Generation of an Autologous Cell System for Immunotherapy of Squamous Cell Carcinoma of the Head and Neck

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Abstract. *Background: To date, there is no tumor antigen known to be sufficiently specific for diagnosis, therapy monitoring and immunotherapy of squamous cell carcinoma of the head and neck (SCCHN). The aim of our study was to generate an autologous immune response against SCCHN in vitro for further characterization of SCCHN-specific tumor markers and adoptive immunotherapy. Materials and Methods: As sources for tumor antigens (Ags) for the restimulation of autologous immune cells, cell lines from solid SCCHN were established and characterized. Forty-five percent of 40 tumors of different SCCHN specimens were maintained for more than 20 cell generations in culture. Results: One primary cell line, SCCHN-GHD, newly established from a hypopharynx carcinoma, was further characterized as a telomerase-positive, immortalized cell line with epithelial cell characteristics. It was found to be tumorigenic in SCID mice. Conclusion: This new SCCHN-GHD cell line is competent as a target for lysis by autologous immune cells and for the restimulation of autologous tumor-specific immune cells. Subsequent characterization of tumor antigens will be performed.*

Squamous cell cancer of the head and neck (SCCHN) is the sixth most common malignancy worldwide, representing 90% of all head and neck cancers. Despite progress in the therapy of SCCHN, patient survival has not improved in over 30 years. The 5-year survival still remains at 30% to 40% in the advanced stages (1). The current primary therapy of surgery followed by single or combined chemo-radiotherapy leaves occult tumor cells in the periphery that remain undetectable in the body (minimal residual disease, MRD) and may result in local or distant relapse (13, 14). In order to introduce immunotherapeutic agents, suitable tumor antigens are needed. Screenings on a large panel of normal and malignant tissues revealed that epithelial cell markers and a variety of so-called tumor markers are not specific for tumors of the head and neck, as they are also expressed on non-malignant tissues and are simultaneously expressed in different malignant epithelia (2, 4, 5, 7-9, 12). Immune responses against tumor cells are strictly dependent on the mutual recognition of autologous cell interaction molecules on the surface of tumor and immune effector cells. For the development of efficient anticancer therapies, research needs to focus on autologous systems with tumor targets and immune effectors obtained from the same patient.

The aim of our study was to establish an autologous *in vitro* system to generate a cytotoxic immune response against tumor cells of the head and neck with regard to the further characterization of SSCHN-specific antigens (4).

Materials and Methods

Initiation and propagation of SCCHN cultures. After written informed consent, biopsies of 40 SCCHN were obtained intraoperatively. These aseptically-treated samples were rinsed in phosphate-buffered saline (PBS), cut into pieces of 0.5 mm in diameter and distributed directly to culture dishes (Nunc, Germany) to initiate explant outgrowth cultures. The tumor pieces were fixed by drying the open dishes for 10 min. Tumor-infiltrating lymphocytes (TILs) were removed by incubation of the tumor

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pieces for 24 h in a culture medium containing 100 U/ml recombinant human interleukin-2 (rhIL-2) (Boehringer, Germany). Migrated TILs were cryogenically preserved. Another portion of tumor was minced with scissors and transferred to a stir flask. For 2 h the tumor pieces were disaggregated with 0.2% (w/v) trypsin (PAA, Germany), plus 0.2% (w/v) collagenase IV (Sigma, Germany) at 37˚C. The growth medium was Dulbecco's modified Eagle's medium, DMEM (GIBCO, Germany).

During the first weeks after culture initiation, 50% of the culture medium was replaced with fresh growth medium at 4- to 7-day intervals. After formation of adherent cell layers, the cells were recultured by trypsinization (trypsin and 0.2% (w/v) ETDA (GIBCO)). Contaminating fibroblasts were removed by short incubation with 0.2% (w/v) EDTA only. Vigorous pipetting left epithelial cells adherent, whereas fibroblasts became non-adherent and were removed.

Tumorigenicity of primary SCCHN cell lines in SCID mice. The animal experiments were approved by the Ethics Commission, according to German federal law. SCID mice (GSF, Munich, Germany), 6 weeks old, were given subcutaneous injections of 4×10^6 cultured tumor cells suspended in 300 μl of sterile PBS. The cells were tested at the 20th and 30th passage. The mice were killed when the tumors were 5 to 10 mm in diameter. The tumors were fixed in formaldehyde, embedded in paraffin and stained with hematoxylin and eosin.

