Liposomal Transfection of Squamous Carcinoma Cells of the Head and Neck with IL-2 and B7 Plasmids Inducing an Autologous Immune Response In Vitro

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Abstract. New treatment strategies need to be developed to face the increasing incidence and mortality of squamous cell carcinoma of the head and neck (SCCHN), as the overall survival rate remains poor, with minor therapeutic progress having been achieved over the past forty years. One major goal could be to restore a damaged immune system by intratumoral injection of IL-2-genes that permanently provide non-toxic IL-2-protein concentrations at the tumor site, sufficient to activate cellular immunity in vivo. We showed that the transfection of SCCHN cell lines with IL-2-plasmids, encapsulated in DOTMA/Col, in vitro resulted in the synthesis of bioactive IL-2-protein for up to 30 days by the tumor cells themselves. The transcription of secondary cytokines (IL-6, IL-8, GM-CSF, TNF-α) and the expression of immunomodulatory surface molecules (MHC Class II, ICAM1) were enhanced. The IL-2-modified tumor cells were effectively lysed by autologous peripheral blood lymphocytes (PBLs). The immune response was enhanced by B7.1-gene-cotransfection and/or preactivation of PBLs with exogenous IL-2. We demonstrated that in vitro liposome-mediated IL-2-gene-transfection of SCCHN cells is an effective method to stimulate an autologous immune response and is, therefore, promising for clinical application.

In cancer, the grade of immunodeficiency seems to be highly variable. For squamous cell carcinoma of the head and neck (SCCHN), it is demonstrated that tumor tissue is well infiltrated by various types of immune cells. Investigations of SCCHN have shown an intense antitumor immune reaction associated with the infiltration of CD8+ T cells (1, 2). The extent of the lymphocytic infiltration in SCCHN is closely correlated to the prognosis of the patient’s survival (3). SCCHN belongs to the cancer specimens that exert a weak immunogenicity. In most SCCHN, tumor-infiltrating lymphocytes (TILs) are not activated and do not expand well in vivo (4). The immunological dysfunction of these TILs, however, is reversible, as TILs isolated from SCCHN are able to exert normal lytic functions against tumor cells in vitro (5). The aim of various experimental and therapeutic approaches is to overcome this tumor-mediated blockade of the immune response. The main interest is the application of immunomodulating molecules, e.g. cytokines, as they participate in antitumor immune responses by activating cellular immunity. IL-2 is known to play a major role (6, 7) as it activates immune cells capable of lysing autologous or allogenic tumor cells (8). In general, tumor cells themselves do not secrete IL-2, or they only secrete IL-2 at a low level. After being activated by IL-2, T cells undergo clonal expansion when further receiving appropriate stimuli, i.e. the binding of an antigen by the T cell receptor (TCR) or the binding of co-stimulatory molecules (9). If not activated, T cells are rendered inactive (10, 11).

Treatment with recombinant rIL-2 has induced definite tumor regression in the cases of renal cell carcinoma, melanoma and colorectal cancer (12, 13). Although it has been shown to be effective in some patients, the effects of IL-2 application are limited by the toxicity of high-doses of IL-2, which is associated with a capillary leakage syndrome (12). In patients with SCCHN, peritumoral or locoregional injection of high-dose rIL-2 was not effective (14).
Complete or partial responses without toxic effects were only observed after application of low-dose IL-2. Intratumoral injection of polyethylene glycol-modified rIL-2 (PEG-IL-2), or peritumoral or intranodal rIL-2-injection appears to be beneficial only for a small portion of SCCHN (15). The data of objective response rates are encouraging for a wide range clinical application of rIL-2. The final treatment schedule has not been defined yet. The short tissue half-life of the IL-2-protein urges the need for a prolonged duration of IL-2 presence in the tissue. Permanent IL-2-production could be achieved by gene transfer into the tumor cells. Tumor cells, engineered to secrete biologically-active IL-2-protein, may deliver IL-2 to the tumor site at appropriate concentrations and thus circumvent the disadvantages of systemic rIL-2-therapy. Our current study evaluated the efficacy of liposome-mediated transfection of IL-2-genes into SCCHN tumor cells and investigated the functional changes in tumor cells and the subsequent improvements in the immune recognition.

