Therapeutic Properties of DNA-based Fibroblast and Dendritic Cell Vaccines in Mice with Squamous Carcinoma

INSUG O-SULLIVAN1, TAE SUNG KIM2, AMLA CHOPRA1 and EDWARD P. COHEN1

1Department of Microbiology and Immunology, University of Illinois College of Medicine, Chicago, IL 60612, U.S.A.; 2School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Republic of Korea

Abstract. Background: Dendritic cells (DC) express class I/II MHC-determinants and co-stimulatory molecules required for T cell activation. In a mouse model of squamous cell carcinoma (SCC), we compared the immunogenic properties of allogeneic DNA-based fibroblast vaccines, which are taken up and processed by DC of the host, and syngeneic DNA-based DC vaccines, which present antigens directly. The incentive was the important practical advantages of using fibroblasts rather than DC in generating vaccines for the immunotherapy of SCC. Materials and Methods: The fibroblast vaccine was prepared by transfer of genomic DNA-fragments (25 kb) from relatively small numbers (107=64 mm3 tumor) of SCCVII/SF cells into LM cells, a mouse fibroblast cell line (H-2 k). SCCVII/SF cells are a highly aggressive squamous carcinoma cell line of C3H/He mouse origin (H-2k). As the transferred DNA spontaneously integrates into the genome of the recipient cells, and is replicated as the cells divide, the number of transfected fibroblasts could be conveniently expanded for repeated immunizations. Syngeneic DC, rather than fibroblasts, were also used as the recipients of DNA from the SCC. C3H/He mice, highly susceptible to growth of SCCVII/SF cells, were immunized with either the DNA-based fibroblast or the DNA-based DC vaccine and the antitumor immune responses were compared. Results: Robust CD8+ T cell-mediated antitumor immunity sufficient to deter the growth of the neoplastic cells was generated in mice immunized with the transfected fibroblasts, but not in mice immunized with the transfected DC. Conclusion: These data raise the possibility that an analogous strategy could be used to treat squamous carcinoma patients with minimal residual disease after primary therapy. Like other neoplasms, squamous carcinoma cells (SCC) form weakly immunogenic tumor-associated antigens (TAA). The tumor-associated serpin SCC antigen-1 (1), p53, Ki-67 and E-cadherin (2, 3), EGFR LI (4), EpCAM (5) and survivin (6) are among those identified as potential targets of cytotoxic T lymphocytes (CTL). It is likely that these are only several representations of an undefined, and possibly large number of TAA expressed by SCCVII/SF cells. Malignant cells are notoriously genetically unstable (7-12). Antitumor immune responses following immunization with effective tumor vaccines directed toward the TAA expressed by malignant cells can be of sufficient magnitude to prolong the lives of tumor-bearing animals and patients. There is a compelling rationale for the use of immunotherapy as an adjunct to the conventional treatment of cancer patients, including patients with squamous carcinoma of the head and neck (SCCHN).

Cancer vaccines have been prepared by modifying the patient’s tumor cells to augment their immunogenic properties. Cancer cells are the richest source of tumor-specific TAA. When successful, whole tumor vaccines can induce immunity to multiple TAA expressed by the malignant cells. To increase their inherently weak immunogenic properties, vaccines were developed by introducing expression-competent genes encoding foreign major histocompatibility complex (MHC) determinants (13-15), co-stimulatory (16) or cell adhesion molecules (17), cytokines (18, 19) or non-pathogenic viruses (20, 21) into the patient’s malignant cells. In animal models, gene-modified tumor cell vaccines were efficient stimulators of antitumor immune responses. Vaccines have also been prepared by introducing tumor cell-derivatives into DC, where TAA are expressed (22-24). DC are "professional antigen presenting" cells. They express surface determinants such as MHC class I and class II molecules for antigen presentation and co-stimulatory molecules for T cell activation. DC also possess migratory properties to facilitate interaction with T cells, primarily in regional lymph nodes. These characteristics led to the widespread use of DC in experimental immunotherapy protocols in animals and in clinical trials in cancer patients (25-30).
Among other immunotherapeutic approaches, clinical studies are underway using autologous DC that were "fed" tumor cell lysates (25, 26), or DC transfected with tumor-derived RNA (27, 28). Vaccines have also been prepared by fusing allogeneic DC with tumor cells from the patient (29, 30). The rationale is that TAA will be expressed in a highly immunogenic form by the modified cells, and that antitumor immune responses will be generated in patients immunized with such DC-based vaccines. Certain of these approaches, however, may require substantial quantities of tumor tissue, restricting their use to patients with relatively large cancer burdens.

