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Combination gene therapy using multiple immunomodulatory genes transferred by a defective infectious single-cycle herpes virus in squamous cell cancer

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Herpes simplex type 2-defective infectious single-cycle (DISC) viruses are attenuated viruses that were originally produced as viral vaccines; however, these viruses are also efficient gene transfer vehicles. The main goals of this study were to examine determinants of the gene transfer by using DISC virus for squamous cancer and to evaluate the antitumoral efficacy of vaccination with tumor cells modified by DISC viruses carrying a combination of immunomodulatory genes (interleukin-2 (IL-2), granulocyte-macrophage colony-stimulating factor (GM-CSF), B7-1) in a model of squamous cell cancer (SCCVII) in C3H/HeJ mice. SCCVII cells transduced by DISC viruses (multiplicity of infection of 10) carrying the IL-2 or GM-CSF gene produced nanogram quantities of IL-2 or GM-CSF per 10^6 cells. Irradiated (5,000 cGy, 10,000 cGy) cells secreted levels of GM-CSF or IL-2 that were comparable with nonirradiated cells. *In vivo* vaccination using tumor cells transduced *ex vivo* with DISC-IL2 or DISC-GMCSF resulted in protection against subsequent tumor challenge ($P < .01$), with DISC-GMCSF-transduced, irradiated tumor cells showing the greatest effects ($P < .001$). Marked growth arrest also was noted in established tumors after direct injection of DISC-GMCSF ($P < .001$). These data demonstrate that (a) DISC virus is capable of efficient gene transfer, (b) GM-CSF-secreting genetically modified tumor vaccine protects against tumor cell challenge and suppresses tumor growth, and (c) intratumoral injection of DISC-GMCSF significantly suppresses the growth of established tumors. These results not only confirm clinically relevant gene transfer but also demonstrate that the gene transfer is an effective anti-cancer therapy. **Cancer Gene Therapy (2000) 7, 1279–1285**

Key words: Squamous cell cancer; gene therapy; tumor vaccine; defective infectious single-cycle virus; granulocyte-macrophage colony-stimulating factor.

Cancer of the head and neck region represents 6.6% of all new cases of malignant disease in United States.[1] One-half of patients with squamous cell cancer of the head and neck present with advanced stage disease, and only approximately one-third of these patients survive 5 years with presently available therapies.[2] The cohort of head and neck cancer patients with advanced disease awaits the development of novel therapies to improve loco-regional control and survival while limiting functional morbidity. Several gene therapy strategies have been evaluated as treatments of squamous cell carcinomas of the head and neck,[3–5] including the use of tumor vaccines consisting of irradiated, nondividing tumor cells genetically engineered to secrete cytokines.[4–9] Many gene transfer vehicles have been used in this regard, including retroviral vectors, adenoviral vectors, and physical means, in attempts to enhance host tumor surveillance by expressing immunostimulatory molecules at the site of putative tumor antigens (Ags).[10–13] Replication-defective amplicon vectors based on herpes simplex virus type 1 (HSV-1) also have been used and have many characteristics highly desirable for such gene transfer. They have the ability to accommodate large inserts, to transduce a variety of cell types, and to transduce slowly replicating or even nonreplicating cells.[14–16]

The defective infectious single-cycle (DISC) virus vector is a HSV-2 virus that is deficient in the gene coding the envelope glycoprotein H (gH), which is essential for the virus to infect cells. When packaged in a complimentary cell line, the DISC viruses produced can infect and transfer their genetic material to a cell, but the viral progeny subsequently produced are noninfective. The safety of such DISC viruses in preclinical studies has been well documented.[17,18] Furthermore, these viruses were originally designed to be viral vaccines and as such are in clinical trials.[19] Current studies

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are examining DISC viruses modified to contain genes encoding interleukin-2 (IL-2), granulocyte-macrophage colony-stimulating factor (GM-CSF), and B7-1, which are immunomodulatory agents showing great promise in the treatment of cancer.[16,20–23] The main goals of this study were to study the determinants of gene transfer to squamous cancers (SCCVII) using DISC viruses and to evaluate the utility of vaccination with DISC viruses carrying a combination of immunomodulatory genes as cancer therapy.

