Interleukin 2 Gene Transfer Prevents NKG2D Suppression and Enhances Antitumor Efficacy in Combination with Cisplatin for Head and Neck Squamous Cell Cancer

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ABSTRACT

Cisplatin has been the most promising single chemotherapeutic agent used against head and neck squamous cell cancer to date. However, dose-related toxicity has been one of the major limiting factors in cisplatin-based therapies, because high doses are required for obtaining a significant antitumor effect. To face the challenge of this limiting factor, a novel interleukin 2 (IL-2)-based combination strategy has been developed. Here we show that the strategy of combination of cisplatin with nonviral IL-2 gene therapy resulted in significant antitumor effects while avoiding dose-limiting toxicity in a head and neck squamous cell cancer murine model. Cisplatin systemic therapy alone suppressed NKG2D expression in lymphocytes. The use of local regional IL-2 gene transfer prevented NKG2D suppression. The combination strategy demonstrated a clear synergistic interaction between cisplatin and IL-2, and NKG2D-based cytotoxicity manifested by increased tumor specific lysis from CTLs and natural killer cells. Moreover, the combination of cisplatin and IL-2 gene therapy greatly enhanced apoptosis and growth inhibition in the treated tumors. This novel combination strategy holds promise for the treatment of head and neck cancer, and the mechanism of NKG2D in activating natural killer and CTL receptors provides a foundation for additional investigation, and development of immune modulation and chemotherapy regimens.

INTRODUCTION

In the year 2000, an estimated 40,300 patients in the United States will be diagnosed with HNSCC, representing 4% of all tumor diagnoses. Whereas surgical resection and definitive radiotherapy remain the cornerstone of therapy for patients presenting with early stage disease, patients presenting with locally advanced stage III and IV tumors remain a significant therapeutic challenge. Chemotherapy plays an important role in the neoadjuvant or adjuvant setting, and management of advanced and metastatic disease. The challenge of treating HNSCC has been to target specific therapies to maximize efficacy and minimize toxicity.

Cisplatin is one of the most active single chemotherapeutic agents against HNSCC. Multiple Phase II studies of cisplatin monotherapy claim response rates ranging from 14% to 41%, with a pooled average of 28% (2, 3). The failure of cisplatin monotherapy is apparently because of development of cisplatin systemic toxicity, as high doses are required to obtain a significant antitumor effect (4–6) and the immunosuppression, such as inhibition of T-cell proliferation and decreases of NK cell activity (7, 8). The mechanism of cisplatin-induced immunosuppression is not fully clear.

It has been well documented that the host immune system plays a major role in recognition and destruction of tumor cells, and local regional immunosuppression allows the advancement of tumors (9, 10). The majority of patients with HNSCC are immunosuppressed, and the level of immune system function correlates with the rate of nodal and distant metastases and a higher mortality rate (1, 11). This immunosuppression is characterized by depressed mitogen responsiveness and reduced cytolytic activity, as well as decreased cytokine production of tumor-infiltrating lymphocytes relative to regional lymph node lymphocytes and peripheral blood lymphocytes (11, 12).

Cell-mediated cytotoxicity is an important immune response to cancer. The mechanisms of cell-mediated cytotoxicity involve direct cell-cell contact between a killer cell and a target tumor cell. The most important activities of cell-mediated cytotoxicity for killing tumor cells in vivo are NK cells and CTLs. Tumor cells can be attacked in the body by activated NK cells, which become active tumor cell killers, and activated CD8+ T lymphocytes, which become CTLs by an MHC-mediated cell-killing system. The molecular mechanism allowing NK cells and CTLs to be activated and to attack tumor cells has not been well elucidated. NKG2D is a recently described activating receptor expressed by both NK cells and CTLs. It has a broader role in cell-mediated immune responses (12, 13). In humans, the NKG2D receptor binds to the polymorphic nonclassical MHC molecules MICA and MICB, which are expressed frequently on tumor cells (14). In the absence of antigen presentation, the interaction of MICA/MICB with NKG2D may promote antitumor responses of NK cell and CD8+ T cells with αβ-receptors (14). A mouse (m) NKG2D has been identified that displays a high degree of similarity with the human NKG2D. Murine NKG2D is expressed on resting and activated NK and CD8+ T cells (15).

