Abstract. Background: Cytokine profiles of permanent cell lines of head and neck squamous cell carcinoma (HNSCC) were analyzed to define the cytokine levels secreted in the absence of immune cells. Materials and Methods: Cytokine profiles of IL-4, IL-6, IL-8 and IL-10 were analyzed in the supernatants of 4 different permanent HNSCC cell lines using the Bio-Plex human cytokine assay system. Results: In HNSCC, IL-6 and IL-8 are involved in oncogenic processes, while IL-4 and IL-10 suppress proper immune responses in the tumor microenvironment. Our data indicate that, in the absence of tumor-infiltrating immune cells, HNSCC secretes high levels of the proto-oncogenic cytokines IL-6 and IL-8, but no significant levels of the immune suppressors IL-4 and IL-10. Conclusion: The data strongly suggest that the intercellular crosstalk between cells of HNSCC and tumor-infiltrating immune cells in vivo is required to stimulate an increased production of immune suppressive mediators in head and neck cancer.

Head and neck squamous cell carcinoma (HNSCC) is one of the most frequent cancers in the world but, over the last 40 years, standard treatment has only marginally improved the 5-year survival rate of patients with HNSCC. It is supposed that tumor production of various immune suppressive mediators contribute to the massively impaired immune functions in patients with head and neck cancer (1, 2).

Cytokines regulate manifold intercellular communication processes, whereas cells of HNSCC are known to develop molecular strategies to evade the growth inhibitory effects of cytokines present in the tumor microenvironment. Thus, the HNSCC malignant transformation process is strongly associated with an altered response to cytokine stimulation (3). In addition, alterations in immune, inflammatory as well as angiogenic responses within the HNSCC microenvironment play a critical role in tumor aggressiveness and its response to chemo- and radiation therapies, as well as its influence on cells of the immune system (1, 4).

The prominent cytokines identified in the HNSCC microenvironment are interleukin-4 (IL-4), IL-6, IL-8, IL-10, granulocyte macrophage-colony-stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), prostaglandin E2 (PGE2) as well as basic fibroblast growth factor (bFGF) (5-8). A better understanding of the cytokine regulatory routes in HNSCC is essential to increase the clinical perspective of patients with this tumor type. Cytokine analysis of permanent HNSCC cell lines allows investigation of the capacity of HNSCC-derived cytokine secretion in the absence of cells of the immune system.

In this study, the secretion levels of cytokines IL-4, IL-6, IL-8 and IL-10 were analyzed in the supernatants of different permanent HNSCC cell lines. Traditionally, IL-6 is known to stimulate the liver to produce acute phase proteins or to trigger the proliferation of B-lymphocytes. However, IL-6 was also shown to possess pro- as well as anti-inflammatory properties and thus dysregulations of IL-6 cytokine signaling often contribute to various kinds of cancer (9, 10). IL-4 and IL-10 are known to participate in the down-regulation of interferon-γ (IFN-γ) and Th1 immune responses (11). In HNSCC, IL-6 and IL-8 are involved in tumorigenic processes such as angiogenesis and metastasis, while IL-4 and IL-10 are known to drive immune suppressive functions within the tumor microenvironment.

Materials and Methods
Permanent HNSCC cell lines BHY (DSMZ Germany, (12)), ANT-1 (13), PCI-1 and PCI-13 (hypopharyngeal cancer, a gift of Dr. T. Whiteside, Pittsburgh, PA, USA) were cultured in DMEM-medium (Dublecco’s Modified Eagle Medium, Gibco) supplemented with 10% FCS, 1 mM glutamine and 0.1 mM sodium pyruvate. All the compounds were purchased endotoxin tested.

Cytokines were determined in at least two independent duplicate measurements using the Bio-Plex Cytokine Assay (Bio-Rad),
according to the instructions provided by the manufacturer. The Bio-Plex cytokine assay allows analysis of multiple cytokines in a small volume of cell culture supernatant over a broad dynamic range (from 0–32,000 pg/ml). The principle of this assay is similar to a capture sandwich immunoassay, but it uses spectrally addressed polystyrene beads coated with the corresponding antibodies. The cytokine assay was analyzed by a specialized microplate reader system (Bio-Plex Array Reader; Bio-Rad), and the revealed data were calculated using the Bio-Plex Manager software.

Results
The permanent HNSCC cell lines BHY, ANT-1, PCI-1 and PCI-13 were cultured for 48 hours at 37°C to a confluence of about 70-80%. After 48 hours of cell growth, the supernatants were collected and the concentrations of cytokines IL-4, IL-6, IL-8 and IL-10 were determined using the Bio-Plex Cytokine Assay (BioRad).