Staining for flow cytometer analysis. 10^5 cells were incubated for 20 min on ice in PBS and 2% (v/v) fetal calf serum (FCS) with 200 ng of primary antibodies (Abs). After washing with PBS, the cells were similarly incubated with secondary Ab (FITC- or PE-labeled F(ab)2 goat anti-mouse IgG). Propidium iodide (PI) was added before the cells were analyzed using a FACS Calibur flow cytometer and the CellQuest analysis program (BD, Germany). The Abs were purchased from Dianova (Germany), Pharmingen (Germany) and Immunotech (France). The anti-Ep-CAM and anti-Her2/neu Abs were kindly provided by Dr. Lindhofer (GSF, Munich, Germany).

Indirect immunofluorescence. SCCHN cells were cultivated on Lab-Tek chamber slides (Nalge Nunc, USA) fixed with 2% (v/v) paraformaldehyde for 20 min at 4˚C, permeabilized with 0.5% (v/v) Triton-X100 (Sigma) for 5 min at room temperature and stained with anti-CK19 Ab (Progen, Germany).

Telomerase-PCR-ELISA. Telomerase activity in the extracts of tumor cell lines was detected by a photometric enzyme immunoassay, according to the Telomeric Repeat Amplification Protocol (TRAP) from Boehringer Mannheim, Germany.

IFN-γ-treatment. 10^5 cells were incubated with 100 U/ml recombinant human interferon-γ (rhIFN-γ) (Boehringer) for 24 h and then stained for analysis by flow cytometry.

Stimulation of bulk PBMC with autologous tumor cells. A total of 2×10^7 peripheral blood mononuclear cells (PBMC) were subjected to autologous restimulation experiments. 10^6 PBMC per well were coincubated with 10^5 irradiated (50 Gy) autologous SCCHN-GHD cells in a 24-well plate (Nunc) with 2 ml of culture medium. Twenty U/ml hIL-2 were added after 48 h. The medium was not changed

for 10 days after culture initiation. Ten days after activation, the PBMC were harvested, enumerated and restimulated in the same way. After 5 rounds of restimulation, 104 irradiated (50Gy) allogenic PBMC from 3 different healthy volunteers, at a ratio of 1:1:1, were added (mixed lymphocyte reaction).

Limiting dilution assay. For cloning tumor-specific cytotoxic T-lymphocytes (CTL), PBMC were seeded after 6 rounds of restimulation on 10x96-well plates (Nunc) at densities of 1-10 cells/well. At 10-day intervals autologous tumor cells, allogenic feeder cells (PBMC), both irradiated at 50 Gy, and 10 U/ml rhIL-2 were added until most of the cultures senesced. Cultures with proliferating cells were further expanded in 48-well and 24-well plates and tested for cytotoxic activity.

Non-radioactive cytotoxicity assay. Tumor cells lysis by autologous immune cells was evaluated with a cytotoxicity assay based on time-resolved fluorometry (Wallac, Finland). In brief, 10^6 target cells per ml were loaded for 30 min at 37˚C with 2 μl of a fluorescence enhancing ligand (BATDA) which hydrolyses within the cells to a form no longer passing the membrane. After washing 5 times, 4000 of loaded target cells were plated per well in round-bottomed 96-well plates in duplicate. Effector cells were added at *E:T* ranges between 6:1 and 100:1. Internal assay controls for spontaneous release, maximum release and background were included. The plates were incubated for 2 h at 37˚C. After cytolysis of the target cells, the ligand was released. The plates were centrifuged for 5 min at 500 μg. Twenty μl of the supernatant from each well were transferred to a flat-bottomed plate and 200 μl of Europium (Eu)-solution per well were added. After incubation for 15 min at room temperature, Eu and the ligand form a highly fluorescent chelate. The fluorescence signals measured in a time-resolved fluorometer, Victor (Wallac), correlate directly with the amount of lysed cells.

Detection of cytokines. To determine the concentrations of cytokines present in the supernatants, commercially available ELISA kits for prostaglandin E_2 (PGE_2) and tumor growth factor-β (TGF-β) were used (Boehringer Mannheim). Tumor necrosis factor-α (TNF-α) protein was evaluated in a 4-h MTT assay (Boehringer) using the limiting effects of TNF-α on the survival of AW99 cells.