IL-2 is naturally produced by T cells, and serves as an important growth and activation factor for CTL, macrophages, NK cells, and B lymphocytes. Treatment with IL-2 has produced definite tumor regression in the cases of renal cell carcinoma, melanoma, and colorectal cancer (16). The peritumoral injection of IL-2 activates local and systemic immune cells in the patients with HNSCC (9, 17). We have developed an animal model with HNSCC to study local delivery of nonviral IL-2 gene transfer strategies and demonstrated antitumor efficacy in both single and combination gene therapy treatments (18–20). Our current study is to investigate whether nonviral IL-2 gene transfer overcomes immunosuppression and enhances antitumor effect when used in combination with cisplatin. Depending on these results, our study will additionally investigate the molecular mechanisms by which CTLs and NK cells recognize and kill target cells.

MATERIALS AND METHODS

Cisplatin and Human IL-2 Plasmid. Cisplatin was purchased form Bristol-Myers Squibb Co. (Princeton, NJ). The IL-2 plasmid used in this study was derived from pUC19, in which the selectable marker for ampicillin resistance...
were added to give E:T ratios of 3:1, 10:1, 33:1, and 100:1, in a final volume available monoclonal antibody ELISAs (IL-2; R&D Systems). The antibody (PharMingen) was used at a concentration of 0.2 mg/ml. Each of the mice received 12.5 μg dotma:cholesterol-formulated IL-2 or control plasmid per injection in desired groups.

**Animal Model.** Animal experiments, including designations for survival outcomes, were approved by the University of Maryland at Baltimore Animal Care and Use Committee. A syngeneic orthotopic murine model for HNSCC that we developed previously and described was used for these experiments (18). Floor of mouth tumors were established in C3H/HeJ mice by percutaneous injection of 5 × 10^6 SCCVII cells using sterile techniques under a laminar flow hood on day 1. The animals were maintained in standard housing until day 5 when tumors were measured in three dimensions using calipers, and the first treatment was administered under direct visualization after surgical exposure. Cisplatin was given via i.p. injection at different doses ranging from 3 mg to 9 mg/kg, whereas dotma:cholesterol-formulated IL-2 or control plasmid was administered via intratumoral injection at the dose of 12.5 μg per injection and repeated via a percutaneous route on day 9. Animals were sacrificed on day 13; tumors, neck lymph nodes and, spleens were harvested, and blood, liver, and kidney samples were taken.

**Measurement of Cytokine.** Post-treatment tumor masses were harvested and minced. A consistent 6 x 6 x 6 mm^3 volume of tissue was placed in 3.8 cm^3 wells containing 1 ml of DMEM plus 10% fetal bovine serum. After 24 h, conditioned medium was harvested from the explant cultures, and the presence of cytokines was measured using commercially available monoclonal antibody ELISAs (IL-2; R&D Systems).

**Cytotoxic T-Lymphocyte Assay from Regional Lymph Node.** Regional neck lymph nodes were collected from the animals and prepared as described previously (18). Using CTIL medium, lymphocytes were washed twice then plated into a 24-well plate at a concentration of 4 × 10^6 cells/well. Mitomycin-treated SCCVII cells were used as stimulator cells and plated into each of the wells at a concentration of 1 × 10^5 cells in 1 ml of medium. Murine IL-2 (PharMingen) was then added to each well at a concentration of 1 ng/well, and the cells were incubated for 7 days. SCCVII target cells were prepared by incubation for 1 h with 3^1*Cr, followed by three washes in culture medium.

**NK Cell Assay from Splenocyte.** Splenocytes were harvested and centrifuged to obtain splenocytes. Cells were washed in HBSS, spun down, and resuspended in HBSS. Splenocytes were separated on Ficoll-Hypaque and again centrifuged before resuspension in CTIL medium. Yac-1 target cells were labeled with 51Cr and coincubated with four dilutions of effector cell to yield four different E:T cell ratios (100:1, 33:1, 10:1, and 3:1). The resulting supernatant was extracted and counted on a gamma counter 12 h after coincubating in 96-well plates.

**Tumor Apoptosis Assay.** Harvested tumors were fixed in 10% neutral buffered formalin and then dehydrated with step-by-step ethanol. The samples were embedded with paraffin into a paraffin block. Paraffin-embedded samples were cut at 5 μm using a Leica microtome device. The flattened sections were put on superfrost slides for apoptosis study.

