an important role in the overall antitumor effects of the hIL-2 plasmid formulation by augmenting the immune system and may prove beneficial against microscopic regional nodal or distant metastasis.

Although we demonstrated that direct injection of the cationic lipid hIL-2 plasmid formulation can significantly delay tumor progression in an orthotopic head and neck murine cancer model, there is a lack of complete cure, despite a treatment-specific induction of hIL-2 and secondary cytokines. Future studies will address the issues of long-term survival advantage of the hIL-2 plasmid formulation and will evaluate the role of hIL-2 in combination with other therapeutic genes using the nonviral cationic lipid delivery system to improve efficacy of gene therapy treatment in combination with surgery. Our experiments in the murine model demonstrate that this novel nonviral hIL-2 plasmid formulation can be well tolerated and used effectively in combination with surgery, delaying tumor progression by treatment-specific immune stimulation. The significant delay in tumor progression associated with hIL-2 formulation in combination with subtotal surgical resection establishes a role for this strategy as applied to large tumors. Regarding the potential clinical application, this nonviral hIL-2 formulation may prove useful as an adjuvant therapy combined with surgery when gross or microscopic residual disease is present.

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