Apoptosis of SCCHN-GHD cells. 10^4 cells were treated for 3 days with 10 μg/ml of apoptosis-inducing anti-CD95 Ab, clone B-G30 (Diaclone Research, France). After incubation in 0.5 ml 0.1% w/v sodium citrate buffer, containing 50 μg/ml PI and 0.1% v/v Triton X-100 for 24 h, DNA degradation was monitored in the red fluorescence channel using a FACS Calibur cytometer.

Sensitivity to TNF-α-induced apoptosis was tested by treatment of 10^4 cells with 1 pg/ml to 10 ng/ml rhTNF-α (Sigma) at log10 dilutions. After a 2-day incubation, cell survival was measured by a commercially available MTT assay (Boehringer).

Results

Generation of primary cell lines from SCCHN. Forty tumors of SCCHN (T3-T4) were placed in culture. Disaggregation of the tumors resulted in rapid formation of adherent cell layers. Fifty-five percent of the tumor cultures were discarded because of contamination after
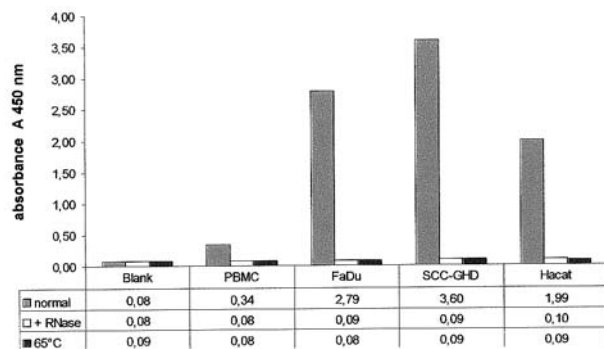
Figure 1. *Specific detection of telomerase activity. 2000 cells (shaded box) were subjected to Telomerase-PCR-ELISA. Controls included procedure without cells (blank), with peripheral mononuclear blood cells (PBMC) and with the epithelial cell lines FaDu and Hacat, RNase treatment (white box) and heating at 65°C (black box). Telomerase activity is expressed as absorbance units at 450 nm from duplicate wells.*

Figure 2. *Tumor formation of SCCHN-GHD cells in SCID mice. (A) Epithelial morphology of tumors in SCID mice 3 weeks after injection of SCCHN-GHD cells (HE, x 400). (B) Ep-CAM expression (thick line) on SCID-mice-passaged SCCHN-GHD cells, at 4 weeks of reculture, isotype control (thin line) (FACSCalibur, BD).*

culture initiation, despite intensive antibacterial and antifungal treatment. Some tumors also yielded small, round-shaped and non-adherent cells, which senesced after several weeks in culture. The proportion of tumors that still grew in culture as established lines varied with the tissue of origin: 6 out of 19 SCCHN of the larynx, 3 out of 9 SCCHN of the oral cavity, 4 out of 6 SCCHN of the oropharynx and 6 out of 6 SCCHN of the hypopharynx. In summary, 19 (45%) of the cultivated SCCHN persisted in subsequent passages.

Phenotypic characterization of the primary cell line SCCHN-GHD. The newly-established cell line SCCHN-GHD, derived from a patient with hypopharyngeal carcinoma, is described in more detail as it was used as the target cell line for the induction of an autologous immune response. In initial cultures, each cell passage was equivalent to 1 cell generation, increasing in proliferating cultures to 3 to 5 cell generations. Analysis of phenotypic marker expression on SCCHN-GHD cells (25th passage) by flow cytometry revealed the pattern described below. The epithelial origin of SCCHN-GHD cells was confirmed as they stained positive for Ep-CAM. The intensity of the Ep-CAM expression did not depend on the confluency of the cells in culture. Western blot analysis furthermore revealed the synthesis of different glycosylated forms of Ep-CAM molecules (11).

