Increased cytokine secretion in head and neck cancer upon p38 mitogen-activated protein kinase activation

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Abstract. Head and neck squamous cell carcinoma (HNSCC) is one of the most frequently diagnosed cancers. It is believed that tumor production of various immune suppressive mediators contributes to massively impaired immune functions, but the underlying signal transduction pathways are mostly unknown. Phosphorylation levels of MAP (mitogen-activated protein) kinase p38 were analyzed in permanent cell lines as well as in solid tumor tissue of HNSCC using flow cytometry and SDS-PAGE. Cytokine secretion was determined using the Cytometric Bead Array Flex Set system. MAP kinase p38 was shown to be activated in HNSCC by phorbol 12-myristate 13-acetate. Activation of p38 led to decreased cell proliferation and increased secretion of cytokines IL-6 and IL-8 in HNSCC. Our data provide novel insights into the origin of the HNSCC microenvironment. A better understanding of these molecular mechanisms in HNSCC is essential for novel drug development and improvement of the clinical perspective of this tumor type.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is one of the most frequently diagnosed cancers in the world, and over the last 40 years standard treatment has only marginally improved the 5-year survival rate of patients with this tumor type. Cells of head and neck cancer are known to develop molecular strategies to escape efficient immune responses. It is supposed that tumor production of various immune suppressive mediators contributes to massively affected immune functions, but the molecular mechanisms responsible for these malignant transformation processes are mostly unknown (1,2).

Mitogen-activated protein (MAP) kinase p38 has been demonstrated to be overexpressed in human gastric cancer and has been suggested to play an important role in tumorigenesis and the regulation of various transcriptional activators such as activated protein 1 (AP-1) and nuclear factor κB (NF-κB) (3). The regulation and function of p38 MAP kinase have been shown to be stimuli- as well as cell type-specific and are mostly still unknown in head and neck cancer (4-6).

MAP kinase p38 was first isolated as a 38-kDa protein which was phosphorylated in response to stimulation with lipopolysaccharide (7,8). A vast variety of extracellular stimuli such as UV light, inflammatory cytokine tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), or growth factors such as colony stimulating factor-1 (CSF-1) trigger the activation of p38. In HNSCC, various cytokines such IL-6 and IL-8 are involved in oncogenic processes such as angiogenesis and metastasis. It is thought that the intercellular crosstalk between cells of HNSCC and tumor-infiltrating immune cells *in vivo* is responsible for an increased production of immune-suppressive mediators in head and neck cancer (9). Thus, the HNSCC malignant transformation process is strongly associated with an altered response to cytokine stimulation (2,9-14).

We demonstrated in this study that MAP kinase p38 is involved in the secretion of cytokines IL-6 and IL-8 in HNSCC. Activation of MAP kinase p38 by phorbol 12-myristate 13-acetate (PMA) resulted in decreased cell growth of permanent HNSCC cell lines and led to an increased secretion of HNSCC-relevant cytokines IL-6 and IL-8.

In summary, our data suggest that MAP kinase p38 participates in the development of the immunosuppressive and tumor-promoting microenvironment as an important parameter of the HNSCC immune escape.

Materials and methods

Cell culture and preparation of supernatants. Permanent HNSCC cell lines BHY (DSMZ, Germany) (15) and PCI-13 (hypopharyngeal cancer, Pittsburgh Cancer Institute) were cultured in DMEM (Dulbecco's modified Eagle's medium; Gibco, CT, USA) supplemented with 10% FCS (fetal calf serum), 1 mM glutamine and 0.1 mM sodium pyruvate. Phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, Seelze, Germany) was added to a final concentration of 200 ng/ml to stimulate p38 activation in permanent cell lines of HNSCC. To inhibit p38 activation, the pyridinyl imidazole inhibitor SB203580 (10 μM) (Jena Bioscience; Jena, Germany) was added 24 h before cell stimulation with PMA.