Materials and Methods

Cells and culture conditions. The primary cell line, SCC-GHD, was newly established from a squamous cell carcinoma of the hypopharynx. The human SCCHN cell line, PCI-1, was kindly provided by Dr. T. Whiteside (University of Pittsburgh Cancer Institute, USA). The epithelial cell lines 22A, FaDu, HLac78 and the IL-2-dependent T cell line, CTLL-2 (ATCC), were purchased from ATCC (Rockville, MD, USA). All cell lines were maintained in DMEM, supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin (all from Gibco, Karlsruhe, Germany). Autologous PBLs were isolated from venous blood by Ficoll-Hypaque centrifugation. Viable cells were counted by trypan blue dye exclusion and immediately used for experiments.

Plasmids. The PIL0697 expression plasmid, with an inserted full length cDNA clone encoding the human IL-2 under the control of the CMV-promoter, was provided by GeneMedicine (Houston, TX, USA). The expression plasmid for B7.1 transfection was constructed and kindly supplied by Dominique Zehnpfennig (GSF, Finnland). In brief, 106 target cells (T) were loaded for 30 min at room temperature. During incubation for 15 min at room temperature, Eu and BATDA form a highly fluorescent chelate. The fluorescence signals only observed after application of low-dose IL-2. Intratumoral injection of polyethylene glycol-modified rIL-2 (PEG-IL-2), or peritumoral or intranodal rIL-2-injection appears to be beneficial only for a small portion of SCCHN (15). The data of objective response rates are encouraging for a wide range clinical application of rIL-2. The final treatment schedule has not been defined yet. The short tissue half-life of the IL-2-protein urges the need for a prolonged duration of IL-2 presence in the tissue. Permanent IL-2-production could be achieved by gene transfer into the tumor cells. Tumor cells, engineered to secrete biologically-active IL-2-protein, may deliver IL-2 to the tumor site at appropriate concentrations and thus circumvent the disadvantages of systemic rIL-2-therapy.

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Transfection of the IL-2- and B7.1-genes into SCCHN cell lines. Tumor cells were transfected with the IL-2-encoding expression plasmid by lipofection. Two hours prior to the transfection, 2x105 cells were seeded in a 24-well plate (Costar, Cambridge, MA, USA) in 1 ml of complete DMEM. The cells were then washed with serum-free medium (OPTIMEM) from Gibco. For transfection, 0.78 mg of IL-2-plasmids plus DOTMA/Chol were diluted in 2.5 ml sterile H2O, mixed gently and incubated for 30 min at room temperature. Aliquots of the reconstituted plasmid-liposome-solution in OPTIMEM were transferred onto the tumor cells, followed by 3-h incubation at 37°C and 5% CO2. Then, 2 ml of DMEM were added to each well. B7.1-plasmids were transfected by Lipofectamine, according to the manufacturer’s protocol (Gibco).

ELISA for IL-2 secretion. The concentrations of IL-2-protein released by transfected tumor cells into the supernatants were determined by a commercially available enzyme-linked immunosorbent assay (ELISA) from Boehringer Mannheim, Germany. The results are given in pg/ml/2x105 cells after different times of incubation and/or at various amounts of transfected IL-2-plasmids in 2 ml culture medium.

Bioassay for IL-2-protein produced by IL-2-transfected tumor cells. The biological activity of IL-2-protein secreted by transfected tumor cells was measured by a proliferation assay of the IL-2-dependent T cell line, CTLL-2 (ATCC), with culture supernatants serially diluted from 1:5 to 1:10000. After 3-day incubation, the proliferation of CTLL-2 cells was calculated in a commercially available MTT-assay (Boehringer Mannheim).

RNA extraction and reverse-transcription polymerase chain reaction (RT-PCR). Total RNA from the cell lines was extracted using TripureTMIsolation Reagent and digested by RNase-free DNase (both Boehringer Mannheim). 1.5 µg RNA were subjected to RT, which was carried out according to the protocol published by Chaubal et al. (17). Primers for cDNA amplification were designed as follows: 5'-ATTCCATGGCCACGGTGTA-3' (f:forward) and 5'-GGCTGTCCTCAC-CACCTTCTT-3' (r: reverse) for GAPDH, yielding a 631-bp fragment, 5'-GATC-CATTGACACTGCAG-3' (f) and 5'-GCTGTCCTCA-CATCTGGCAACCCTACAACAG-3' (r) for IL-1β (400 bp), 5'-CACACAGA-CAGCCACTCACCTC-3' (f) and 5'-GGTCACCGAACAAGGAGAGTAGAGG-3' (r) for IL-6 (518 bp), 5'-GCAGCTCCTGTGTGGAAGGTCGAG-3' (f) and 5'- GCCATCTGGCAACCCTACAACAG-3' (r) for IL-8 (350 bp), 5'- GTGGCGCTCGAGCATCTGCAGC-3' (f) 5'-CCTGGACTGGCTC-3' (r) for TNF-α (237 bp). The primers were all purchased from ARK Scientific, Darmstadt, Germany.