In prior studies (31-35), we tested vaccines prepared by transfer of unfractionated DNA-fragments derived from malignant mouse neoplasms (breast, melanoma, SCC) into LM cells, a highly immunogenic mouse fibroblast cell line. Mouse fibroblasts, rather than DC, were chosen as the DNA recipients for several compelling reasons. The use of a fibroblast cell line, rather than DC, enabled the recipient cells to be modified in advance of DNA transfer to augment their immunogenic properties. For example, the cells could be modified to secrete a Th-1 cytokine such as IL-2 and to express foreign (allogeneic) MHC determinants, to stimulate uptake by DC of the host. As the transferred DNA integrates spontaneously into the genome of the recipient cells and is replicated as the transfected cells divide, the number of vaccine cells can be increased as desired for multiple immunizations. DNA from surprisingly small amounts of tumor tissue is sufficient to prepare the vaccine, enabling treatment at an early stage of the disease, before the tumor becomes, large, bulky and unresponsive to immune-based therapies, increasing the likelihood of success.

Here, in an orthotopic mouse model of SCC, we compared the immunogenic properties of a vaccine prepared by transfer of tumor-derived genomic DNA fragments from SCCVII/SF cells into allogeneic mouse fibroblasts with a vaccine prepared by transfer of DNA fragments from the same tumor into syngeneic DC. SCCVII/SF cells are a highly malignant squamous carcinoma cell line derived from a neoplasm that originated in a C57BL/6 mouse, were also from the American Type Culture Collection (ATCC; Manassas, VA, USA). B16F1 cells, a melanoma cell line from a neoplasm that originated in a C57BL/6 mouse, were also from the ATCC. All the cell cultures were maintained at 37°C in a humidified 7% CO2/air atmosphere in DMEM (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO, USA) and antibiotics (Life Technologies, Grand Island, NY, USA).

Modification of LM fibroblasts to secrete IL-2. As a means of augmenting their non-specific immunogenic properties, the fibroblasts, used as recipients of DNA from the SCC, were modified to secrete the Th-1 cytokine, IL-2 (LM-IL-2 cells), as described previously (34). A replication-defective retroviral vector (pZipNeoSVIL-2, from M.K.L Collins, University College, London, UK) specifying IL-2 was used.

Modification of LM fibroblasts to express MHC class I H-2Kb determinants. To stimulate uptake by DC, the fibroblasts were modified before transfection to express allogeneic class I determinants (LM-IL-2Kb), as described previously (36).

Isolation of bone marrow-derived DC from C3H/He mice. DC were obtained from the bone marrows of C3H/He mice, according to the method described previously (37).

DNA isolation. Unfractionated genomic DNA was isolated from SCCVII/SF cells with the aid of DNeasy isolation kits (Qiagen,
was then added drop-wise to the DC, followed by incubation at 37°C for 3 hours in a 7% CO2/air atmosphere. As controls, the same procedure was followed except the DNAs were added to Lipofectamine 2000 and then to 2.0x10⁷ transfected cells were analyzed for specific fluorescence in a FACS.

Transfection of DC with plasmid DNA specifying green fluorescent protein (GFP). To determine if the transferred DNA was expressed as protein, 1x10⁶ bone marrow-derived DC were transfected with 1 µg plasmid DNA (pTracer-CMV2; Invitrogen, Carlsbad, CA, USA) specifying GFP. One µg of pTracer-CMV2 in 1.25 ml Opti-MEM was mixed with 60 µl Lipofectamine 2000 (Invitrogen) diluted in 1.25 ml Opti-MEM. The DNA-Lipofectamine mixture was then added drop-wise to the DC, followed by incubation at 37°C for 3 hours in a 7% CO2/air atmosphere. Five days later, the transfected cells were analyzed for specific fluorescence in a FACS.

Preparation of the DC-based vaccine. Transfection of DC with genomic DNA fragments from the SCC. The DC-based vaccine was prepared by transfer of genomic DNA fragments from SCCVII/SF cells into bone marrow-derived DC from C3H/He mice. The DC were washed twice at room temperature with Opti-MEM medium (Gibco, BRL). After, 5x10⁶ DC/ml in 2.5 ml DMEM were added to 60-mm Petri dishes (Falcon, Franklin Lakes, NJ, USA). Fifty µg sheared (25 kb) genomic DNA fragments from SCCVII/SF cells in 1.25 ml Opti-MEM were mixed with 60 µl Lipofectamine 2000 (Invitrogen) diluted in 1.25 ml Opti-MEM. The DNA-Lipofectamine mixture was then added drop-wise to the DC followed by incubation at 37°C for 3 hours in a 7% CO2 /air atmosphere. The cells were washed with Opti-MEM and then resuspended in the same medium for use in the experiments.

