Antitumor Vaccination in Patients with Head and Neck Squamous Cell Carcinomas with Autologous Virus-Modified Tumor Cells

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ABSTRACT

Prognosis of patients with advanced head and neck squamous cell carcinomas (HNSCC) is still poor. Therefore, we analyzed whether antitumor vaccination with a virus-modified autologous tumor cell vaccine is feasible and safe in HNSCC patients. Furthermore, we determined the influence on disease-free survival and overall survival and the vaccination-induced antitumor reactivity. In a nonrandomized pilot study, 20 patients were vaccinated postoperatively. Vaccine was prepared from the tumor cell cultures of patients by infection of the cells with Newcastle Disease Virus, followed by γ-irradiation, and vaccine was applied up to five times. Antitumor immune reactivity was determined in the skin by delayed type hypersensitivity skin reaction and in the blood by enzyme-linked immunospot assay. Establishment of tumor cell cultures was successful in about 80% of the cases. After vaccination, we observed no severe side effects. Percentages of survival of vaccinated patients with stage III and stage IV tumors (n = 18) were 61% at 5 years. Immune monitoring revealed significant increases of antitumor delayed type hypersensitivity reactivity especially in disease-free patients, and in a significant proportion of vaccinated patients the presence of tumor-reactive T-cells in the peripheral blood even 5 to 7 years after the last vaccination. Postoperative vaccination with virus-modified autologous tumor cells seems to be feasible and safe and may improve the prognosis of HNSCC patients with advanced tumors. This could be supported by antitumor immune responses that we observed especially in long-term surviving patients.

INTRODUCTION

Head and neck squamous cell carcinomas (HNSCCs) are the sixth most common neoplasms in the world, and their incidence is still increasing (1, 2). Despite progress in surgical techniques and an improved application of radio- and chemotherapy, the mortality of patients with these tumors is still high because of local recurrences, therefore demanding the development of new treatment modalities.

HNSCCs are characterized by their ability to induce changes in the micro-environment (3) as well as in the immune system of the host leading to reduced T-cell levels and reduced T-cell proliferation in response to mitogens (4, 5). This may be in part caused by the release of immunosuppressive substances such as transforming growth factor β and interleukin-10 (6, 7). The impact of immune suppression in HNSCC patients on the clinical course of disease is indicated by the association between reduced T-cell function and poorer survival (4). Nevertheless, the possibility of developing immunotherapeutic approaches as a treatment for HNSCC has gained interest because it offers the potential for tumor specificity with low side effects (8, 9). Certain proteins are selectively overexpressed in HNSCC (10), making these tumor antigens possible targets for immune recognition and reaction. In an animal model, it was shown that postoperative vaccination with irradiated, modified tumor cells could cure about one-third of the animals even after establishment of lymph node metastases (11). Furthermore, application of recombinant vaccinia virus expressing interleukin (IL)-2 as a tumor vaccine significantly improved survival of animals bearing HNSCC-tumors (6). Very recently, intradermal vaccination of six HNSCC patients with irradiated autologous tumor cells admixed with bacillus Calmette-Guérin followed by adoptive transfer of polyclonally activated lymph node cells resulted in CD4(+) and CD8(+) responses to the tumor (8). However, in this trial no objective antitumor response was observed.

For optimal efficacy, tumor cell vaccines require the addition of danger signals (12) by adjuvants. For this purpose, we used Newcastle Disease Virus (NDV), an avian paramyxovirus, which has antineoplastic and immune stimulatory properties (13). The virus selectively replicates in the cytoplasm of tumor cells (14) and induces T-cell costimulatory activity in the tumor cells (15). In the infected cells it induces danger signals such as double-stranded RNA, type 1 interferons (IFNα and IFNβ) and the chemokines RANTES (regulated on activation, normally T cell-expressed and -secreted) and IFN-γ-inducible protein-10 (16). Therefore this virus is a promising candidate for antitumor vaccination. This is supported by its successful preclinical evaluation in relevant animal tumor models (17).

The present pilot study was undertaken to evaluate a NDV-modified autologous tumor vaccine in HNSCC patients. The primary aims were to determine feasibility of this approach and to assess possible side effects. In addition, antitumor reactivity in vaccinated patients as well as a potential clinical benefit were evaluated.