MATERIALS AND METHODS

Mice

Female C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, Me), 6–8 weeks of age, were maintained in specific pathogen-free conditions and were used for experiments at ≥8 weeks of age. The SCCVII tumor is a poorly immunogenic cell line previously used in immunocompetent mice, and has been shown to closely parallel the clinical and biological nature of head and neck cancer.[24] The experimental protocol was reviewed and approved by the Institutional Animal Care Committee of the Memorial Sloan-Kettering Cancer Center.

Cell culture

The SCCVII cell line was a gift from Ditmar Seeman (University of Florida, Gainesville, Fla). It is a cutaneous squamous cell cancer that spontaneously arose in the C3H/HeJ mouse. For *in vitro* studies, cells were grown in minimal essential media supplemented with 10% fetal calf serum at 37°C in a 5% CO_2 humidified atmosphere and subcultured twice a week.

Viruses: Preparation of DISC herpes virus vector

DISC virus vectors were derived from the wild-type strain of HSV-2 known as strain HG52 and modified by deletion of the *gH* gene. It was constructed and prepared as described previously.[18,25] The gH-deleted HSV-2 strain HG52 was propagated and titrated on a complementing Vero cell line that expresses the HSV-2 *gH* gene. Human *IL-2*, murine *GM-CSF*, or murine *B7-1* genes were inserted into the genome of HSV-2 strain HG52 in place of the gH gene using the recombinant vector PIMR3. The resulting recombinant viruses were termed DISC-IL2, DISC-GMCSF, and DISC-B7, respectively. The functional copies of these cytokine genes are under control of a cytomegalovirus immediate-early promoter. The multiplicity of infection (MOI) for the experiments described herein were based on plaque-forming unit (PFU) assays on CR1 cells. DISC virus stocks were stored at −80°C until use.

In vitro *experiments: Transduction of SCCVII cells and demonstration of IL-2 and GM-CSF production*

SCCVII murine squamous cell carcinoma cells were plated in 6-well, flat-bottom plates at a density of 1×10^6 cells/well. After 12 hours, the cells were irradiated: control cells received no irradiation, whereas the other groups were irradiated with 5,000 cGy and 10,000 cGy. The wells were then infected with DISC-IL2 or DISC-GMCSF vectors for 2 hours at MOIs of 0, 1, 5, 10, and 20 by adding the appropriate concentration of virus in 1 mL of medium. After 2 hours of exposure to virus, the wells were washed three times with phosphate-buffered saline (PBS) and 2 mL of medium was added after the final wash. All assays were performed in duplicate, and control wells were treated with medium alone. After 24 hours of incubation, the supernatants were collected and centrifuged at 1500 rpm for 5 minutes. Subsequently, GM-CSF and IL-2 levels were quantified by enzyme-linked immunosorbent assay (ELISA) as described previously (R&D Systems, Minneapolis, Minn).

In vivo *experiments*

In vivo *effect of gene transfer of multiple immunomodulatory transgenes on subsequent tumor challenge.* C3H/HeJ mice were randomly divided into eight groups ($n = 10$/group). In the test groups, the animals were vaccinated by subcutaneous (s.c.) injection of irradiated SCCVII cells (volume of 50 μL) that had been exposed to various combinations of DISC viruses carrying immunomodulatory transgenes; animals in the control group received a s.c. injection of PBS or irradiated tumor cells alone. In previous experiments, we have demonstrated that tumor cells infected with DISC virus carrying the β-galactosidase gene produced no detectable amounts of IL-2 or GM-CSF, and vaccination with tumor cells so modified produced effects that were no different from vaccination with irradiated, noninfected tumor cells. Other groups studying colorectal cancers[26] or hemopoietic malignancies[17] made this same observation. Therefore, in these experiments, to simplify the study design, irradiated tumor cells were used as controls. The mice were vaccinated every 2 days to a total of three doses. The SCCVII cells used for the vaccination were first irradiated to a dose of 10,000 cGy using a ^{137}Cs source. Next, 1×10^6 of the SCCVII cells were exposed to one of the various DISC vectors at a MOI of 10. A s.c. injection was delivered in a volume of 50 μL to the various experimental groups as follows: group I (control), PBS alone; group II, irradiated SCCVII cell alone; group III, DISC-IL2-transduced SCCVII; group IV, DISC-IL2- and DISC-B7-transduced SCCVII; group V, DISC-GMCSF-transduced SCCVII; group VI, DISC-GMCSF- and DISC-B7-transduced SCCVII; group VII, DISC-GMCSF- and DISC-IL2-transduced SCCVII; and group VIII, DISC-GMCSF-, DISC-IL2-, and DISC-B7-transduced SCCVII. During all injections of tumor cells or viruses, the animals were anesthetized with pentobarbital (50 mg/kg intraperitoneally). After 1 week, the mice were challenged with 1×10^5 SCCVII tumor cells (volume of 50 μL) injected into each flank. The animals were examined daily, and body weights and tumor volumes were recorded three times a week. Tumor volume was assessed by assuming the shape to be that of a prolate spheroid with semi-axes "a" and "b" being one-half of the two maximal dimensions, and volume equal to $4/3\pi ab^2$.