Preparation of the fibroblast-based vaccine. Transfection of LM fibroblasts with sheared genomic DNA fragments from relatively small numbers of SCCVII/SF cells. LM-IL-2 cells modified to express allogeneic H-2Kb determinants (LM-IL-2Kb) were transfected with genomic DNA fragments from SCCVII/SF cells, using Lipofectamine 2000 (Invitrogen) to aid DNA uptake, according to the manufacturer’s instructions. In brief, 30 µg of DNA from SCCVI/SF cells were mixed with 3 µg pcDNA6/HisA (Invitrogen), a plasmid specifying a gene conferring resistance to blasticidin. Next, the DNAs were added to Lipofectamine 2000 and then to 2.0x10⁷ LM-IL-2Kb cells divided into four 100-mm plastic cell culture dishes 24 hours previously. Eighteen hours later, the cells were divided into sixteen 100-mm dishes and incubated for 14 days in fresh growth medium containing 5 µg/ml blasticidin. The surviving blasticidin-resistant cells (at least 2.6x10⁴ colonies in each instance) (LM-IL-2Kb/SCC) were pooled and maintained as a cell line for use in the experiments. As controls, the same procedure was followed except that the fibroblasts were transfected with DNA fragments from B16F1 cells, a melanoma cell line mixed with pcDNA6/HisA (LM-IL-2Kb/B16F1 cells) or with pcDNA6/HisA alone.
plates (B-D Pharmingen, San Diego, CA, USA; ELISPOT Mouse IFN-γ Set). The plates were coated with 100 μl of the capture Ab (5 μg/ml in PBS). Stimulator SCCVII/SF cells were then added at a responder: stimulator ratio of 10:1, as described previously (37).

**Immunization of C3H/He mice with fibroblasts or DC transfected with DNA from SCCVII/SF cells (LM-IL-2Kb/SCC and DC/SCC respectively).** The immunogenic properties of LM-IL-2Kb/SCC and DC/SCC were compared in C3H/He mice. In the case of mice immunized with LM-IL-2Kb/SCC, the mice received two s.c. and i.p. injections at weekly intervals of 2x10⁶ LM-IL-2Kb/SCC at each injection site. As a control, the same protocol was followed except that LM-IL-2Kb/B16 cells were substituted for LM-IL-2Kb/SCC. In the case of mice immunized with DC/SCC, 2x10⁶ DC/SCC were injected i.v. according to the same schedule. One week after the last injection, 1x10⁸ viable SCCVII/SF cells were injected s.c. into mice in either group. The median survival time (MST±SE) of tumor-bearing mice in each group was determined by life-table methods and by Kaplan-Meier log-rank analysis. p<0.05 was considered significant.

**Results**

**Modification of LM mouse fibroblasts for the secretion of IL-2 and the expression of allogeneic H-2Kb class I determinants.**

SCC, like other types of neoplastic cells, forms an array of weakly immunogenic TAA. The rationale for the generation of therapeutic tumor vaccines is that the immunogenic properties of TAA can be enhanced if they are expressed by highly immunogenic cells.

A vaccine for SCC was prepared by transfer of genomic DNA fragments from SCVII/SF cells, a highly aggressive SCC, into a mouse fibroblast cell line. For comparison, a vaccine was prepared by transfer of DNA fragments from the same cell type into bone marrow-derived DC from syngeneic C3H/He mice. To augment their non-specific immunogenic properties before DNA transfer, the fibroblasts were modified to secrete IL-2 and to express allogeneic class I determinants (H-2kb). IL-2 promotes the maturation of cytotoxic T lymphocytes. Allogeneic class I determinants stimulate uptake of the vaccine by DC of the host (38, 39).

A multi-step approach was used to prepare the fibroblast vaccine. For the first step, a plasmid (pZipNeoSVIL-2), which specified human IL-2 along with a gene that conferred resistance to the neomycin analog G418, was introduced into the cells. (Like mouse IL-2, human IL-2 stimulates the proliferation and maturation of mouse T cells.) After selection in medium containing sufficient quantities of G418 to kill one hundred percent of non-transfected cells, the surviving antibiotic-resistant cells were analyzed for IL-2 secretion. An IL-2-specific ELISA was used for this purpose. The results indicated that 1x10⁶ transfected fibroblasts formed approximately 200 pg IL-2/48 hours. Culture supernatants of fibroblasts transfected with the IL-2-negative vector (pZipNeoSV(X)), which conferred antibiotic resistance alone, and non-transfected LM-IL-2Kb cells failed to contain detectable quantities of IL-2. IL-2 secretion by fibroblasts transfected with pZipNeoSVIL-2 was a stable property of the cells. Equivalent amounts were detected in the culture supernatants after more than 3 months of continuous culture. The introduction of DNA from the SCC into LM-IL-2 cells did not affect the levels of IL-2 secretion (these data are not presented).