MATERIALS AND METHODS

Patients. Twenty patients with pathologically confirmed HNSCC (maxillary sinus n = 1, oral cavity n = 4, oropharynx n = 4, larynx n = 6, hypopharynx n = 4, CUP (carcinoma with unknown primary tumor) n = 1; Table 1) were recruited from December 1995 to April 1997 to receive antitumor vaccination. Eligibility criteria included a Karnofsky performance scale of ≥60 and normal baseline hematologic parameters 2 weeks before the first vaccination (hemoglobin, total granulocyte count, platelet count, creatinine, transaminases, and thromboplastin time), a patient age >18 years, and a written informed consent. Exclusion criteria were pregnancy, severe pulmonary, cardiac, or other systemic disease associated with an unacceptable operative risk, presence of an acute infection, autoimmune disorders, or other malignancies. Each patient received maximal surgical resection (20 of 20) followed by radiotherapy in 16 of 20 cases (total dose 50.4–64 Gy). Vaccination therapy was started 3 months after surgery, when radiotherapy was completed, and the subsequent preconditioning period was finished. Patients were required to use a medically accepted form of birth control during the study. The study was approved by the institutional review board, in accordance with the Helsinki Declaration of 1975, as revised in 1983. All patients were closely evaluated during follow-up to determine tumor relapse and overall survival. Data were analyzed after a median follow-up period of 93 (83–99) months.

Immunologic data obtained from vaccinated patients by IFN-γ-ELISPOT (enzyme-linked immunospot) assay were compared with those of 42 nonse-
lected HNSCC patients, who were operated on at our department between September 2000 and May 2004.

Autoimmune Tumor Cell Culture and Characterization. Tumor cell cultures were established in our laboratory as described previously (18). In brief, tumor samples were mechanically dissected within 2 hours after resection. The cell suspension was cultured in DMEM (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany) and antibiotics. We only used pretested serum batches from countries in which bovine spongiform encephalopathy has never been diagnosed. Mycoplasma contamination was excluded by 4',6-diamidino-2-phenylindole staining (Roche Diagnostics, Mannheim, Germany) and cells were routinely tested for lack of fungal and yeast contaminations. We characterized all short-term cultures for their epithelial origin by the immunohistochemical detection of tissue-specific markers, using antibodies recognizing a broad spectrum of cytokeratins, and for expression of MHC I molecules.

Preparation of Newcastle Disease Virus-Modified Autologous Tumor Cell Vaccine. Per vaccine, 1 × 10^7 tumor cells were incubated for 1 hour with 64 hemagglutinating units of the avirulent strain Ulster of NDV. Successful infection was proven by immunohistochemical staining with monoclonal antibody anti-NDV-HN recognizing the viral protein hemagglutinin-neuraminidase, kindly provided by Dr. Iorio (Worcester, MA). For a delayed type hypersensitivity (DTH) test, virus-modified autologous tumor cells were used. Finally, cells were irradiated with 200 Gy. In case of the Newcastle Disease Virus-modified autologous tumor cell (ATV-NDV) vaccine, a dose of 400,000 IU of recombinant IL-2 (Chiron, Ratingen, Germany) was added to the vaccine before administration.

Prevaccination Treatment, Vaccination Procedure and DTH Test. During a preconditioning period of 12 weeks, patients received 10^6 IU IL-2 four times a week, which was administered subcutaneously in the abdominal wall. IL-2 treatment started when radiation therapy was completed or in case of no adjuvant radiotherapy directly after surgery. Subsequent vaccination consisted of up to 5 applications with 1 × 10^6 ATV-NDV cells intradermally in the upper thigh (alternately left and right). In addition, 1 × 10^6 IU IL-2 were subcutaneously applied four times over a time period of 2 days at the vaccination site. Vaccinations 1 to 4 were given in 3 weeks intervals followed by a 6 months interval for vaccination 5. To determine antitumor reactivity against unmodified tumor cells before and at the end of the vaccination period, challenge tests were done with 1 × 10^6 nonvirus-modified cells in the upper thigh 2 days before the first and 2 days before the last vaccination. DTH reactivities at the vaccination and at the challenge site were recorded 24 hours after injection by measuring the area of induration.

