**Objective:** To investigate the therapeutic efficacy of fused dendritic-tumor cell hybrids against murine squamous cell carcinoma (SCC).

**Design:** Squamous cell carcinoma VII is a poorly immunogenic murine SCC tumor in C3H/HEN (H-2<sup>K</sup>) mice. Subdermal tumors were established by inoculation in the mid abdomen of mice. Tumor diameters were measured with a Vernier caliper and used as an indication of treatment efficacy. Survival studies were performed on mice with 3-day pulmonary metastasis or subdermal tumors. Dendritic cells were generated from bone marrow and cultured for 8 days. Dendritic cells were harvested and mixed with cultured tumor cells in a 1:1 ratio. Cell fusion was achieved by exposing the cell mixture to an alternate electrical current to bring cells into alignment and close together, followed by a short direct electrical current pulse.

**Subjects:** Female C3H/HEN mice aged 8 to 12 weeks.

**Interventions:** Mice with 3-day established SCCVII tumors were vaccinated by inguinal intranodal injection of fusion cells (0.3 × 10<sup>6</sup> per side). To support the development of antitumor immunity, mice were given adjuvant injections intraperitoneally. Anti-OX40R monoclonal antibodies or interleukin 12 were used. Treatment groups included no treatment, anti-OX40R monoclonal antibodies or adjuvant IL-12 alone, fusion cells alone, and fusion cells with adjuvant treatment.

**Main Outcome Measures:** Tumor size and overall survival.

**Results:** Mice treated with adjuvant treatment or fusion cells alone did not show a statistical difference in tumor growth when compared with controls. In contrast, fusion cells with adjuvant treatment demonstrated a significant decrease in tumor size when compared with nontreated mice (P < .001). Treatment with fusion cells also resulted in increased survival in the pulmonary metastasis and subdermal tumor models.

**Conclusion:** Immunotherapy with fused dendritic-tumor cell hybrids can significantly affect 3-day established sSCC VII tumor growth.


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**HEAD AND NECK SQUAMOUS cell carcinoma (HNSCC) comprised an estimated 39,000 newly diagnosed cases and 11,000 deaths in the United States during 2005.** Over the past few decades, the treatment of HNSCC has seen advances in surgical techniques, radiation modalities, and chemotherapy regimens. Advanced-stage HNSCC often requires a combination of these treatment options, and these advancements have led to organ preservation and an improvement in loco-regional control. Despite these improvements, the primary cause of cancer failure remains distant metastatic disease.

One approach to addressing this issue is immunotherapy. The goal of immunotherapy is to sensitize T cells to recognize and kill tumor cells. This approach would be especially useful in addressing distant metastatic disease in which current treatment options are not as effective. Within the past decade, studies have identified the dendritic cell to be a critical component in eliciting a T-cell response against neoplastic cells. Dendritic cells are the most specialized antigen-presenting cell known because of their unique characteristics to uptake, process, and present antigens to T cells. Because of this, immunotherapy approaches have been focused on using the properties of dendritic cells. One such approach is the use of fused dendritic-tumor cell hybrids created by electrofusion.

Electrofusion is a process in which 2 different parent cells are fused together to form a new hybrid cell. This hybrid cell retains properties of both parent cells. In the case of dendritic-tumor cell fusion, the new hybrid cell retains the antigen-presenting properties of the dendritic cell to present the full complement of anti-
gens from the tumor cell. This would include both known and unknown tumor antigens. Fused dendritic-tumor cell hybrids have been shown to have immunotherapeutic effects after a single vaccination in a number of murine carcinomas including breast cancer, sarcoma, and melanoma. The immunotherapeutic efficacy induced by these fusion cells were dependent on the presence of both CD4+ and CD8+ T cells. An important finding was the need for an adjuvant treatment for these effects to be seen. Neither adjuvant treatment alone nor fusion cells alone were able to achieve similar results.

To date, no preclinical studies, to our knowledge, using immunotherapy with dendritic-tumor cell fusion against squamous cell carcinoma (SCC) have been reported. Investigation of the immune mechanisms induced by fusion vaccines in a murine SCC model will help develop future clinical trials. Our hypothesis is that a fused dendritic-tumor cell hybrid can induce an immunotherapeutic effect against established murine SCC tumor.

### METHODS

#### ANIMALS

The Animal Research Committee of The Cleveland Clinic approved this study. All animals used in this study received humane care in compliance with the “Guide for the Care and Use of Laboratory Animals,” as outlined by the National Institutes of Health. Institutional guidelines regarding animal experimentation were followed. The Cleveland Clinic Animal Care facility is accredited by the American Association for the Accreditation of Laboratory Animal Care.