For the second step, the IL-2-secreting fibroblasts were modified to express allogeneic MHC H-2Kb class I determinants. A plasmid (pBR327H-2Kb) specifying H-2Kb class I determinants was introduced into the cells, along with a puromycin-resistant plasmid (pBabePuro), used for selection. After incubation in medium containing sufficient quantities of puromycin to result in the death of one hundred percent of non-transfected cells, the surviving antibiotic-resistant fibroblasts were pooled and maintained as a cell line (LM-IL-2Kb). Quantitative immunofluorescent analysis of transfected fibroblasts stained with PE-conjugated mAbs for H-2Kb determinants was used to measure the expression of H-2Kb determinants. As a control, PE-labelled IgG2a isotype serum was substituted for PE-conjugated mAbs for H-2Kb determinants. As indicated (Figure 1), 99 percent of the transfected fibroblasts stained with PE-conjugated H-2Kb and H-2Kb mAbs. (LM fibroblasts, of C3H/He mouse origin, express H-2Kb determinants constitutively).

To further characterize the cells used as DNA recipients, the fibroblasts were also stained with PE-conjugated B7-1, B7-2, or ICAM-1 determinants. As indicated (Figure 1), the fibroblasts expressed both H-2Kb and the co-stimulatory molecule B7-1 constitutively. B7-2 and ICAM-1 determinants were not detected. An analogous procedure was used to detect the expression of H-2Kb, anti-H-2Kb, B7-1, B7-2, or ICAM-1 determinants by the DC used as recipients of DNA from SCCVII/SF cells (described, below). The results (Figure 1) indicated that, with the exception of H-2Kb, the DC, of C3H/He mouse origin, stained positively for each determinant. Seventy-five±5 percent of the cells stained positively with mAbs for CD11c, a membrane-associated determinant characteristic of DC. At least 99% of the DC were viable (0.4% trypan blue staining) at the time they were used in the experiments. Like IL-2 secretion by the fibroblasts, the introduction of DNA from SCCVII/SF cells (or from B16 cells) into the DC had no significant effect upon the expression of these determinants (these data are not presented).

**Tumor progression was inhibited in mice immunized with modified fibroblasts transfected with DNA from SCCVII/SF cells (LM-IL-2Kb/SCC).** Immunization of mice with DC transfected with DNA from SCCVII/SF cells (DC/SCC) failed to inhibit tumor growth. The immunogenic properties of LM-IL-2Kb/SCC and DC/SCC were investigated in C3H/He mice.
mice. C3H/He mice are extremely susceptible to the growth of SCCVII/SF cells, a highly aggressive squamous carcinoma cell line. (Mice injected s.c. with as few as 1x10^5 SCCVII/SF cells die from progressive tumor growth in approximately 25 days. The cells' generation time is 18.3 hours.)

In the study, naive C3H/He mice received two s.c. and i.p. injections at weekly intervals of 2x10^6 LM-IL-2Kb/SCC at each injection site. One week after the second injection, the mice were injected s.c. with 1x10^5 SCCVII/SF cells. As controls, the same procedure was followed except that the mice were injected with modified fibroblasts transfected with DNA from B16 cells, or the mice were not injected. The immunogenic properties of DC/SCC were determined by injecting 1x10^6 DC/SCC i.v. at weekly intervals into C3H/He mice, or as a control, with DC transfected with DNA from B16 cells, followed by an injection s.c. of an equivalent number of SCCVII/SF cells one week later. In prior studies (37), we found that transfer of DNA from KLN205 cells, a SCC cell line, generated a vaccine that inhibited tumor growth in immunized DBA/2 mice. As indicated (Figure 2), mice immunized with LM-IL-2Kb/SCC followed by the injection of SCCVII/SF cells survived significantly (p<0.05) longer than mice immunized with the transfected DC. Two mice in the group immunized with modified fibroblasts