**RESULTS**

Inhibition of Tumor Growth by Combined Cisplatin with IL-2 Treatment

For determination of the therapeutic efficacy of the combination of cisplatin with dotma:cholesterol-formulated IL-2, we used a syngeneic orthotopic mouse model with established HNSCC. Tumor-bearing mice were treated and monitored for tumor growth. As shown in Fig. 1, IL-2 gene therapy and cisplatin at varied concentrations of 3 mg, 5 mg, 7 mg, and 9 mg/kg when used alone showed statistically significant decreases in tumor size as compared with no treatment (P = 0.004–0.02 and 0.01, respectively). The combination of IL-2 gene therapy and cisplatin at varied concentrations demonstrated statistically significant decreases in tumor size when compared with cisplatin alone (P = 0.012–0.028). Each incremental dose of cisplatin in combination showed statistical superiority to the combination at the next lower dose up to a concentration of 9 mg/kg (P = 0.004–0.045). The combination IL-2 gene therapy and cisplatin demonstrated significant therapeutic benefit over IL-2 gene transfer alone at all of the doses (P = 0.027 and 0.022). The combination of formulated control plasmid and cisplatin showed no increased antitumor efficacy versus cisplatin alone, and it was significantly less therapeutic than combination IL-2 gene therapy and cisplatin (P = 0.013–0.018). There was no statistical significance of antitumor effects between formulated control plasmid alone and no treatment group (data not shown).

**IL-2 Transgene Expression**

To determine whether increased local expression of IL-2 was the mechanism for the enhanced therapeutic benefit of combination IL-2 gene therapy and cisplatin, ELISA assays were used to quantify IL-2 expression in the local tumor environment. As shown in Fig. 2, cisplatin alone showed no statistically significant increase in IL-2 expression when compared with no treatment controls. Dotma:cholesterol-formulated control plasmid in combination with cisplatin was replaced with a gene for kanamycin resistance. The expression cassette for IL-2 was contained in plasmid pIL0555 (mouse IL-2) under transcriptional control of the cytomegalovirus. An “empty” plasmid (pVCM0612), identical to pIL0555 while only lacking the IL-2 cDNA sequence, was used as a control for analysis of IL-2 gene-specific effects. Plasmids were propagated in Escherichia coli strain DH 5a, 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).

**Formulations.** The formulation selected used dotma as the catonic lipid and cholesterol as the colloid to optimize the delivery of cytomegalovirus-promoter-driven expression plasmid for IL-2. Small unilamellar vesicles (catonic liposomes) composed of the dotma and neutral lipid cholesterol in a 1:1 m ratio were prepared by microfluidization. The resulting dotma: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. Each of the mice received 12.5 μg dotma:cholesterol-formulated IL-2 or control plasmid per injection in desired groups.

**Murine NK2GD Assay.** Regional local lymph nodes were collected from each of the groups studied. Total cellular RNA was prepared from the lymphocytes of collected lymph nodes. The lymphocytes were lysed with 1 ml TRZol reagent (Life Technologies, Inc.) and then centrifuged with 0.2 ml of chloroform at 12,000 x g for 15 min at 4°C. The aqueous phase was transferred to a fresh tube. RNA was precipitated with mixing isopropyl alcohol and redissolved with diethyl pyrocarbonate-treated water.

RT-PCR was performed according to the instruction of enhanced RT-PCR kit (Sigma). Briefly, RNA was mixed with the primers (β-actin: sense primer 5'-TACCACAGGCATTGTAGG-3', antisense primer 5'-AAT-AGTGGATGCCTGGCCTG-3'; and mNK2GD: sense primer 5'-CGACCT-CAAGCCGACAAAGTG-3', antisense primer 5'-TGTGCTGATGGGTAATG-3') and reagents. The designed primers of mNK2GD covered 667 bp of 1142-bp full-length cDNA. The amplification was performed in the thermal cycler (Hybaid). PCR products were electrophoresed in 1.5% agarose gels.