Generation of Dendritic Cells and T-Lymphocytes. Dendritic cells (DCs) were generated from peripheral blood-derived monocytes by incubation for 7 days in serum-free X-VIVO (BioWhittaker, Walkersville, MD) supplemented with human granulocyte-macrophage colony-stimulating factor (Behring-Werke, Marburg, Germany) and IL-4 (PromoCell, Heidelberg, Germany) as described previously (19). They were purified from contaminating cells by the use of anti-CD3-, anti-CD19-, and anti-CD56—coupled magnetic beads (Dynal, Oslo, Norway) and pulsed with 20 hours for lysates with 200 μg protein/mL from autologous tumor cells or autologous peripheral blood mononuclear cells (PBMC), T-cells were cultured by incubation of PBMC for 7 days in RPMI 1640 (Invitrogen, Karlsruhe, Germany) supplemented with 10% human AB serum (PromoCell), IL-2 (100 units/mL), and IL-4 (60 units/mL) followed by overnight incubation in medium without cytokines and magnetic bead depletion of contaminating CD19+ B-cells, CD15+ myeloid cells, CD56+ NK-cells, and NK-T-cells.

IFN-γ ELISPOT Assay. IFN-γ producing T-lymphocytes were determined as described previously (19). DCs were pulsed with lysates either from autologous tumor cells or from autologous PBMC (negative control). Pulsed DCs were co-cultured with 1 × 10^5 autologous T-cells for 40 hours in a ratio of 1:5. The number of IFN-γ spots was measured automatically with the KS ELISPOT software (Carl Zeiss Vision, Hallbergmoos, Germany). Individuals were designated as responders when the numbers of spots in the presence of DCs pulsed with tumor lysate were significantly higher than in negative control wells. A two-sided student’s t test was used to determine statistical significance. The frequency of tumor-reactive T cells was calculated as follows: (spot numbers in wells with tumor lysate activated DCs – spot numbers in negative control wells)/T-cell numbers per well.

Fluorescence-Activated Cell Sorter Analysis. Cultured tumor cells (2 × 10^6) were stained with mouse antihuman monoclonal antibody specific for HLA-A, -B, -C (hybridoma W6/32, kindly provided by G. Moldenhauer, DKFZ, Heidelberg, Germany) and FITC-conjugated secondary goat antimouse antibody (Dianova, Hamburg, Germany). Cytometric analysis was done with a FACSscan with CELLQuest software (BD Bioscience, Heidelberg, Germany). Typically 100,000 events were collected, and data were expressed as dot plots.

Immunohistochemistry. Immunohistochemical staining was done on HNSCC cells and on cryostat sections of the frozen specimens. Fixation and staining were carried out as described previously (20). Primary antibodies used were anti-Cytokeratin 18 (clone CY-90, Sigma, Munich, Germany), anti-pan-Cytokeratin antibodies recognizing a broad spectrum of cytokeratins (clone MNF 116 recognizing cytokeratins 5, 6, 8, 17, and 19 and clone 34BE12, recognizing cytokeratins 1, 5, 10, 14, both obtained from Dako, Hamburg, Germany), anti-PECAM-1 (PharMingen, Hamburg, Germany), antifactor VIII (Dako), and anti-MHC I (clone W6/32, Dako).

Statistical Analysis. Correlation between immunologic end points and clinical outcome or treatment and control group were determined by a two-sided t test. P values of 0.05 were considered significant.
RESULTS

Patients and Study Design. The study design, including a preconditioning period and preparation of the autologous, virus-modified tumor vaccine for 20 patients, is illustrated in Fig. 1. Clinical data of these patients are listed in detail in Table 1 and clinical characteristics of the vaccination group are listed in Table 2. Surgical removal of the tumor was followed by optional radiotherapy. To compensate immunosuppressive effects of radiotherapy, which may lead to a decrease of T-cell levels (4, 5), IL-2 was administered in the abdominal wall during a preconditioning period of 12 weeks. Afterward, patients received up to 5 vaccinations because of tumor relapse or insufficient tumor cell growth in vitro. In six cases, patients received additional palliative chemotherapy after tumor relapse.