SCCHN-GHD cells stained positive for tumor antigen Her2/neu, interaction molecule MHC class I and adhesion molecule LFA-3 (CD58). They did not express MHC class II molecules, costimulatory molecules B7.1 (CD80) and B7.2 (CD86) or cell adhesion molecule ICAM-1. SCCHN-GHD did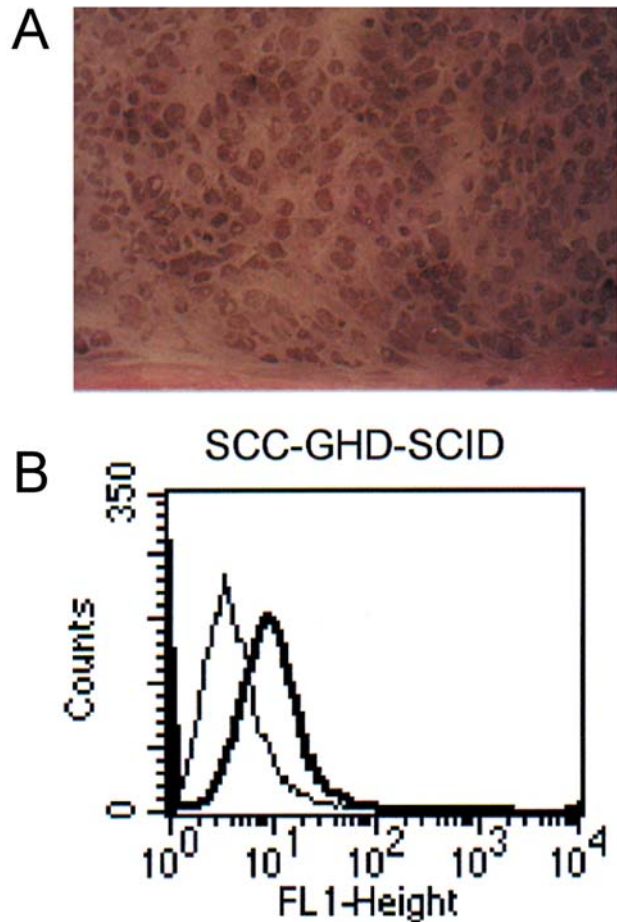 not express markers for T cells (CD3), B cells (CD23), granulocytes and monocytes (CD11b, CD14, CD16) or dendritic cells (CD33, CD83). Fibroblastic contamination of the stable SCCHN-GHD cultures was excluded, as the cells stained negative for the anti-fibroblastic marker AS02. The morphological pattern was constant during the whole subcultivation period.

Immortalization of the SCCHN-GHD cell line. Immortalization of cells is related to arresting telomere shortening by an increase of telomerase activity (3, 12). In a specific telomerase assay, the SCCHN-GHD cells were screened for telomerase activity. Controls included a procedure without cells (blank), with PBMC and with the epithelial cell lines FaDu and Hacat. The SSC-GHD cells achieved a high activity of telomerase, still seen in similiar epithelial tumor cell lines (Figure 1). For the control, in
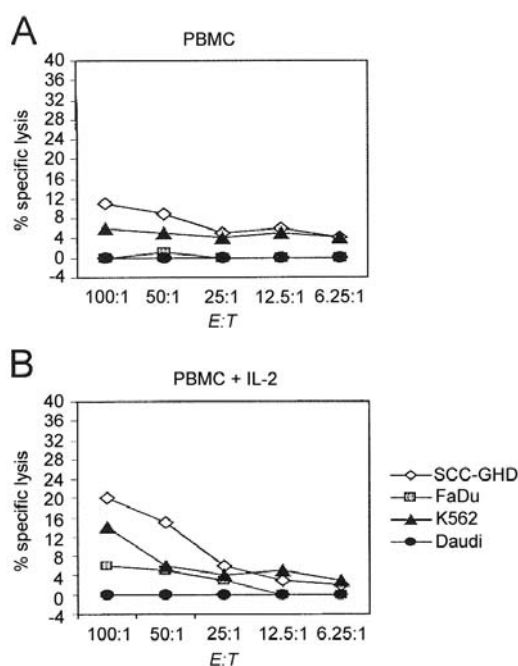
Figure 3. *Cytotoxicity of autologous PBMC against SCCHN-GHD tumor cells evaluated in a 2-h Europium-release assay. (A) Non-stimulated PBMC. (B) PBMC preincubated with rhIL-2. Lysis of autologous tumor cells was compared to lysis of the allogenic epithelial FaDu cell line. K562 and Daudi target cells were used for evaluation of MHC–restriction of the immune response. Internal assay controls revealed spontaneous release of 10 to 14% for all cell lines. One of 3 performed experiments is shown. The data represent mean % specific lysis of duplicate wells.*

normal peripheral blood cells only a basal level of telomerase activity was detectable. RNase treatment (white box) and heating at 65˚C (black box) eliminated the telomerase activity.

Formation of secondary tumors in SCID mice. Malignancy *in vivo* was tested by subcutaneous injection of SCCHN-GHD cells into SCID mice. Within 20 days, the SCCHN-GHD cells had produced tumors that were 10x20x25 mm in size, with fluid accumulation in the center and encapsulated by connective tissue. In sections of SCCHN-GHD-derived secondary tumors, histologically typical SCCHN with densely packed, cube-shaped cells were seen (Figure 2A). From each secondary tumor, Ep-CAM-positive cells grew out in culture (Figure 2B).