In vivo *effects of DISC-GMCSF on established tumors.* C3H/HeJ mice were randomly divided into three groups. In each group, bilateral s.c. flank tumors were established by injecting 1×10^6 (volume of 50 μL) SCCVII tumor cells. The animals were monitored on a daily basis for tumor growth until the implanted tumors reached 0.5 cm in maximum diameter. From prior experience, it takes ~5 days for a 0.5-cm tumor nodule to form. At that time, one of the following preparations was injected every 2 days to a total of three doses intratumorally (i.t.): group I (control), PBS alone; group II, 5×10^6 PFU heat-inactivated DISC-GMCSF (70°C × 5 minutes); group III, 5×10^6 PFU DISC-GMCSF (volume of 50 μL). The animals were examined daily, and body weights and tumor volumes were recorded as described above.

Determination of in vivo *GM-CSF production.* Flank tumors were established in C3H/HeJ mice by injecting 1×10^6 SCCVII tumor cells s.c. When the tumor nodules became palpable (~0.5 cm in diameter), animals were treated in the following groups: group 1 (control), PBS alone; group 2, 5 ×

10^6 PFU heat-inactivated DISC-GMCSF; group 3, 5×10^6 PFU DISC-GMCSF. Two animals in each group were sacrificed at 24, 48, and 72 hours after i.t. injection and tumors were harvested for analysis. Each tumor was weighed, and tumor lysates were prepared for determination of GM-CSF levels by ELISA (R&D Systems).

Determination of i.t. infiltration of $CD4^+$, $CD8^+$, and $CD45R^+$ cells. Mice with established tumors (1 cm in diameter) were injected with three doses of PBS (group 1), 5×10^6 PFU heat-inactivated DISC-GMCSF (group 2), or 5×10^6 PFU DISC-GMCSF (group 3) given i.t. 2 days apart. Animals were sacrificed 7 days after the last i.t. injection and their tumors were harvested for analysis of the surface phenotype of tumor-infiltrating cells. Tumor cell suspensions were prepared by mincing the tumors and digesting them in collagenase (0.1%) for 45 minutes at 37°C and then washing them in PBS. Cells were then stained with anti-murine CD4 (09005A; PharMingen, San Diego, Calif) (1:100), anti-murine CD8 (01044A; PharMingen) (1:100), and anti-murine CD45R (01125A; PharMingen) (1:100) monoclonal antibodies. A fluorescein isothiocyanate-labeled antibody was used to detect $CD8^+$ cells. $CD4^+$ or $CD45R^+$ cells also were stained with a phycoerythrin-labeled antibody. Flow cytometric analysis was performed using a FACScan (Becton Dickinson, San Jose, Calif) and the percentages of $CD8^+$, $CD4^+$, and $CD45R^+$ cells were calculated in all tumor tissue.

Statistical analysis

All data are expressed as the mean ± SE. Comparisons between groups were made using a two-tailed Student's *t* test. An analysis of variance test, where appropriate, was used to identify statistical significance for multiple comparisons.