Female C3H/HEN (H-2b) mice were purchased from the Bio-logic Testing Branch, Frederick Cancer Research and Development Center, National Cancer Institute, Frederick, Maryland. The animals were housed in a specific pathogen-free environment and were used for experiments at the age of 8 to 12 weeks.

#### TUMORS

Squamous cell carcinoma VII (SCC VII) is a spontaneously arising SCC tumor in C3H/HEN (H-2b) mice. Despite the expression of MHC class I molecules, it is poorly immunogenic and highly metastatic. Squamous cell carcinoma VII cells were maintained in complete medium made of Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% heat-inactivated fetal calf serum, 2mM levoglucosamidine, 0.1mM non-essential amino acids, 1mM sodium pyruvate, 100 µg/mL of streptomycin sulfate, 100 µg/mL of penicillin G potassium, 0.5 µg/mL of amphotericin B, 30 µg/mL of gentamicin sulfate (all from BioWhittaker, Walkersville, Maryland) and 50mM 2-mercaptoethanol (Sigma, St Louis, Missouri). Cultured SCC VII cells were harvested after brief exposure to 0.25% trypsin and 0.02% EDTA (BioWhittaker). Tumor cells were washed twice and re-suspended in Hanks balanced salt solution for animal inoculation. Before fusion, tumor cells were exposed to 5000 Gy of radiation to prevent in vivo replication and stained green with intracellular carboxyfluorescein diacetate succinimidyl ester according to manufacturer’s instructions (Molecular Probes, Eugene, Oregon).

#### DENDRITIC CELL GENERATION

Dendritic cells were generated by harvesting the ilibula and tibia bone marrow of 8- to 12-week-old C3H/HEN mice. Unfractionated bone marrow cells were cultured for 6 days in a proliferative cocktail of FMS-like tyrosine kinase 3 (10 pg/mL) and interleukin (IL)-6 (10 µg/mL). On day 6, cells were harvested and placed into complete medium containing granulocyte macrophage colony-stimulating factor (10 pg/mL) and IL-4 (10 ng/mL). Toll-like receptor agonist lipopolysaccharide LPS (50 ng/mL) and CpG (5µM) were added to the culture on day 7. The cells were harvested, counted, and used for fusion 24 hours later.

### ELECTROFUSION

Dendritic cells and irradiated (5000 cGy) tumor cells were first washed in a prefusion media of 5% glucose, 0.5mM magnesium acetate tetrahydrate, 0.1mM calcium acetate tetrahydrate, and 0.3% bovine serum albumin (pH 7.2) at a ratio of 1:1. Cells were then resuspended in fusion media of 5% glucose, 0.5mM magnesium acetate tetrahydrate, and 0.1mM calcium acetate tetrahydrate. Routinely, total cell concentration was between 10 × 10⁶/mL to 15 × 10⁶/mL.

Electrofusion was performed using the ECM 2001 generator (BTX Instrument Division, Genetronics, San Diego, California). A custom designed concentric fusion chamber was used to hold and fuse the cell suspension. Alignment of the cells was accomplished by applying an alternating current (160 V/cm) to the suspension for 10 seconds. Alignment was confirmed with direct visualization using a microscope. Immediately following the alternating current application, a direct current pulse (1533 V/cm) was applied for 99 microseconds. The cell suspension then underwent a tapering of the same alternating current for 9 seconds.

The fusion cells were harvested and incubated overnight in complete medium. After the cells were cultured overnight, adherent cells were harvested after brief exposure to trypsin and suspended in Hanks balanced salt solution for in vivo use. Verification of fusion efficiency was performed using fluorescein-activated cell sorter (FACS) analysis. After fusion, adherent hybrid cells were then labeled with the red phycoerythrin-conjugated monoclonal antibodies to cell markers found exclusively in dendritic cells. Since tumor cells were stained green with carboxyfluorescein diacetate succinimidyl ester, fused dendritic-tumor cell hybrids are recognized by double-positive staining by FACS analysis.

### ESTABLISHMENT AND TREATMENT OF 3-DAY ESTABLISHED TUMORS

Tumors were established subdermally by injection of 0.5 × 10⁶ SCC VII cells into the mid abdomen. After 3 days, fusion tumor vaccine was given by intranodal route. This was performed while the animal was anesthetized with 0.8 mg of pentobarbital intraperitoneally. The inguinal lymph nodes were surgically exposed, and 0.3 × 10⁶ fusion cells in 10 µL per side were injected intranodally with a 29-gauge needle. The surgical wound was reapproximated with surgical clips. Once mice recovered from anesthesia, a vaccine adjuvant anti-OX40 monclonal antibody (mAb) (150 µg/1 day) or IL-12 (0.2 µg/d × 4 days) was administered intraperitoneally. Perpendicular tumor diameter was measured at regular intervals using a Vernier caliper. Size was then recorded as the mean of these 2 measurements. Mice were humanely killed when tumor size reached a mean of 15 mm according to animal handling guidelines.