Production of immunomodulatory proteins. Supernatants of 3-day cultures of untreated SCCHN-GHD cells were monitored for soluble cytokines. Low amounts of PGE_2 and TGF-β in high concentrations were detected. The supernatants of the SCCHN-GHD cells did not induce

apoptosis of the TNF-α-sensitive cell line AW99, implicating that SCCHN-GHD cells do not produce soluble TNF-α.

Cytotoxicity of autologous immune cells against SCCHN-GHD target cells. For the exploration of an autologous immune response, the newly-generated SCCHN cell line was subjected to lysis by autologous bulk PBMC in a non-radioactive cytotoxicity assay.

The basal level of cytotoxicity of the unstimulated bulk PBMC against the newly-established autologous tumor cell line SCCHN-GHD was 5%. The cytotoxicity increased to 9% at an *E:T* ratio 50:1 and to 11% at an *E:T* ratio 100:1 (Figure 3A). Lysis of autologous tumor cells was twice as high as lysis of K562 cells, which was about 5% at all *E:T* ratios up to 100:1.

Preactivation of autologous PBMC with rhIL-2 increased the cytotoxicity against autologous SCCHN-GHD cells 2-fold (Figure 3B) compared to the cytotoxicity of unstimulated PBMC. In this experiment, the lysis of K562 cells was increased too, but was half as high as the lysis of autologous tumor cells. No cytotoxicity of unstimulated bulk PBMC against the allogenic epithelial cell line FaDu and against NK cell-resistant Daudi cells was achieved. IL-2 activation slightly increased only lysis of the allogenic FaDu cells. These data indicate that autologous bulk PBMC include a portion of MHC-restricted immune cells inducing destruction of the autologous SCCHN-GHD tumor cells.

Generation of autotumor reactive CTLs. Freshly-isolated bulk PBMC were restimulated weekly by autologous SCCHN-GHD cells. In Figure 4A, the feature of SCCHN-GHD tumor cells and autologous PBMC 6 days post-incubation is shown. Each restimulation cycle achieved T cells with a bean-shape typical for activation at day 3 to 5 after adding tumor cells (Figure 4B). After lysis of the autologous tumor cells, activated T cells arrested and became round. In order to obtain cytotoxic cell lines or clones with specificity against autologous tumor cells, weekly-restimulated bulk PBMC were subjected to limiting dilution. In 90% of the culture, cells senesced after 2 months of restimulation. In 58 cultures, proliferating cells were achieved and repeatedly tested for cytotoxicity against autologous tumor cells and K562 cells. Two months after limiting dilution, 8 cultures did not reveal any cytotoxicity against autologous tumor cells. In most of the cultures only a low level of cytotoxicity against autologous tumor cells was detected besides low to high unspecific cytotoxicity. Sixteen cultures revealed autotumor specific cytotoxicity greater than 10% and higher than unspecific cytotoxicity (data not shown). In 2 cultures, a high level of specific cytotoxicity against tumor cells and low unspecific lysis of K562 was detected, which will be expanded for further experiments.
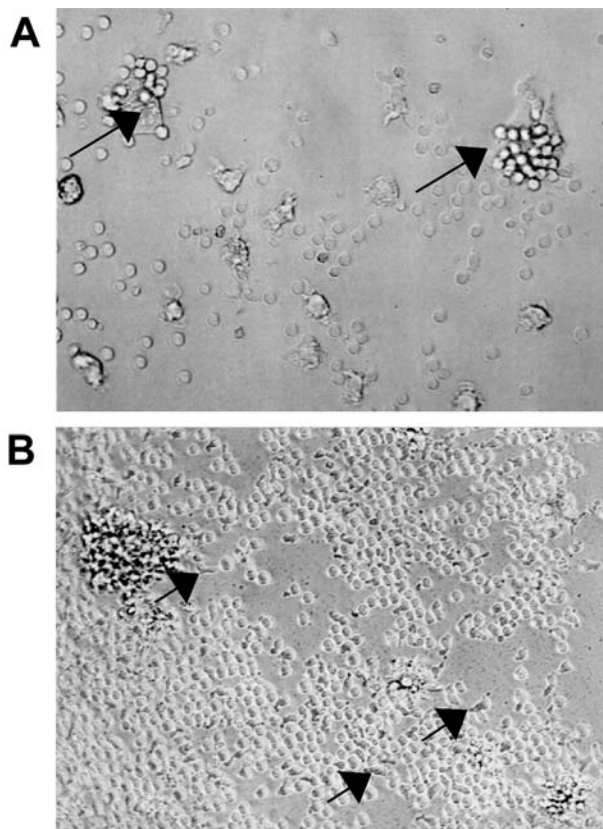
Figure 4. *Bulk PBMC after 6 days (A) (invert-microscopy, x 600) and after 2 months of restimulation by autologous SCCHN-GHD tumor cells (B) (invert-microscopy, x 320). Arrows indicate rosetting of tumor cells by autologous PBMC (A) and activated T cells (B).*