Figure 1. Expression of co-stimulatory, MHC and cell adhesion molecules by the cells used as recipients of DNA from SCCVII/SF cells. 1x10^6 cells were incubated with FITC-labelled mAbs for B7-1 (FITC-labelled CD80), B7-2 (PE-labelled CD86), FITC-labelled H-2Kb or PE-labelled H-2Kk determinants, or with FITC-labelled mAb for ICAM-1. After labelling, the cells were analyzed for specific immunofluorescence by flow cytometry. Cells were considered positive if the mean fluorescence index (MFI) was at least 3-fold greater than cells stained with FITC or PE-labelled isotype control serum. More than 10,000 cells were analyzed in each instance. Inset: FACS analysis of LM-IL-2Kb/SCC cells stained with FITC or PE-conjugated mAbs for H-2Kb or H-2Kk-determinants.
transfected with DNA from SCCVII/SF cells, followed by the challenging injection of SCCVII/SF cells, failed to develop tumors. They appeared to have rejected the SCC and survived indefinitely. In contrast, the survival of mice injected with the transfected DC, followed by the injection of SCCVII/SF cells, was not significantly different than that of mice injected with PBS rather than the vaccine.

To determine if the immunity in mice immunized with modified fibroblasts or DC transfected with DNA from B16 cells developed immunity to SCCVII/SF cells, the same protocol was followed except that the mice were immunized with fibroblasts or DC transfected with DNA from B16 melanoma cells (LM-IL-2Kb/B16 or DC/B16, respectively) before the injection of the SCCVII/SF cells. As indicated (Figure 2), none of the mice immunized with DC/B16 cells before tumor challenge survived longer than mice in the group injected with PBS. In contrast, until 75 days, when they started to die from progressive tumor growth, mice injected with fibroblasts transfected with DNA from B16 cells survived significantly longer than mice in any of the other groups except mice immunized with LM-IL-2Kb/B16 cells before SCCVII/SF cells. Whether or not B16 cells and SCCVII/SF cells share determinants in common was not determined.

Thus, the immunity in mice immunized with modified fibroblasts transfected with DNA from SCCVII/SF cells was directed primarily toward SCCVII/SF cells, consistent with the expression of unique TAA by the transfected cells. In each instance, the immunizations were without apparent harm. Tumors failed to form in mice injected with either the transfected fibroblasts or the transfected DC alone. There was no evidence of the induction of an autoimmune disease. Since the fibroblasts used as the DNA recipients expressed allogeneic MHC determinants (H-2Kb), it is probable that, like other foreign tissue grafts, the cellular vaccine was rejected through uptake and antigen processing by DC.

Augmented spleen cell-mediated immunity toward SCCVII/SF cells in C3H/He mice immunized with LM-IL-2Kb/SCC. ELISPOT IFN-γ assays were used to determine the
proportion of spleen cells responsive to SCCVII/SF cells in mice immunized with the transfected fibroblasts. Naïve C3H/He mice were injected s.c. three times at weekly intervals with 5x10^6 LM-IL-2Kb/SCC cells. One week after the last immunization, spleen cells from immunized mice were co-incubated with mitomycin C-treated SCCVII/SF cells as described previously before the ELISPOT IFN-γ assays were performed. p<0.05 for the number of spots/10^6 spleen cells in cultures of spleen cells from mice immunized with LM-IL-2Kb/SCC cells co-incubated with SCCVII/SF cells vs. the number of spots/10^6 spleen cells in cultures incubated without SCCVII/SF cells. p<0.05 for the difference in the number of spots/10^6 spleen cells in cultures of cells from mice immunized with LM-IL-2Kb/SCC cells co-cultured with SCCVII/SF cells relative to the number of spots in cultures from mice immunized with non-transfected cells co-cultured with SCCVII/SF cells. p<0.05 for the difference in the number of spots in cultures of cells from mice immunized with LM-IL-2Kb/SCC cells co-cultured with SCCVII/SF cells relative to the number of spots in cultures from mice immunized with DC/SCC co-cultured with SCCVII/SF cells. B) Generation of spleen cells cytotoxic toward SCCVII/SF cells in mice immunized with LM-IL-2Kb/SCC or DC/SCC cells (51Cr-release assays). The protocol described in the legend to Figure 3A was followed. One week after the last injection, spleen cells from the immunized mice were co-cultured for 5 days with (mitomycin C-treated) SCCVII/SF cells (ratio of spleen cells to SCCVII/SF cells = 30:1). After incubation, the non-adherent cells were collected and co-incubated for 5 hours with 51Cr-labelled SCCVII/SF cells before the specific cytosis was determined. As controls, spleen cells were obtained from mice immunized with non-transfected LM-IL-2Kb cells, with non-transfected DC or the spleen cells were from naïve mice. Approximately 15% of the maximum 51Cr-release was released spontaneously.