RESULTS

In vitro *demonstration of GM-CSF and IL-2 production in SCCVII cells*

Production of GM-CSF or IL-2 from SCCVII cells exposed to the DISC-GMCSF or DISC-IL2 viruses, respectively, at MOIs of 1, 5, 10, and 20 was determined by ELISA. IL-2 production ranged from 2000 to 9000 pg/million cells and GM-CSF production ranged from 1800 to 7000 pg/million cells, depending upon the MOI. SCCVII cells before DISC-GMCSF or DISC-IL2 infection produced no GM-CSF or IL-2. Of particular interest was the observation that cells subjected to irradiation (5,000 cGy, 10,000 cGy) secreted levels of GM-CSF or IL-2 that were comparable with the levels seen for nonirradiated cells (Fig 1). This confirms efficient DISC virus-mediated gene transfer even to nonreplicating cells.

In vivo *effect of gene transfer of multiple immunomodulatory transgenes on subsequent tumor challenge*

Animals were vaccinated by s.c. injection of irradiated SCCVII cells (volume of 50 μL) that had been exposed to the DISC virus carrying one of various immunomodulatory transgenes. At 1 week after the third dose, the mice underwent a tumor challenge with 1×10^5 SCCVII tumor cells (volume of 50 μL) injected into each flank.

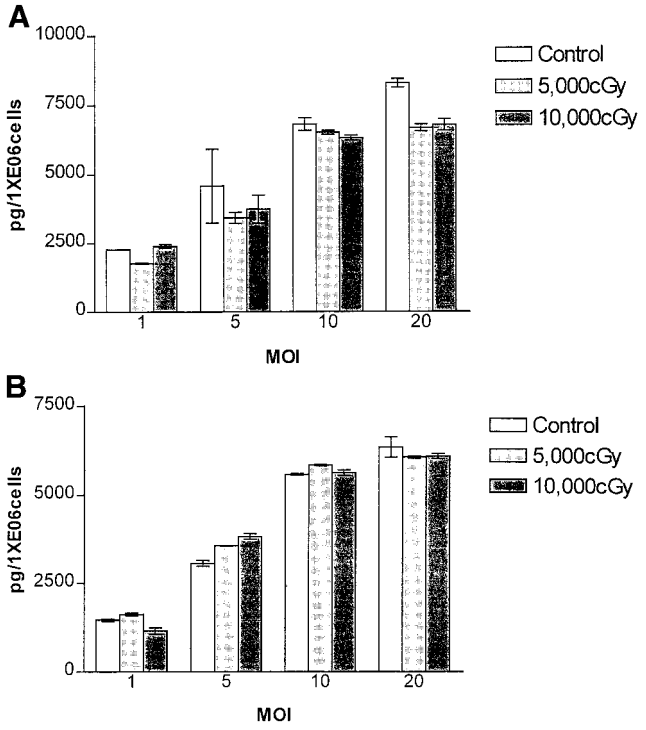

Figure 1. IL-2 **(A)** and GM-CSF **(B)** production by SCCVII cells modified by DISC-IL2 and DISC-GMCSF, respectively. Detection was by ELISA at 24 hours posttransduction. SCCVII cells before DISC-GMCSF or DISC-IL2 infection produced no GM-CSF or IL-2. Of particular interest was the observation that cells irradiated at different doses (5,000 cGy, 10,000 cGy) secreted levels of GM-CSF or IL-2 that were comparable with the level observed for nonirradiated cells. All data are expressed as mean ± SE.

Immunization with DISC-containing immunomodulatory genes significantly reduced tumor growth (Fig 2, A and B). This resulted in a significant improvement in survival ($P < .001$) (Fig 3). There was no significant effect of vaccination with irradiated SCCVII cells. Among the multiple immunomodulatory transgene vaccination groups, treatment regimens containing GM-CSF showed the greatest protection against tumor growth. Most importantly, vaccination with GM-CSF alone proved to be equally efficient in preventing tumor development and growth as a combination of GM-CSF with IL-2, B7, or both.