Flow cytometry analysis. Cells were incubated with a first antibody (Ab) (Dako, Germany) directed anti-mouse for 15 min on ice, then washed twice with PBS plus 10% (v/v) FCS and incubated with a secondary anti-mouse IgG-FITC (Dako) conjugated Ab for 15 min. After another washing, flow cytometry was performed using a FACSCalibur flow cytometer and the CellQuest analysis programme (Becton Dickinson, Heidelberg, Germany).

Non-radioactive cytotoxicity assay. Lysis of parental and transfected tumor cells by autologous PBLs was evaluated with a cytotoxicity assay based on time-resolved fluorometry (Wallac, Turku, Finland). In brief, 10⁶ target cells (T) were loaded for 30 min at 37°C with 2 µl of a fluorescence-enhancing ligand (BATDA). After gently washing 5 times, 4x10⁵ of loaded targets were plated per well in round-bottom 96-well plates in duplicate. Effector cells (E) were added at E:T ranges from 6:1 to 100:1. Internal assay controls for spontaneous, maximum and background release were included. The plates were incubated for 2 h at 37°C. Meanwhile the BATDA ligand is released into the supernatant after cytolysis of the target cells. The plates were centrifuged for 5 min at 500 x g. Twenty µl of the supernatant from each well was transferred into a flat-bottom plate and 200 µl of Europium (Eu) solution was added per well. During incubation for 15 min at room temperature, Eu and BATDA form a highly fluorescent chelate. The fluorescence signals
then measured in a time-resolved fluorometer (Victor from Wallac) correlate directly with the amount of lysed cells.

**Results**

**Liposomal IL-2-gene-transfected SCCHN cell lines secrete bioactive IL-2.** SCCHN cell lines were transiently transfected first with a constant amount of 0.12 mg/ml hIL-2-plasmids formulated in DOTMA/Chol. Secretion of IL-2-protein by parental and IL-2-transfected cell lines was analyzed by ELISA. As shown in Figure 1, the parental cells did not produce soluble IL-2 after 72 h of incubation. IL-2 was detected in the supernatants of transiently IL-2-transfected SCCHN cell lines with varying amounts of IL-2-protein secretion between the cell lines. The SCC-GHD cell line achieved more than 4 ng/ml IL-2 (high-producer). 22A and HLac78 cells produced 0.3 and 0.1 ng/ml IL-2, respectively (low-producer). FaDu cells did not synthesize IL-2 in this experimental setting (non-producer).

After transfection with IL-2-DNA/DOTMA/Chol complexes, the IL-2-protein secreted by SCC-GHD cells was tested for bioactivity. Supernatants derived from IL-2-modified SCC-GHD cells enhanced proliferation of the strictly IL-2-dependent T cell line CTLL-2, thus implicating that IL-2-protein from genetically-engineered SCC-GHD cells is bioactive. CTLL-2 proliferation was not found by testing supernatants from SCC-GHD parental cells.

**Kinetics of IL-2-protein secretion by IL-2-transfected tumor cells.** We were interested whether transient transfection of SCCHN cells with IL-2-DNA/liposome complexes resulted in IL-2-protein production long enough to induce and sustain antitumor cytotoxicity. After liposome-mediated transfection with 0.03 or 0.06 mg/ml IL-2-DNA, SCC-GHD cells were cultured for 30 days. After every 9 days in culture, the cells were harvested and were recultured after 1:2 dilution. The supernatants were harvested at 3-day intervals and tested for IL-2 secretion by ELISA. At both transfection concentrations an increase in the amount of synthesized IL-2-protein, ranging from 1ng/ml to 3.8 ng/ml IL-2, up to day 9 post transfection was found (Figure 2). After the first reculture of the transfected cells, the IL-2-protein amount increased to various levels. After the second reculture, IL-2-protein secretion rose again but did not reach the initial IL-2-protein levels. IL-2-protein was measured at low levels (0.5 ng/ml) up to day 30 post transfection.