**Statistical Analysis.** The significance of differences was determined by Mann-Whitney analysis of StatMost program.
showed statistically significant increases in IL-2 expression as compared with no treatment controls \((P = 0.04)\). We have demonstrated previously that dotma:cholesterol-formulated bacterial plasmids enhance cytokine expression and induce low level immune responses in our head and neck tumor models \((19)\). However, the combination of dotma:cholesterol-formulated IL-2 plasmid with cisplatin at varied concentrations showed a significant increase in local IL-2 expression versus the formulated plasmid controls but did not show increased IL-2 expression when compared with formulated IL-2 gene transfer alone. All of the treatments containing IL-2 were superior to all of those that did not have an IL-2 gene transfer component \((P = 0.01)\). Therefore, enhanced local IL-2 expression is not a mechanism for the apparent synergistic effect of combination IL-2 gene therapy and cisplatin.

**Cell-mediated Cytotoxicity in Combined Cisplatin with IL-2 Treatment**

**CTL Activity.** 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 Fig. 3a, cisplatin in combination with IL-2 appeared to be superior to all of the groups in synergistic CTL activity. At an E:T cell ratio of 100:1, low levels of CTL activity were found in single cisplatin treated group (27% target cell lysis), combined cisplatin and control plasmid group (31% cell lysis), and no treatment group (27% cell lysis). Although the IL-2-treated animals demonstrated an increased level of CTL activity (38% target cell lysis), the greatest activity of CTL occurred in the group treated with combined IL-2 and cisplatin (52% target cell lysis). These results demonstrated a clear synergistic interaction between cisplatin and IL-2 with a marked increase in activating CTL responses against the tumors.

To additionally determine whether the antitumor immunity was associated with the presence of tumor-specific CD8⁺ CTLs and/or CD4⁺ “helper” T lymphocytes, monoclonal antibodies against either CD8 or CD4 were incorporated as blocking reagents in CTL assay. As seen in Fig. 3b, the monoclonal antibody against CD8 effectively blocked the CTL responses against target cells, whereas the antibody against CD4 was relatively ineffective. This result indicates that the antitumor response observed was primarily mediated by CD8⁺ CTLs.

**NK Cell Activity.** NK cells were harvested from splenocytes to evaluate their ability to lyse tumor cells after the various treatment regimes. As shown in Fig. 4, cisplatin alone did not induce a significant increase in NK cell activity compared with no treatment controls. Limited NK cell activity was found in both cisplatin alone (20% target cell lysis) and no treatment (21% cell lysis) groups when looking at an E:T cell ratio of 100:1. However, the combination of lipid formulated control plasmid with cisplatin did demonstrate statistically significant increases in NK cell activity when compared with either no treatment controls or to cisplatin alone \((P = 0.05)\). Despite this, a gene-specific effect was demonstrated in that formulated IL-2 gene transfer in combination with cisplatin at varied concentrations was statistically superior to control plasmid with cisplatin at varied concentrations, as well as cisplatin alone and no treatment. These data support a synergistic immune activation of the combination IL-2 gene therapy and cisplatin treatment strategy.

**Induction of Apoptosis in Combined IL-2 with Cisplatin Treatment**

To investigate whether apoptosis is induced and enhanced by the treatment of combined cisplatin with IL-2, tumors were analyzed by the *in situ* terminal deoxynucleotidyltransferase-mediated UTP end-labeling assay. The most extensive induction of apoptosis was observed in the group treated with combined cisplatin at 5 mg/kg and...
IL-2 in which 36% of cells were identified as apoptotic (Fig. 5). A statistically significant increase in apoptotic cells was found in combined cisplatin and IL-2 group compared with control plasmid at 5 mg/kg ($P = 0.009$), cisplatin at 5 mg/kg ($P = 0.009$), IL-2 ($P = 0.009$), and no treatment ($P = 0.009$). Control plasmid alone did not increase apoptosis as compared with no treatment controls (data not shown). The mice treated with IL-2 gene transfer alone demonstrated no significant increase in apoptotic cells compared with no treatment controls. These results indicate that dotma: cholesterol-formulated IL-2 in combination with cisplatin can augment apoptosis in established tumors.

Activation of mNKG2D by IL-2 in Cisplatin-based Chemotherapy

NKG2D is an activating receptor expressed by both NK cells and CTL. To investigate whether mNKG2D is expressed on the lymphocytes of collected lymph nodes in single IL-2 and cisplatin-treated groups or in combination, RT-PCR analysis was performed using RNA extracted from different groups. The expression of NKG2D was detected in the groups of IL-2 in combination with cisplatin, IL-2, no treatment (with established tumor), and normal control mice (Fig. 6). The mice treated with either cisplatin or cisplatin in combination with dotma:cholesterol-formulated control plasmid had no detected expressions (Fig. 6). These results suggest that cisplatin-based chemotherapy suppresses the expression of NKG2D, whereas dotma:cholesterol-formulated IL-2 gene transfer prevented cisplatin-induced suppression of mNKG2D.