In vivo *effects of DISC-GMCSF on established tumors*

Because of the above-mentioned findings, we chose to test only DISC-GMCSF in the experiment with established tumors. SCCVII tumors were established in s.c. tissue on the flanks of C3H/HeJ mice. Once tumors had reached ~5 mm in diameter, PBS, 5×10^6 PFU heat-inactivated DISC-GMCSF, or 5×10^6 PFU DISC-GMCSF virus was injected i.t. As seen in Figure 4, inhibition of tumor growth was seen in the DISC-GMCSF-treated group ($P < .001$). In contrast, heat-inactivated DISC-GMCSF showed no significant tumor-suppressive effect.

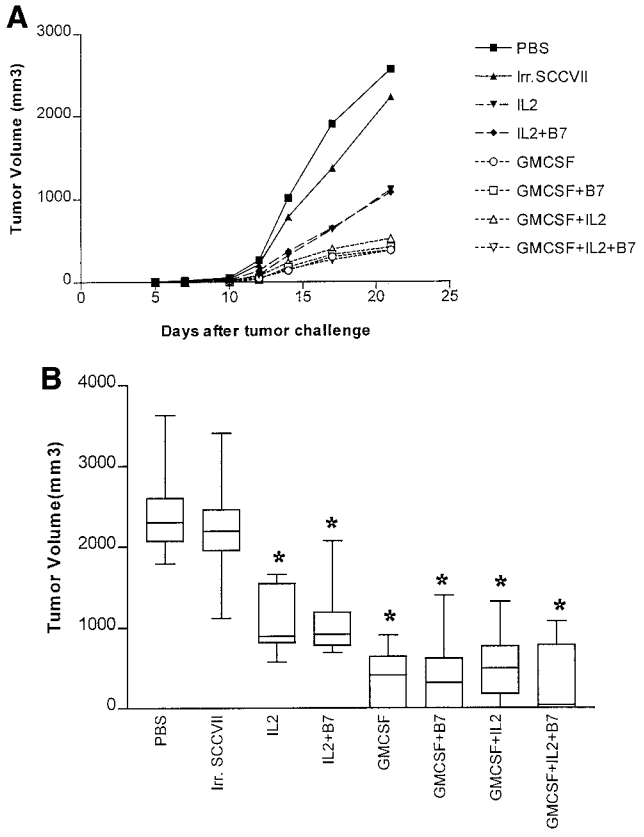

Figure 2. Effect of vaccination with SCCVII cells transduced with various DISC viral constructs on tumor growth in a syngeneic tumor model. **A:** All treated groups were significantly different from groups treated with PBS or irradiated SCC. Among the multiple immunomodulatory transgene vaccination groups, combinations that included GM-CSF showed the greatest antitumoral protection. **B:** Box plots demonstrating the effect of vaccination with SCCVII cells modified with various DISC constructs. Data indicate tumor size at day 21 after tumor inoculation in C3H/HeJ mice. $*$, $P < .01$ for each treated group versus PBS or irradiated SCC controls. PBS, PBS controls; Irr. SCCVII, irradiated SCCVII cells; IL2, DISC-IL2-transduced SCCVII cells; IL2+B7, DISC-IL2- and DISC-B7-transduced SCCVII cells; GMCSF, DISC-GMCSF-transduced SCCVII cells; GMCSF+B7, DISC-GMCSF- and DISC-B7-transduced SCCVII cells; GMCSF+IL2, DISC-GMCSF- and DISC-IL2-transduced SCCVII cells; GMCSF+IL2+B7, DISC-GMCSF-, DISC-IL2-, and DISC-B7-transduced SCCVII cells.

Determination of in vivo *GM-CSF production*

Tumors that were injected with PBS or the 5×10^6 PFU heat-inactivated DISC-GMCSF virus had GM-CSF levels that were below detectable limits, as seen in Figure 5. The group that underwent DISC-GMCSF i.t. injection had significant concentration levels, which exceeded 4500 pg/0.5 g of tumor tissue, but the production decreased markedly over time.

Determination of i.t. infiltration of $CD8^+$, $CD4^+$, and $CD45R^+$ cells

The proportion of $CD4^+$ and $CD8^+$ cells seen in the tumors is shown in Figure 6, A and B. It is interesting to note that the injection of DISC-GMCSF resulted in a significant elevation in the percentage of $CD8^+$ cells in the tumors compared with treatment with PBS or heat-inactivated DISC-GMCSF ($P < .05$). Also, there was significant elevation of the percentage of $CD4^+$ cells in comparison with the PBS-treated group. The i.t. infiltration of $CD45R^+$ cells was not significantly increased compared with control groups.