**Liposomal transfection increases cytokine gene transcription in IL-2-transfected tumor cells.** The signals for IL-2-specific DNA and IL-2-specific RNA, evaluated by IL-2-specific PCR and RT-PCR analysis, were significantly increased in SCCHN after transient transfection with IL-2-DNA/DOTMA/Chol complexes (data not shown). Analysis of immunomodulatory cytokine genes by RT-PCR demonstrated that, in transfected SCC-GHD cells, transcription of IL-6, IL-8, TNF-α and GM-CSF was up-regulated in comparison to non-transfected cells (Figure 3). IL-1β transcription was not influenced by IL-2-DNA-transfection. An up-regulation of secondary cytokines was also seen after IL-2-gene transfection in PCI-1 cells (data not shown).

**Expression of cell surface molecules on tumor cells after liposome-mediated IL-2-gene transfection.** As shown by FACS analysis, the parental SCC-GHD cells highly expressed MHC class I as well as ICAM-1 molecules, whereas the co-stimulatory B7.1 molecules were weakly-expressed and MHC class II molecules were not expressed at all (Figure 4). Following liposomal transfection with IL-2-plasmids, no significant difference in the expression of the MHC class I and ICAM-1 molecules between parental and transfected cells was observed. However, B7.1 and MHC class II expression was up-regulated in IL-2-transfected tumor cells. On HLac78 cells the same expression pattern was detected after IL-2-gene transfection (data not shown).

**Enhanced cytotoxicity of autologous PBLs against IL-2-gene-transfected tumor cells.** In a 3-h non-radioactive Europium-release cytotoxicity assay, whether or not IL-2-gene-transfected SCC-GHD cells had an effect on the cytotoxicity of freshly isolated autologous PBLs, relative to the cytotoxicity against parental tumor cells, was analyzed in vitro. Control cells for MHC restriction and antigen-specificity were included. The allogenic SCCHN cell line FaDu, the MHC class I-negative K562 cell line and NK cell-resistant Daudi lymphoma cells did not induce cytotoxic activity in PBLs (Figure 5). In contrast, autologous PBLs were more
prepared to kill parental and IL-2-transfected SCC-GHD cells than to kill allogenic target cells. Here, the killing efficacy was further enhanced at E:T ratios equal to or greater than 50:1 when autologous PBLs were co-incubated with autologous IL-2-gene-transfected tumor cells.

Incubation of autologous PBLs with a 1:1 mix of SCC-GHD cells either transfected by IL-2- or by B7.1-genes resulted in an antitumor cytotoxicity comparable to that seen with tumor cells transfected with the IL-2-gene alone.

Pre-activation of autologous PBLs with rIL-2-protein 24 h prior to Europium-release assay revealed either no killing (Daudi) or some low unspecific allogenic cell killing (FaDu and K562) at E:T ratios equal to or greater than 50:1, whereas the killing of IL-2-transfected autologous tumor cells again was almost twice as high as the killing of parental cells.
The cytotoxic activity was markedly increased when a mix of IL-2- and B7.1-transfected tumor cells was subjected to lysis by pre-activated PBLs. It was higher than the specific cytotoxicity measured with tumor cells transfected with IL-2-genes alone.

**Discussion**

Many strategies of current immunotherapies focus on activation of cells from the acquired and innate immune system by application of immunomodulatory cytokines and/or by alteration of the immunogenicity of the tumor cells themselves (18, 19). Interleukin 2 (IL-2) is a promising candidate for successful immunotherapy with cytokines, as it is known to be one of the most important T cell and LAK cell activator molecules (20, 21). In SCCHN therapy, rIL-2 was applied peritumorally, locoregionally or at the tumor site at high or low doses in several clinical studies (14-16, 23-25). Due to the short half-life of recombinant IL-2 in the tissue, it has been shown that, after local application rIL-2, it only exerts a short activity (6, 7). Application of high doses of rIL-2 at the tumor site or systemic rIL-2 administration was either associated with toxic effects or did not add to the efficiency (14). At present, a new therapeutic strategy for IL-2-immunotherapy could involve the techniques of gene transfer to establish a permanent locoregional IL-2 production. Within appropriate expression vector systems, IL-2-genes could be transfected into the tumor cells in vivo, either separately or together with genes of other immunomodulatory molecules, e.g. costimulatory B7 molecules. In our study, we transfected human SCCHN cell lines with the same IL-2-plasmid DNA used in the clinical study by Wollenberg et al. (24), in order to obtain basic information about the applied liposome-mediated gene transfer method in SCCHN. It could be shown that liposomal-mediated IL-2-gene transfer into tumor cells induces an antitumor immune response resulting in the lysis of tumor cells by autologous effector cells. The bioactivity of the secreted IL-2-protein was further confirmed by stimulation of proliferation of the IL-2-dependent T cell line CTTL-2.