Pulmonary metastases were established by injection of 0.3 × 10⁶ SCC VII into the tail vein of mice. After 3 days, the fusion tumor vaccine was delivered in the same manner as previously described. Mice were then monitored on a routine basis to measure survival.
The experiments described herein are designed to evaluate the therapeutic efficacy of immunotherapy by vaccination with dendritic-tumor cell hybrids. Differences in the tumor size between experimental groups were evaluated with the nonparametric Wilcoxon rank-sum test (SigmaStat; Systat Software Inc, San Jose, California). Thus, the likelihood of the observed results being due to chance alone was determined. \( P \leq .05 \) was considered statistically significant. Each tumor growth group consisted of 5 animals. This is the minimum number of mice for which 1 overlap may be considered significant. According to the proposed statistics, in groups consisting of 5 animals, significance would be achieved in overlaps of 0 or 1 between experimental groups. However, if only 4 animals per experimental group are used, significance may only be achieved without any overlap whatsoever.

RESULTS

After the 8-day dendritic cell culture as described in the "Methods" section, we were able to generate a mean of \( 30 \times 10^6 \) cells per mouse. These cells demonstrated high expression of molecules typical of matured dendritic cells (CD86, I-A^k, intercellular adhesion molecule-1 [ICAM-1], and OX40L). The up-regulation of these molecules demonstrate that these mature dendritic cells are especially equipped to present antigen to the immune system.8,11 The FACS analysis is shown in Figure 1.

Before performing therapeutic experiments using dendritic and tumor cells, the capacity of irradiated (5000 cGy) SCCVII to proliferate in vivo was investigated. A total of \( 1 \times 10^6 \) cells of either live or irradiated SCCVII cells were inoculated subdermally. Figure 2 shows the growth of these cells over time. Animals inoculated with irradiated tumor showed tumor regression after an initial limited growth. These animals had no signs of tumor at 6 months after irradiated tumor inoculation.

VERIFICATION OF DENDRITIC-TUMOR CELL FUSION

Dendritic–SCCVII tumor cell fusion was performed as described in the “Methods” section, and fusion rates were routinely above 20%. Fusion was confirmed using FACS analysis. Tumor cells were stained with the green fluorescent carboxyfluorescein diacetate succinimidyl ester before fusion. Fusion cells were then analyzed by exposure to red fluorescent phycoerythrin-labeled antibody.
to exclusively dendritic cell surface markers. Cells that expressed dual fluorescence represent fused dendritic-tumor cells. The FACS data of a nonfused dendritic-tumor cell mixture as a negative control is shown for comparison (Figure 3).

Fusion cells were stained with Giemsa to allow for direct visual confirmation of successful fusion. Multinucleated cells represent successful fusion. Figure 4 is a Giemsa-stained cytospin preparation of fused dendritic-SCCVII cells. A Giemsa-stained cytospin preparation of a dendritic-tumor cell mixture is shown for comparison.

### IMMUNOTHERAPY AGAINST 3-DAY ESTABLISHED SCCVII SUBDERMAL TUMOR

The treatment of 3-day established SCCVII subdermal tumor was investigated. Treatment groups included no treatment as a negative control, IL-12 alone, anti-OX40R mAbs alone, fusion cells alone, fusion cells + IL-12, and fusion cells + anti-OX40R mAbs. Subdermal tumor growth is shown in Figure 5. Only fusion cells + IL-12, and fusion cells + anti-OX40R mAbs consistently demonstrated a significant difference when compared with the nontreated group (P < .001). All results were confirmed in repeated experiments. Figure 6 demonstrates long-term survival in mice after fusion vaccine treatment of 3-day established subdermal tumors. Survival of mice treated with fusion cells + OX40R mAbs and fusion cells alone were 60% and 40%, respectively.

### SURVIVAL AFTER TREATMENT OF 3-DAY ESTABLISHED SCCVII PULMONARY METASTASIS

Survival of 3-day established SCCVII metastases was investigated because this more closely mimics the clinical scenario of HNSCC metastasis. Treatment groups included no treatment as a negative control, anti-OX40R mAbs alone, fusion cells alone, and fusion cells + anti-OX40R mAbs. Mice treated with fusion cells + OX40R mAbs and fusion cells alone demonstrated survival of...
100% and 80% at 75 days, respectively. No mice in the control group and adjuvant treatment only group survived beyond 43 and 60 days, respectively (Figure 7).