Characterization of Autologous Tumor Vaccine, Feasibility, and Safety of Application. To obtain vaccine in sufficient quantity and quality, ATV-NDV was prepared for each patient from autologous HNSCC cell cultures. Between December 1995 and April 1997, when patients where included in the study, establishment of primary HNSCC cultures was possible in 77.8% (49 of 63) of tumor samples. As shown in Fig. 2A, HNSCC cells showed homogenous expression of the epithelial marker CK18 (20 of 20) and no staining for markers of other cell types that were present in the original tumor tissue such as endothelial cells, as evidenced by negative FVIII-staining. As shown by either immunohistochemistry and/or fluorescence-activated cell sorter analysis, all cultured tumor cells expressed MHC class I molecules (20 of 20), which are important for presentation of tumor-associated antigens (Fig. 2B).

After vaccination, we did not observe any serious adverse events nor was there evidence of autoimmune phenomena such as vasculitis, rheumatoid arthritis, or lymphatic disorders. Two patients developed flu-like symptoms (e.g., fever) and two patients presented with a marked induration at the vaccination site >5 cm². Mild fatigue and palpable indurations at the vaccination site were reported frequently, but no hematologic toxicities were observed.

Clinical Course. To determine a possible influence of antitumor vaccination on the clinical outcome of the patients, disease-free and overall survival were assessed (Table 1 and 2). After a median follow-up period of 93 months (83–99 months) 12 of 20 patients are still alive, and among these 11 of 20 are without recurrent tumors. Mean disease-free was 59.65 months (±38.89) and mean overall survival 67.8 months (±32.59). With regard to the prognostically unfavorable subgroup of stage III and stage IV tumors (n = 18), percentages of overall survival were 100% at 1 year, 67% at 3 years, and 61% at 5 years (Table 2). Even for stage IV tumors, the observed 5-year survival rate of 50% (7 of 14) is remarkably high, especially taking into consideration that two patients within this group did not die from their HNSCC tumor. Interestingly, all patients suffering from stage II and III tumors are still disease-free and alive.

Antitumor Immune Reactivity in Skin and Peripheral Blood of Vaccinated Patients. In six patients, analysis of DTH skin reactions to challenges with unmodified, irradiated tumor cells could be obtained. Figure 3A shows changes in indurations before the vaccinations started (1. challenge) and at the time of the last vaccination (2. challenge). Five of six patients presented with clearly increased indurations. All of these patients are still disease-free and alive. In contrast, DTH reactivity from patient 3 dramatically decreased at the end of the vaccination period. At this time, the patient developed a
rectum carcinoma, from which he died 1 year later. However, the increased reactivity to unmodified tumor cells in five of six still healthy patients is of particular relevance because it indicates systemic reactivity to autologous tumor cells as they may have remained in the patient.

In addition, DTH reactivities to NDV-modified tumor cells could also be obtained from 10 patients at the 1st and either the 4th or 5th vaccination. A subclassification in disease-free and relapsed patients (Fig. 3B) revealed a substantial DTH increase in disease-free patients compared with relapsed patients ($P < 0.05$).

Vaccination may induce or augment generation of tumor-reactive T-cells in cancer patients, which were shown to confer antitumor immunity (19). Nine of 12 vaccinated, long-term–surviving patients could be analyzed 5 to 7 years after the last vaccination for the presence of tumor-reactive memory T-cells and were compared with a control group of 42 nonselected HNSCC patients. The IFN-γ ELISPOT assay, which was used to quantitate antitumor T-cell reactivity, was entirely autologous and used DCs as antigen-presenting cells (19). PBMCs of vaccinated patients contained high frequencies of tumor-reactive T cells in six of nine cases analyzed compared with only 5 of 42 in the nonvaccinated control group (Fig. 3C). Thus, a significantly higher number of vaccinated patients contained tumor-specific T-lymphocytes in their PBMC ($P < 0.03$) 5 to 6 years after the last vaccination and were in a state of long-term complete remission.

**DISCUSSION**

In this pilot study, we show that postoperative antitumor vaccination of 20 HNSCC patients with an autologous virus-modified tumor vaccine is feasible and safe and is associated with remarkably high 5-year survival rates in stage III and IV tumors. Vaccination caused an augmentation of tumor-specific immunity as detected by antitumor DTH reactions especially in disease-free patients. Moreover, in 67% of long-term surviving patients analyzed we detected tumor-reactive T cells even 5 to 7 years after the last treatment. Because no blood samples were collected before vaccination, we cannot exclude that these patients may have already possessed tumor reactive T cells before vaccination. However, in a control group consisting of 42 operated HNSCC patients, a significantly lower proportion of only 12% contained tumor-reactive T cells in their peripheral blood postoperatively.