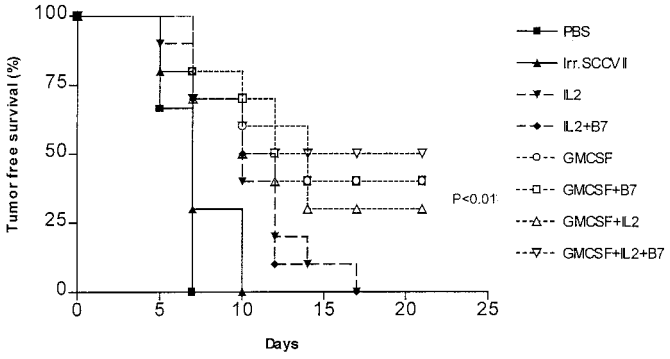

Figure 3. Effect of vaccination with SCCVII cells transduced with various DISC viral constructs on tumor-free survival in a C3H/HeJ syngeneic tumor model. The groups tested are described in the legend to Figure 2. The tumor-free survival of mice from the GM-CSF group was significantly better than that seen for the mice from the IL-2 group (log rank test, $P < .001$: GMCSF versus PBS or Irr. SCCVII, GMCSF+B7 versus PBS or Irr. SCCVII, GMCSF+IL2 versus PBS or Irr. SCCVII, or GMCSF+IL2+B7 versus PBS or Irr. SCCVII).

DISCUSSION

The prognosis of patients with advanced stage head and neck squamous cell cancer remains poor despite aggressive therapies combining surgery, chemotherapy, and radiation therapy. Preclinical studies evaluating viral vector-mediated gene transfer in the treatment of head and neck squamous cell cancer have reported promising preclinical results.[3–5] Among these gene therapy strategies are efforts to augment the immune response of the host to cancer by using tumor vaccines[27–30] to increase the tumoricidal activity of the immune system and to generate lasting immunological memory against tumor cells. One method for using genetically modified tumor vaccines for enhancement of anti-cancer immunity involves introducing genes encoding immunostimulatory molecules into tumor cells in the hope that these compounds will be expressed and secreted in proximity to the tumor cells and putative tumor Ags. Thus, they may provide immunostimulatory signals at the site of the evolving immune response to obviate or reduce the need for $CD4^+$ T cells, which are thought to be a limiting factor in the generation of an effective antitumoral response.[11,27,31] The genes GM-CSF, IL-2, and B7-1 were chosen for the current experiments because they have been shown to be promising immunostimulatory molecules in other models.[27,28,32,33] The main function