COMMENT

These data represent the first immunotherapy treatment model against murine SCC using fused dendritic-tumor cell hybrids. The use of fused dendritic-tumor cell hybrids with an adjuvant treatment (IL-12 or anti-OX40R mAbs) were shown to significantly affect tumor growth when compared with experimental controls that included fusion cells alone, adjuvant treatment alone, and no treatment. We theorize that the use of an adjuvant treatment demonstrated improved efficacy when compared with fusion cells alone because the success of an immune response depends on 3 signals. The first is the presentation of the antigen on the major histocompatibility complex molecule to the antigen receptor on the T cell. The second signal is ligation of costimulatory molecules such as CD80 and CD86. The third signal is supplied by the adjuvant treatment, which appears to be important in developing an effective antitumor response. Furthermore, significant survival of mice was seen when given fusion cells with or without OX40R mAbs when compared with controls. Although there was some effect seen with fusion cells alone in the pulmonary metastases model, there is an increase in efficacy when fusion hybrids are given with OX-40 mAbs.

The use of IL-12 and anti-OX40R mAbs as adjuvant treatments were based on previous studies using fusion cells in other tumor models. The mechanism of action was described in a recent publication that found IL-12 and OX40R ligation leads to interferon secretion from T cells. Although the fusion cells strongly provide signals 1 and 2, without the addition of the third signal supplied by the adjuvant treatment, T cells may not expand and sustain the antitumor response.13

Furthermore, we report the feasibility of using a new method of dendritic cell generation from bone marrow. This process was modified from the previous bone marrow–derived methods and involves proliferating DC in FMS-like tyrosine kinase 3 ligand and IL-6 for 6 days. Further maturation in vitro is done using the toll-like receptor agonists LPS and CpG 24 hours before fusion. These dendritic cells demonstrated fusion capacity with SCCVII and immunotherapeutic effects against established tumors. This new dendritic cell culture method may potentiate the immunotherapeutic effects of fusion cells alone, since there were significant effects noted as compared with controls. This effect of fusion cells alone was not observed in other tumor models using traditional dendritic cell culture methods.13
One advantage to using dendritic-tumor cell hybrids is that it is not dependent on the identification and isolation of specific tumor antigens. Rather, the unaltered tumor antigens derived from the tumor component of the fusion cell is processed and presented to the immune system by the dendritic cell component of the fusion cell. Thus, the immunotherapy approach using dendritic-tumor cell hybrids is applicable to tumors in which these tumor antigens are not well defined, such as SCC. The process of electrofusion should be applicable to all mammalian cells. The fusion of human dendritic cells and melanoma tumor cells has been performed and studied. These fusion cells were able to present antigen to both CD4+ and CD8+ T cells. The feasibility of fusing human SCC tumor cells with dendritic cells has also been reported, suggesting that this immunotherapy would be feasible for patients with HNSCC.

With the use of this treatment model, further analysis can be done on the immune mechanisms responsible for the significant decrease in tumor growth. We are currently investigating cytokine secretion from specific immune T cells obtained by fusion hybrid stimulation. The specific immune T cells will be generated from tumor-draining lymph nodes. These studies will also include immune T cells enriched for CD4 and CD8, since we hypothesize that both cell populations will demonstrate activity from fusion cell stimulation. Efforts are currently being directed in improving this response with other adjuvant treatments. Furthermore, attempts at optimizing the dendritic cell antigen-presenting characteristics via in vitro manipulations during the proliferation dendritic cell culture are ongoing. Analysis of immune cell differentiation due to fusion cell treatment is also being studied. As these mechanisms are further delineated and our understanding of immune responses against SCC improves, treatment of advanced established tumor (ie, 9 days) will be attempted.

In conclusion, these data represent the first preclinical results, to our knowledge, that demonstrate dendritic cell fusion immunotherapy against a murine SCC model and supports using a fusion hybrid vaccine in future human HNSCC clinical trials. Immunotherapy with fused dendritic-tumor cell hybrids can significantly affect 3-day established SCCVII tumor growth. These preclinical data support using fused dendritic-tumor cells as an immunotherapeutic approach against HNSCC.

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Author Contributions: Dr Lee had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Lee. Acquisition of data: Lee, Tamai, Cohen, Teker, and Shu. Analysis and interpretation of data: Lee, Tamai, Cohen, and Shu. Drafting of the manuscript: Lee. Critical revision of the manuscript for important intellectual content: Lee, Tamai, Cohen, Teker, and Shu. Obtained funding: Lee and Shu. Administrative, technical, and material support: Lee, Tamai, and Teker. Study supervision: Lee, Cohen, Teker, and Shu.

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