This is of major importance because HNSCCs are well known for their highly immune-inhibitory nature and have been shown particularly to be deficient in their immune responsiveness (6, 21, 22). To overcome the immune-inhibitory properties of HNSCC, we decided to infect the tumor cells with a particular virus, which induces proinflammatory cytokines. For vaccine production, we used individual intact tumor cells from established tumor cell cultures. This allowed producing a vaccine with a standardized high number of 10 million viable tumor cells. The tumor antigens of this vaccine might include individual tumor antigens derived from mutations or other genetic alterations, and they might be representative for the heterogeneity of tumor antigens in a single tumor. It was shown in a breast cancer study, with tumor cells obtained by enzymatic digestion of the resected tumor tissue, that a clinical benefit was seen only when patients received at least $1.5 \times 10^6$ ATV-NDV cells per vaccine (23).

The use of whole tumor cells from established cultures was feasible and eliminated the need to first identify the respective tumor antigens, which would require sophisticated techniques. Because even multiple applications of such a vaccine did not induce auto-immune disease, this approach can be considered safe.

As virus in the tumor vaccine, we used NDV strain Ulster, based on

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**Fig. 3.** Immune monitoring of vaccinated patients. A, changes in DTH reactions of six vaccinated patients to unmodified tumor cells. † Patient died after 21 months. B, changes in DTH reactions to NDV-modified tumor cells of 10 vaccinated patients after the 1st and the 4th or 5th vaccination. C, frequencies of tumor-reactive memory T cells analyzed by IFN-γ-ELISPOT assay in nine long-term surviving vaccinated patients and 42 nonvaccinated patients. Six of nine vaccinated patients analyzed (1, 9, 11, 15, 16, 20) contain high frequencies of tumor-specific memory T cells compared with only 5 of 42 nonvaccinated patients. * (+) significant differences compared with control lysate [*: $P < 0.05$; (+), $P < 0.1$].
good experiences in various animal tumor models and in previous clinical studies with other forms of cancer (11, 14, 23–25). Recently, interest in the use of tumor-selective replication competent viruses such as NDV, which has already been safely applied to many cancer patients in Europe and the United States, is reviving (13). The immune stimulatory properties of NDV lead to proinflammatory effects at the vaccination site and thus contribute to the augmentation of cytotoxic antitumor effects (26). In accordance with these observations, in our study vaccine application was associated with a local skin response leading to improvement of systemic cell-mediated immune responsiveness as evidenced by antitumor DTH reactivity. Moreover, we detected antitumor T-cell responsiveness in the blood of most long-term surviving HNSCC patients even as little as 5 years after the last vaccination in patients lacking any signs of residual or recurrent tumor disease. Therefore, we assume that the observed antitumor responses were based on memory T cells rather than on effector T cells. With regard to the clinical outcome of the vaccinated patients, we observed a remarkable 5-year survival rate of 61% in the prognostically unfavorable subgroup of stage III and IV tumors. This compares advantageously with recent reports outlining a 5-year survival rate of 38% in 363 HNSCC patients of the same subgroup (155 patients suffering from stage III and 208 from stage IV tumors) after having received the same type of standard therapy consisting of surgery, optional radiotherapy, and in some cases chemotherapy (27). It is of note that in the analysis of Gleich et al. (27), similar survival rates were only seen in younger patients (20–44 years), whereas in our treatment group, only five patients were younger than 45 years of age, and among these only two are still alive. Therefore the favorable 5-year survival rate observed after vaccination therapy cannot be explained by an accidental selection of young patients. An unwanted bias caused by the additional cytostatic therapy in a subgroup of vaccinated patients seems unlikely, because a meta-analysis of 63 trials of locoregional treatment with or without chemotherapy showed a survival benefit of only 4% in favor of chemotherapy (28).

Altogether, the results of our pilot study are remarkable because the treatment is well tolerated, has no major side effects, and therefore does not negatively affect the quality of life of the patients. The present study supports a concept of tumor vaccine combining multiple tumor antigens with NDV-induced danger signals (12–17, 23, 26) thereby facilitating polyclonal antitumor immune responses and the establishment of a specific antitumor memory (19). Our results encourage a further validation in a randomized trial to exclude unknown confounders.

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