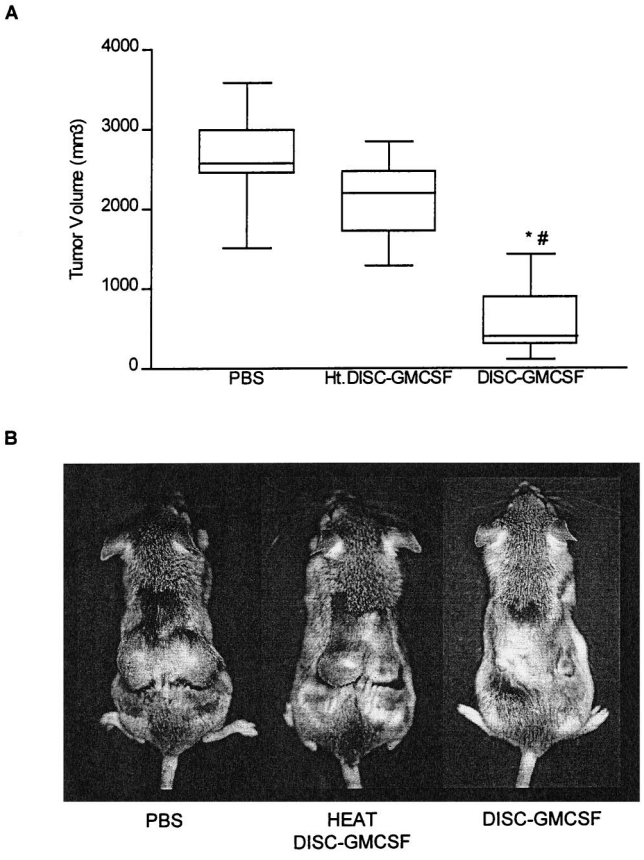

Figure 4. A,B: Effect of DISC-GMCSF injection on an established syngeneic tumor model. At day 14 after the last DISC-GMCSF injection, a great inhibition of tumor growth was seen in the DISC-GMCSF-treated group. In contrast, treatment with heat-inactivated DISC-GMCSF showed no significant tumor suppression effect. *, $P < .001$, control versus DISC-GMCSF; #, $P < .01$, heat DISC-GMCSF versus DISC-GMCSF. All data are expressed as mean ± SE.

of IL-2 is activation and stimulation of major histocompatibility complex (MHC) class II-restricted CD4 cells to mediate efficient induction of a MHC class I-restricted

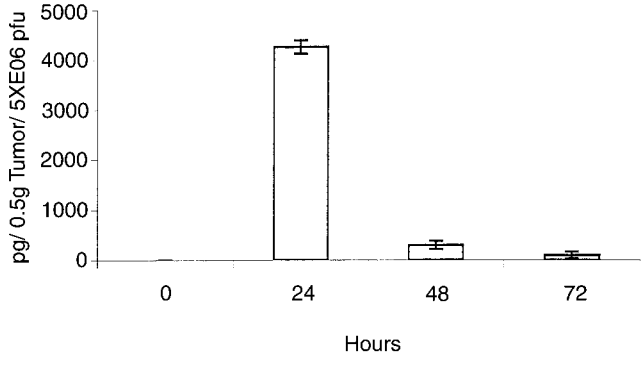

Figure 5. *In vivo* GM-CSF concentration after i.t. DISC-GMCSF injection. The group that underwent the 5×10^6 PFU DISC-GMCSF i.t. injection had significant concentration levels, which exceeded 4500 pg/0.5 g of tumor tissue.

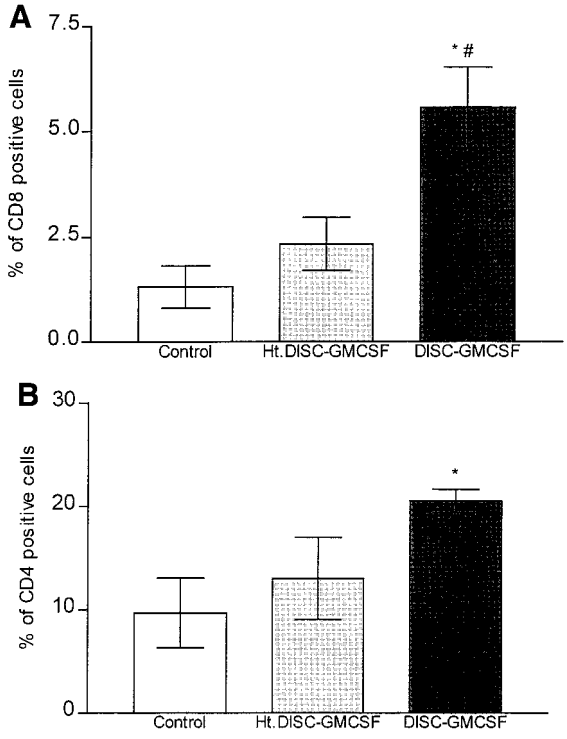

Figure 6. A,B: Flow cytometric analysis for tumor-infiltrating $CD8^+$ and $CD4^+$ lymphocytes. **A:** Treatment of DISC-GMCSF resulted in a significant elevation in the percentage of $CD8^+$ cells in the tumors compared with the PBS or heat-inactivated DISC-GMCSF-treated group. **B:** There was also a significant elevation in the percentage of $CD4^+$ cells compared with the PBS-treated group. *, $P < .05$, control versus DISC-GMCSF; #, $P < .05$, heat DISC-GMCSF versus DISC-GMCSF.