In our hands, IL-2-protein secretion was highly variable between different SCCHN cell lines. High proliferating cell lines, like SCC-GHD or HLac78, produced much more bioactive IL-2 than PCI-1 or FaDu cells (Figure 1), which proliferate at lower rates. The varying amounts of IL-2 biosynthesis may be due to different numbers of IL-2-plasmid copies taken up by the different tumor cell lines. Further information about plasmid uptake and expression is not yet available. In repeated experiments we always observed high, low or no IL-2 production for the appropriate transfected SCCHN cell line. These in vitro data implicate that the efficacy of IL-2 secretion after liposome-transfection may depend on the proliferation characteristics of the individual tumor cells, and this may also occur in vivo after intratumoral administration of IL-2-DNA/liposomes. We further observed that some low-proliferating SCCHN cell lines, like PCI-1, produced IL-2 only after transfection at a low concentration of IL-2-plasmids. This “high-dose inhibition” effect should be taken into consideration for clinical approaches to IL-2-gene therapy.

IL-2-gene transfection augmented the immunogenicity of low immunogenic SCCHN by influencing the expression pattern of surface molecules relevant in the immune response. In our system, the expression of MHC class II and the costimulatory signal molecule B7.1 was highly up-regulated, and MHC class I and ICAM-1 expression was slightly increased (Figure 4). An up-regulation of immuno-
relevant surface molecules was not seen in retroviral-IL-2-modified SCCHN cells by Nagashima et al. (23). In the case of exogenously applied IL-2, the expression of the HLA-class I and ICAM-1 molecules on SCCHN cells were even down-regulated (22). Our data of liposomal-mediated IL-2-gene transfection showed an enhancement of immunogenicity on SCCHN, particularly due to an increased presentation of immunomodulatory signals on the tumor cells themselves, as previously shown by Cayeux et al. in a murine model (26, 27). These results implicate the important role of B7 molecules on tumor cells for the induction of cellular immune responses.

In our experiments, the transcription of secondary cytokine genes (IL-6, IL-8, TNF-α and GM-CSF) was up-regulated after liposomal-IL-2-transfection (Figure 2). IL-1β transcription was not influenced. This implies that IL-2-gene transfer has effects on the paracrine production of multiple cytokines, an effect seen by Weidman et al. and also by Li et al. in a murine model of SCCHN (28). After hIL-2-gene therapy, murine IFN-γ and IL-12 levels were increased. In the retroviral-IL-2 transfection system, an increase of IL-6 was observed (23). As secondary cytokines are also involved in the antitumor response (18), the single gene transfer in general is important for generating a therapeutic effect in surgery for head and neck cancer and also for other tumor specificities.

Our data further indicate that liposomal-mediated IL-2-gene transfection enhances a tumor-specific, MHC-restricted lysis of autologous tumor cells in vitro. As SSC-GHD cells were positive for MHC class I molecules (Figure 4), a CD8+ T cell response might be predicted. Furthermore a T cell immune response becomes evident from our data because of the fact that additional presentation of the costimulatory B7.1 molecule on tumor cells by liposome-mediated transfection and preactivation of autologous PBLs with exogenous IL-2 greatly increased cytotoxicity against tumor cells (Figure 5). This is in accordance with Lang et al. who demonstrated, both in situ and in vitro, that retroviral transfection of B7.1(−) SCCHN cells with B7.1-genes resulted in T cell proliferation and generation of antitumor effector T cells (29, 30). Wollenberg et al. could show in mice that vaccination with B7.1-transfected SCCHN cells prohibited the outgrowth of recurrent tumors (31).

An alternative therapeutic approach, avoiding the need to establish and transfec tumor lines in vitro or the characterization of specific tumor antigens, is the direct in situ transfer of immunostimulatory genes into established tumors. The results of our investigations are promising for such a therapeutic approach since we could show that liposome-mediated IL-2-gene transfer into SCCHN resulted in increased production of bioactive IL-2-protein and induced and sustained effector cell activation in vitro. Furthermore, the transfer and expression of DNA of co-stimulatory molecules like B7 may enhance the immunogenicity of weakly immunogenic tumors, such as SCCHN.

References


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