The same protocol was followed to measure spleen cell-mediated cytotoxic activity toward SCCVII/SF cells in mice immunized with the transfected fibroblasts or the transfected DC. In a standard 51Cr-release cytotoxicity assay, equivalent cytotoxic activity toward SCCVII/SF cells (the percent specific release of isotope) was observed using as effector cells spleen cells from mice injected with LM-IL-2Kb/SCC or spleen cells from mice injected with DC/SCC (Figure 3B). The cytotoxic responses toward SCCVII/SF cells using spleen cells from non-immunized mice or mice immunized with non-transfected fibroblasts as effector cells were significantly (p<0.05) less than those of mice immunized with the transfected cells. The results are consistent with the expression of TAA by the transfected cells.

Long-term immunity toward the SCC mediated by CD8+ T cells was generated in C3H/He mice immunized LM-IL-2Kb/SCC cells, but not in mice injected with DC/SCC. Both ELISPOT IFN-γ and 51Cr-release cytotoxicity assays were
used to determine if long-term immunity toward SCCVII/SF cells was generated in C3H/He mice immunized with LM-IL-2Kb/SCC cells. As previously, naïve C3H/He mice received two s.c. and i.p. injections at weekly intervals of 2x10⁶ LM-IL-2Kb/SCC at each injection site. One week after the second injection, the mice were injected s.c. with 1x10⁵ SCCVII/SF cells. Fifty days later, spleen cells from tumor-free mice (they appeared to have rejected the SCC) were co-incubated for 18 hours with (mitomycin C-treated) SCCVII/SF cells, after which the cells were tested in ELISPOT IFN-γ assays for the presence of T cells responsive to SCCVII/SF cells. As controls, the same protocol was followed except that the mice were injected with (non-transfected) LM-IL-2Kb cells or with LM-IL-2Kb/B16 cells before the injection of SCCVII/SF cells. As additional controls, the mice were injected i.v. with DCs, DC/SCC or DC/B16 cells before the injection of SCCVII/SF cells. p<0.001 for the difference in the number of spots in spleen cell cultures from mice immunized with LM-IL-2Kb/SCC cells co-incubated with (mitomycin-C-treated SCCVII/SF cells) relative to the number of spots in cell cultures without co-incubation. p<0.001 for the difference in the number of spots in cells from mice immunized with LM-IL-2Kb cells relative to the number of spots in cell cultures from mice immunized with LM-IL-2Kb/B16 cells with or without co-incubation. p<0.01 for the difference in the number of spots in cells from mice immunized with LM-IL-2Kb/SCC cells relative to the number of spots in cultures from mice immunized with transfected or non-transfected DCs. B) Generation of spleen cell cytotoxic toward SCCVII/SF cells in tumor-bearing C3H/He mice immunized 50 days previously with LM-IL-2Kb/SCC or DC/SCC cells (³²Cr-release cytotoxicity assays). Naïve C3H/He mice received three s.c. and i.p. injections of 2x10⁶ LM-IL-2Kb/SCC cells at each injection site or three i.v. injections of 1x10⁵ DC/SCC cells. Both injections were at weekly intervals. One week after the last injection, 1x10⁵ SCCVII/SF cells were injected s.c. Fifty days later, spleen cells from the surviving mouse were co-cultured for 5 days with mitomycin C-treated SCCVII/SF cells (ratio of spleen cells to SCCVII/SF cells = 30:1). After incubation, the non-adherent cells were collected and co-incubated for 5 hrs with ³²Cr-labeled SCCVII/SF cells before the specific cytotoxicity was determined. As controls, spleen cells were obtained from mice injected with non-transfected DC or from naïve mice injected with PBS. Approximately 15% of maximum ³²Cr released spontaneously. p<0.02 for the specific release of isotope from SCCVII/SF cells co incubated with spleen cells from mice injected with LM-IL-2Kb/SCC cells vs. that of mice in any of the other groups.
DNA from SCCVII/SF cells. These responses, however, were significantly less than those in mice immunized with the transfected fibroblasts. As for mice immunized with fibroblasts transfected with DNA from B16 melanoma cells, DC transfected with DNA from B16 cells failed to generate T cells responsive to SCCVII/SF cells.

The same protocol was followed to measure the cytotoxic responses toward SCCVII/SF cells in mice immunized fifty days previously with the transfected fibroblasts. As indicated (Figure 4B), the highest response (percent specific lysis) was in the group of mice immunized with the transfected fibroblasts. The responses in mice immunized with DC/SCC were not significantly different from those of mice immunized with DC/B16 cells.