cytotoxic T-cell response. GM-CSF is a major stimulatory cytokine for the maturation and activation of Ag-presenting cells. It also augments the expression of MHC molecules on Ag-presenting cells such as macrophages and dendritic cells. B7-1 is a costimulatory molecule that augments an existing immune response.[27–36] There are theoretical reasons to suppose that the expression of a combination of these molecules would be more effective at eliciting an antitumoral response than the expression of each alone. The premise of the current experiments is that DISC viruses can produce efficient transfer of these genes to tumor cells and elicit antitumoral responses. The results not only confirm clinically relevant gene transfer but also demonstrate that the gene transfer is an effective anti-cancer therapy.

The majority of past gene transfer approaches use adenoviral vectors and retroviral vectors to transfer therapeutic genes.[28,37] Recent work from our laboratory also has demonstrated that HSV amplicon vectors provide efficient gene transfer in head and neck squamous cancer cells and gastrointestinal cancers.[15,38–40] HSVs have a natural affinity for neural tissue, and these vectors systems were originally designed for gene transfer into the nervous system.[23,41] The natural life cycle of the

HSV involves an initial infection of a cutaneous or mucosal surface followed by a lytic replication cycle. Considering its natural cycle, it is not surprising that HSV vectors are efficient vehicles for gene transfer to the epithelial-derived SCCVII cells. Using HSV vectors for gene transfer has many advantages. It allows multiple gene insertion due to its large genome size (150 kbp). It also is capable of rapidly and efficiently infecting a variety of cell types and has the ability to transduce nonreplicating or slowly replicating cells.[15,41] Efficient transduction of irradiated, nondividing tumor cells is a critical factor in the ability to produce a genetically modified tumor vaccine.

Because of some technical obstacles in amplicon production, we examined the possibility of using DISC viruses based on HSV-2. The current results demonstrate that DISC viruses also are efficient gene transfer vehicles. These DISC viruses have many features in common with HSV-1 amplicons in that they can accommodate large insert sizes and infect nondividing cells efficiently and rapidly. The cytomegalovirus early promoter-driven expression of a transgene coding for IL-2 or GM-CSF resulted in high levels of protein production by these transduced cells. These studies demonstrate that DISC-HSV vectors are potentially useful tools for the clinical production of tumor vaccine, because they can transduce irradiated, nonreplicating cells rapidly and efficiently and produce biological effects. These physical properties of HSV translate into important clinical advantages. Freshly isolated tumor cells may be transduced without the need to provide a tissue culture environment conducive to cell replication.

The administration of irradiated SCCVII cells transduced by DISC-GMCSF or DISC-IL2 led to inhibition of tumor growth, whereas tumors treated with irradiated unmodified SCCVII cells continued to progress rapidly. The vaccination of animals with SCCVII tumor cells expressing GM-CSF or IL-2 induced active immunity. A significant percentage of mice that underwent a subsequent challenge with wild-type SCCVII cells did not develop tumors, whereas all control animals developed tumors by day 7 after tumor challenge. Among the animals treated by tumor vaccines exposed to multiple DISC viruses, combinations involving the use of DISC-GMCSF showed the greatest suppressive effects on tumor growth. In the current model, no advantage was gained by adding other immunostimulatory genes to the DISC-GMCSF treatment. Whether combinations of chemokines, costimulatory/adhesion molecules, and cytokines would work better together than each alone in human disease, where more heterogeneous tumors and more disparate tumor locations are encountered, can only be answered by future human trials.

Clinically relevant gene transfer is also possible by direct injection of tumors *in situ*. This is particularly important for head and neck cancers, where patients often will have had surgical resection and radiation as treatment at the primary local site, which is also the most common site of first recurrence. Excision and *ex vivo* tumor transduction to produce a tumor vaccine would be hindered by technical difficulties of operating in this scarred area. The current data indicate that direct injection into the tumor may be sufficient to produce biological effects on established tumors. I.t. injection of DISC-GMCSF resulted in effective *in vivo* production of the cytokine, with clear antitumoral effects. These data, along with the record of safety seen in trials of DISC viruses as antiviral vaccines, support a clinical examination of DISC viruses as a therapy for head and neck cancers.

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