Our data revealed that, among the analyzed cytokines, IL-6 and IL-8 were secreted in huge amounts by all investigated HNSCC cell lines, although cell line-specific alterations in IL-6 and IL-8 secretion levels were observed. High secretion levels of IL-6 of about 3,750 pg/ml were detected in BHY supernatants and lower, but still significant, levels of IL-6 were detected in the ANT-1 (1,490 pg/ml), PCI-1 (270 pg/ml) and PCI-13 (620 pg/ml) cell lines (Figure 1). Determination of IL-8 in these supernatants revealed similar cytokine levels of approximately 1,000 pg/ml (ANT-1), 820 pg/ml (BHY) and 760 pg/ml (PCI-13). Lower IL-8 levels were detected in the supernatants of cell line PCI-1 with an average of about 200 pg/ml (Figure 1).

In contrast to these data, the cytokine IL-4 was found to be secreted only at very low levels, in a range of 1-4 pg/ml, in all the analyzed cell lines, as illustrated in Figure 2. Surprisingly, no significant levels of IL-10 could be detected in the analyzed HNSCC supernatants (Figure 2). In summary, cytokines IL-6 and IL-8 were found to be secreted at markedly higher levels compared to the detected amounts of cytokines IL-4 and IL-10 in the investigated permanent HNSCC cell lines.

Discussion
This study suggests that, in the absence of tumor-infiltrating immune cells, permanent cell lines of HNSCC secrete high levels of tumor-promoting cytokines IL-6 and IL-8, whereas only low spontaneous levels of the immune suppressive cytokines IL-4 and IL-10 could be detected. Deviations in cytokine profiles found in different HNSCC cell lines are
most probably due to specific kinetics (half-life), metabolism, or binding protein modulation parameters. These cell line-specific alterations determine individual tumor characteristics, such as aggressiveness, tumor progression and immunosuppression (1, 2, 4).

Recent data indicate a partial Th2 cytokine bias in HNSCC patients and a more aberrant expression of cytokine expression in the plasma of patients with more advanced disease. These patients reveal increased levels of the Th2 cytokines IL-4, IL-6 and IL-10 and diminished levels of the Th1 cytokines such as IFN-γ (14).

Recently, it has been suggested that those individuals who are genetically-predisposed to produce high levels of IL-6 reveal a reduced capacity to reach the extreme limits of human life, whereas individuals producing high levels of IL-10 are significantly increased among centenarians (15). In fact, the human immune system has evolved to control pathogens and, thus, individuals with genetically-predisposed decreased levels of IL-6 or increased levels of IL-10 might better control inflammatory responses and cancer development (15). Recent data suggest that IL-6 expression induces a down-regulation of the CD80 costimulatory molecule in HNSCC and, thus, participates in an impaired activation of T lymphocytes (10). Furthermore, cytokine IL-6 acts as a major mediator of inflammation and activator of STAT3 (signal transducer and activator of transcription 3) and, thus, inhibits apoptotic processes in cells during the inflammatory process. Unfortunately, these mechanisms serve also to maintain cells progressing towards neoplastic growth, protecting them from cellular apoptotic deletion and chemotherapeutic drugs (16).

Angiogenesis has been linked to increased metastasis formation and decreased survival of patients with HNSCC (17, 18). In HNSCC, angiogenesis is significantly triggered by VEGF as well as IL-8 (19, 20).

Since human solid tumor tissues are known to be infiltrated by various kinds of immune cells, it is necessary to distinguish between directly tumor-derived cytokines and cytokines produced by tumor-triggered immune cells, respectively (21-23). Stimulation of primary HNSCC cultures with exogenous IL-1 resulted in significantly increased levels of various cytokines such as IL-4, IL-6 and GM-CSF, which strongly suggests that the cells of HNSCC secrete cytokines not only to inhibit, but to trigger, local immune cells such as dendritic cells. IL-1 seems to play a crucial role in the regulation of cytokine production by tumor and resident tissue cells (5). Our results strongly support these data that an intercellular crosstalk between the cells of HNSCC and infiltrating immune cells is responsible for the origin of a complete immune suppressive HNSCC microenvironment. The understanding of these molecular networks may be helpful in developing novel therapeutic strategies, which might enable an immunomodulatory intervention in HNSCC in the future.

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**Figure 2.** Expression levels of cytokines IL-4 and IL-10 in supernatants of HNSCC cell lines BHY, ANT-1, PCI-1 and PCI-13. The data are the means of at least two independent duplicate determinations of each HNSCC supernatant.
References


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