CD8+ T cells and NK cells mediated tumor resistance in mice immunized the transfected fibroblasts. The effect of mAbs for CD8+, CD4+ or NK cells added to spleen cell suspensions from mice immunized with LM-IL-2Kb/SCC was used as a means of determining the classes of cells mediating tumor resistance in mice immunized with the transfected fibroblasts. As indicated (Figure 5A), treatment of spleen cells from mice immunized with LM-IL-2Kb/SCC with anti-CD8 mAbs or NK antibodies, but not with CD4+ mAbs, reduced the cytotoxic activity toward SCCVII/SF cells to the greatest extent. Treatment with anti-CD8 mAbs had no significant effect upon spleen cell-mediated cytotoxicity toward SCCVII/SF cells in mice immunized with non-transfected DC or IL-IL-2Kb cells were used instead of spleen cells from mice immunized with the transfected cells. Values represent means±SD of triplicate determinations. [*] p<0.05 for the difference in the number of spots in the presence or absence of the mAb and complement.

Figure 5. CD8+ T cells mediated tumor resistance in mice immunized with LM-IL-2Kb/SCC cells. A) ELISPOT INF-γ assays. 2x10⁶ LM-IL-2Kb/SCC cells were injected s.c. and i.p. three times at weekly intervals at each injection site into C3H/He mice. Other C3H/He mice received three i.v. injections at weekly intervals of 1x10⁶ DC/SCC cells. One week after the last injection, the mice were injected s.c. with 1x10⁶ SCCVII/SF cells. Fifty days later, spleen cells from the immunized tumor-bearing mice were co-incubated for 18 hours with SCCVII/SF cells (E: T ratio = 10:1). mAbs for CD8+, CD4+ or NK1.1 cells and complement were added 30 minutes before ELISPOT INF-γ assays were performed. [*] p<0.05 for the difference in the number of spots in the presence or absence of the mAb and complement. B) Cytotoxicity assays. C3H/He mice were immunized according to the schedule outlined in the legend to Figure 5A. One week after the last injection, the mice were injected s.c. with 1x10⁶ SCCVII/SF cells. Fifty days later, spleen cells from the immunized tumor-bearing mice were co-cultured for 5 days with (mitomycin C-treated) SCCVII/SF cells (ratio spleen cells to SCCVII/SF cells = 30:1). After incubation, the non-adherent cells were collected and co-incubated for 5 hours with ⁵¹Cr-labelled SCCVII/SF cells before the specific cytosis was determined in a standard ⁵¹Cr-release assay. In some instances, as indicated, mAbs for CD8+, CD4+ or NK1.1 cells and complement were added 30 minutes before the cytotoxic activities against ⁵¹Cr-labelled SCCVII/SF cells (E: T ratio = 100:1) were determined. As controls, spleen cells from mice immunized with non-transfected DC or IL-IL-2Kb cells were used instead of spleen cells from mice immunized with the transfected cells. Values represent means±SD of triplicate determinations. [*] p<0.05 for the difference in the number of spots in the presence or absence of the mAb and complement.
Greater numbers of transfected DC than transfected fibroblasts took up DNA from SCCVII/SF cells. One possible explanation for the heightened antitumor immune responses in mice immunized with the transfected fibroblasts, relative to that of mice immunized with transfected DC, is that a greater proportion of fibroblasts than DC took up DNA from the SCC. If a greater number of fibroblasts in the cell suspensions used to immunize the mice expressed TAA, then a heightened antitumor immune response might be expected. To investigate this question, both fibroblasts and DC were transfected with FITC-labelled DNA from SCCVII/SF cells or with a plasmid (pTracer-CMV2; Invitrogen) specifying green fluorescent protein (GFP). Then, the proportion of fluorescent cells in each cell suspension was determined by analysis in a FACS. As indicated (Table I), 90 percent of DC transfected with FITC-labelled DNA stained positively. 

Under similar conditions, 38 percent of transfected fibroblasts stained positively (MFI=3.8 and 1.3, respectively). An analogous approach was used to determine the proportions of DC and fibroblasts that took up plasmid DNA specifying GFP. As indicated (Table I), the proportion of DC that were fluorescent was approximately three-fold higher than that of transfected fibroblasts.

Thus, as indicated by two independent assays, a greater proportion of DC than fibroblasts took up and expressed the exogenous DNA.

**Table I. Uptake and expression of DNA by DCs and LM-IL-2Kb cells transfected with FITC-labelled DNA from SCCVII/SF cells or with a plasmid (pTracer) encoding enhanced green fluorescent protein (EGFP): Percent fluorescent cells.**

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<th>Transfected with FITC-labelled DNA from SCCVII/SF cells</th>
<th>Transfected with pTracer (pEGFP)</th>
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<tr>
<td>DC</td>
<td>90%</td>
<td>37%</td>
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<tr>
<td>LM-IL-2Kb fibroblasts</td>
<td>38%</td>
<td>12%</td>
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Discussion

More than 29,000 new cases of SCCHN were anticipated in 2005 (2.1 percent of the total cancer incidence) and approximately 7,300 patients were expected to die of the disease (40). There is a need for more effective and innovative forms of treatment. Immunotherapy with effective vaccines may increase the likelihood of success, especially if administered at an early stage of the disease.