Discussion

Immunohistochemical and histopathological studies of different tumor types have provided useful information about the presence, distribution and frequency of lymphocyte subsets in human tumors. It has been shown that a complex network of interactions exists between the inflammatory cells and tumor cells within the tumor microenvironment. It is further known that efficient immune responses against tumor cells are strictly dependent on the mutual recognition of autologous cell interaction molecules on the surface of tumor and immune effector cells. Therefore, knowledge about tumor-specific antigens would provide opportunities for the development of specific immunotherapies, which are necessary especially for the treatment of solid tumors. In the field of SCCHN, conventional therapies have not improved the 5-year survival rate to date (1). A major drawback is that antigens so far identified on SCCHN are either not SCCHN-tumor-specific, being expressed on other healthy tissues, or the

expression is too heterogenous to serve as a target for all cancer cells. Disregarding the above-mentioned restrictions, some tumor antigens are currently under investigation in clinical settings. Simultaneously, further research on the identification of SCCHN-specific marker molecules is absolutely necessary.

A conventional experimental design for further characterization of tumor antigens is the analysis of the TCR-repertoire of cytotoxic lymphocytes that were generated against autologous tumor cells (10, 15). From this the sequence of antigenic determinants on tumor cells may be predicted.

The aim of our study was to generate a cytotoxic immune response against tumor cells of the head and neck in an autologous *in vitro* system with regard to further characterization of SCCHN-specific antigens. First, primary cell lines were established from solid tumors of the head and neck as a source for physiologically-active forms of SCCHN-specific antigens. Sixty-three percent of the SCCHN tested grew out, of which 45% could be further expanded. A poor outgrowth of SCCHN of 20% to 30% is usually described (6), which is mainly due to two factors: a) to microbiological contaminations as the tumors are derived from the upper aerodigestive tract; b) to the heterogenous phenotypes of SCCHN, making it difficult to define optimal conditions for maintaining SCCHN cultures. Further research on the specific growth requirements of SCCHN tumor cells in culture is indicated.

One newly-established cell line, SCCHN-GHD, was used as a tumor-antigen-presenting cell for autologous immune cells. The SCCHN-GHD cell line originated from epithelial tumor cells, as confirmed by expression of the epithelial marker molecules Ep-CAM and CK 19. The cells were malignant *in vivo*, as proved by tumor formation in SCID mice.

The immune effector cells, freshly isolated from the peripheral blood, mediated *in vitro* lysis of autologous SCCHN-GHD target cells which was twice as high as the lysis of non-MHC-restricted K562 target cells. Preactivation of the immune cells by IL-2 increased the cytotoxicity against the autologous tumor cells and K562 cells. The specific lysis of autologous tumor cells was twice as high as the lysis of K562 cells. Unstimulated PBMC did not reveal cytotoxicity against allogenic FaDu cells, an epithelial cell line from the head and neck, or against Daudi cells, which are resistant to NK cell-mediated lysis. These data implicated that the autologous PBMC assessed a portion of MCH-restricted immune cells with specificity for autologous tumor cells.

After repeated stimulation of PBMC by autologous tumor cells and after limiting dilution of PBMC, anti-SCCHN-GHD immune effector cell populations were generated, which revealed cytotoxicity against autologous tumor cells only. Increased expressions of IFN-γ, IL-2 and TNF-α after restimulation confirmed that the induction of an immune response was mediated by activated T cells.

Blocking of MHC class I molecules with anti-MHC antibodies prior to restimulation defined that the observed immune response in this newly-established autologous cell system was mediated by CD8$^+$ T cells.

In summary, the establishment of an autologous cell system, as presented here, is an essential step in considering the use of autologous immune effector cells for adoptive immunotherapy in patients with SCCHN and, overall, in the further characterization of tumor-specific antigens in head and neck cancer.

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