DISCUSSION

Both cisplatin and IL-2 have marked effects on tumor size. The combination of cisplatin and nonviral IL-2 gene transfer proves superior to either of the two alone at all of the concentrations of cisplatin. Furthermore, the effect of IL-2 gene therapy is gene specific with increased antitumor effects compared with dotma:cholesterol-formulated empty plasmid used as a control. A key point from the above experiment is that IL-2 gene therapy in combination with cisplatin at 5 mg/kg (a standard low toxic dose) is superior to cisplatin alone at 7 mg/kg (a dose that causes renal toxicity with elevated renal function; data not shown). The combination of IL-2 gene transfer and
cisplatin at 5 mg/kg is equivalent in efficacy to cisplatin alone at 9 mg/kg (a known toxic dose and lethal in >50% of our treated animals). The combination of cisplatin and IL-2 gene therapy demonstrates synergistic antitumor effects and may prove valuable in reducing toxicity of cisplatin or enhancing standard dosing regimens when translated to the human HNSCC patient population. The fact that a nontoxic dose of cisplatin in combination with IL-2 gene therapy can achieve the same tumor response as a lethal dose of cisplatin alone argues for the coadministration of these two agents and future human clinical trials.

The superior antitumor activity of combination cisplatin and IL-2 gene therapy may be explained in part by augmented NK and CTL responses. It is 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 including our previous findings have shown IL-2-activated tumor inhibition in vivo (18, 19, 21). Cisplatin has been the most promising single chemotherapeutic agent used against HNSCC. Its antitumor activity results from intra- and interstrand cross-links in DNA via covalent bonds with the platinum molecule, leading to DNA strand breakage during replication. The synergy of combined cisplatin and IL-2 gene therapy may be attributed to the release of tumor antigen and tumor cell fragments resulting from cisplatin-induced cell death coupled with classic IL-2 activation of NK and CTLs.

The understanding of the precise molecular mechanisms by which NK cells and CTLs recognize and kill target cells continues to evolve. The discovery, both in mouse and human, of NKG2D receptor clarified the molecular basis of NKG2D-related cytotoxicity (13, 15). NKG2D is expression on resting and activated NK cells as well as involved in natural cytotoxicity mediated by normal NK cells against a variety of tumors. We identified NKG2D receptor expression in the regional lymph nodes and lymphocytes of normal and nontreated HNSCC tumor-bearing mice. However, animals treated with cisplatin chemotherapy regimens alone showed a loss of NKG2D expression in the lymphocytes and regional draining lymph nodes. This loss or suppression of NKG2D on the regional lymphocytes is prevented by combining cisplatin with IL-2 gene therapy. The persistent expression of NKG2D in the combination cisplatin and IL-2 gene therapy groups may play a critical role in enhancing the NK and CTL activity against the established HNSCC tumors.

Cytotoxic cells kill tumor using two mechanisms: (a) release of lytic granules (containing perforin and granzymes); and (b) FasL/Fas-initiated apoptosis. CD8+ T cells use both granule and FasL release to kill targets, whereas NK cells use primarily their granules. The FasL is expressed on mature CD8+ and CD4+ T cells after activation. Ligation of Fas induces clustering of the Fas molecules, association with an intracytoplasmic protein, MORT-1, and, ultimately, apoptosis is the target. An increased level of apoptosis observed in the present study correlates well with increased level of CD8+ CTLs in combined cisplatin and IL-2 treatment group. This finding strongly suggests that CD8+ CTLs, not NK cells, play an important role in regulating apoptosis in the present system. It is possible that cisplatin and IL-2 may be synergistically increasing apoptosis, whereas both are effective on their own.

The combination of cisplatin and IL-2 gene therapy as a realistic treatment for HNSCC holds potential. The addition of IL-2 gene therapy to a nontoxic dose of cisplatin allows us to achieve the same antitumor effects of a lethal dose of single agent cisplatin therapy. The possibility of synergistic antitumor effects with the novel combination therapeutic strategy warrants additional investigation and consideration of human clinical trials.

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