In this study, we compared the immunogenic properties of two DNA-based vaccines. Both vaccines were prepared by transfer of DNA fragments from SCCVII/SF cells, an extremely aggressive SCC, into either of two types of recipient cells. This approach was an application of classic studies indicating that the introduction of genomic DNA fragments from one cell type into another results in stable integration of the transferred DNA and alteration of both the genotype and the phenotype of the recipient cells (41-44). We reasoned that an array of weakly immunogenic tumor antigens formed by the SCC would become strongly immunogenic if they were expressed by highly immunogenic cells. The results were consistent with this hypothesis. Mice immunized with transfected, but not with non-transfected, fibroblasts developed immunity to the SCC, as indicated by both in vitro and in vivo measurements of tumor immunity.

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Immunity to the SCC following immunization with the transfected DC was less successful.

Mouse fibroblasts were chosen as the recipients of DNA from the cancer cells for several compelling reasons:

- Allogeneic class I MHC determinants expressed by the fibroblasts stimulate uptake by host DC.
- The recipient cells can be modified to secrete one or more immune-augmenting cytokines, such as IL-2 or GM-CSF, which greatly magnify the immune response.
- Effective vaccines can be generated from surprisingly small amounts of tumor tissue. Sufficient DNA to prepare the vaccine can be obtained from a neoplasm of about 4 mm, enabling treatment at an early stage of the disease.
- As the transfected fibroblasts proliferate under standard cell culture conditions, the number of vaccine cells can be expanded as required for multiple immunizations.
- The transferred DNA includes mutant/dysregulated genes that specify a broad array of antigens that characterize the SCC.
- The vaccine is conveniently prepared at relatively low cost and is therefore applicable to large numbers of cancer patients.

The results indicated that C3H/He mice immunized with the transfected fibroblasts developed robust, cell-mediated immune responses toward the highly aggressive SCC that were capable of deterring tumor growth.

For comparison, we tested the immunogenic properties of syngeneic bone marrow-derived DC transfected with DNA from SCCVII/SF cells. Conceivably, the immunogenic properties of transfected DC would exceed those of transfected fibroblasts. DC are "professional" antigen presenting cells. They express MHC class I/II determinants, co-stimulatory molecules and have migratory properties designed to increase their interaction with CTL and NK cells. In each instance, however, the immunity toward the SCC in mice immunized with the transfected fibroblasts exceeded that of mice immunized with transfected DC. This was especially notable for the survival of immunized mice injected with SCCVII/SF cells, a highly aggressive SCC line. The DNA-based fibroblast vaccine was clearly superior. In spite of the fact that immunization with DC/SCC resulted in
immunity toward SCCVII/SF cells, as determined by ELISPOT IFN-γ and 51Cr-release cytotoxicity assays performed in vitro, the immunity toward the SCC was insufficient. Mice immunized with the transfected DC followed by the challenging injection of SCCVII/SF cells failed to survive longer than mice injected with PBS before injection of the tumor cells.

It would be premature, however, to conclude that the immunogenic properties of transfected fibroblasts exceeded those of transfected DC under all circumstances. Among other considerations, the mice were immunized with a greater number of transfected fibroblasts than transfected DC. The transfected fibroblasts were injected s.c. the transfected DC were injected i.v. Not every transfected DC or transfected fibroblast would be expected to participate in the induction of immunity to SCCVII/SF cells. There was no assurance that the number of cells in the transfected cell populations that expressed TAA were the same. Nevertheless, the heightened antitumor immune responses directed toward the SCC in mice immunized with transfected fibroblasts clearly illustrated the potential of this approach in preparing tumor vaccines.

We conclude that an array of undefined squamous carcinoma-associated antigens was expressed by the allogeneic transfected cells, and that the cells were taken-up by DC of the host. The results raise the possibility that a vaccine useful in cancer therapy can be prepared by transfection of human fibroblasts with DNA from the patient’s malignant cells. DNA from surprisingly small amounts of tumor tissue was sufficient, enabling treatment of patients in whom available tissue is limited. As the TAA expressed by each patient’s neoplasm are likely to contain both common and unique epitopes, to encompass the array of antigens that characterize the patient’s cancer, ideally the vaccine should be prepared with DNA derived from the autologous tumor.

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