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Cell-free supernatants were collected by centrifugation and filtration after 24 h of cell cultivation.

Flow cytometry. Cells were stained with phycoerythrin (PE)-conjugated anti-phospho-p38 MAPK antibodies (BD Biosciences, San Jose, CA, USA) to analyze the phosphorylation of p38. For intracellular staining, cells were permeabilized with saponin buffer phosphate-buffered saline, (PBS: 0.1% saponin, 1% FCS, and 1 M HEPES). Samples were analyzed on a FACSCanto (BD Biosciences, Heidelberg, Germany), and data acquisition was performed using the FACS Diva software (BD Biosciences). The vital dye propidium iodide (PI) was used in conjunction with Annexin V-FITC staining to identify dead cells (Annexin-V-FITC positive, PI positive) and cells which were in an early apoptotic stage (Annexin-V-FITC positive, PI negative), respectively.

Protein analysis. Cell extracts were prepared and solubilized, and protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad GmbH, Munich, Germany) with bovine serum albumin (BSA) as a standard. Aliquots of protein extracts (60 μg) were run on 10% SDS-polyacrylamide gel electrophoresis and were then transferred to a nitrocellulose membrane. Blots were decorated with specific antibodies (Biomol, Hamburg, Germany) as specified in the figure legends.

Cytokine analysis. Cytokines were determined using the Cytometric Bead Array Flex Set system (CBA; BD Biosciences, Heidelberg, Germany) according to the instructions provided by the manufacturer. The CBA Human Flex Set is a bead-based immunoassay capable of analyzing multiple cytokines in a small volume of cell culture supernatant using spectrally addressed polystyrene beads coated with the corresponding antibodies. Similar to a sandwich ELISA, cytokines were bound to specific beads, and a PE-coated detection antibody was bound to the cytokines. The cytokine assay was performed using the FACSCanto and the data analyzed by FCAP Array Software (BD Biosciences).

MTT assay. For the quantitative determination of cellular proliferation, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich, Munich, Germany) was used, which is based on the cleavage of the yellow tetrazolium salt MTT into purple formazan by metabolically active cells. The solubilized formazan product can be photometrically quantitated using an ELISA reader. An increase in the number of living cells results in an increase in total metabolic activity which leads to a stronger color formation.

Results

Expression of p38 in HNSCC. Protein expression of phosphorylated as well as unphosphorylated human MAP kinase p38 was analyzed in cell lines and solid tumors of head and neck squamous cell carcinoma (HNSCC). We analyzed different permanent HNSCC cell lines as well as numerous solid tumors (n=15) some of which are shown as representative samples in Fig. 1. All samples were found to express p38 MAPK, whereas activated p38 could only be detected in different levels in solid tumors but not in permanent HNSCC cell lines (Fig. 1). These data suggest that activation of p38 in HNSCC occurs in response to the manifold stimuli within the tumor environment such as immune cell infiltration (Fig. 1).

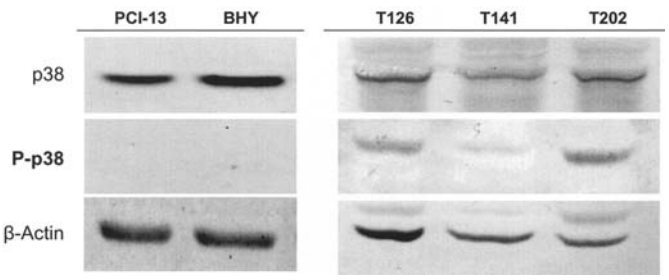

Figure 1. Expression levels of phosphorylated and unphosphorylated p38 in HNSCC. Levels of p38 and phosphorylated p38 (P-p38) were determined in permanent HNSCC cell lines PCI-13 and BHY as well as in various solid tumors as illustrated by three representative examples (T126, T141, T202). Expression of ß-Actin was used as a loading control.

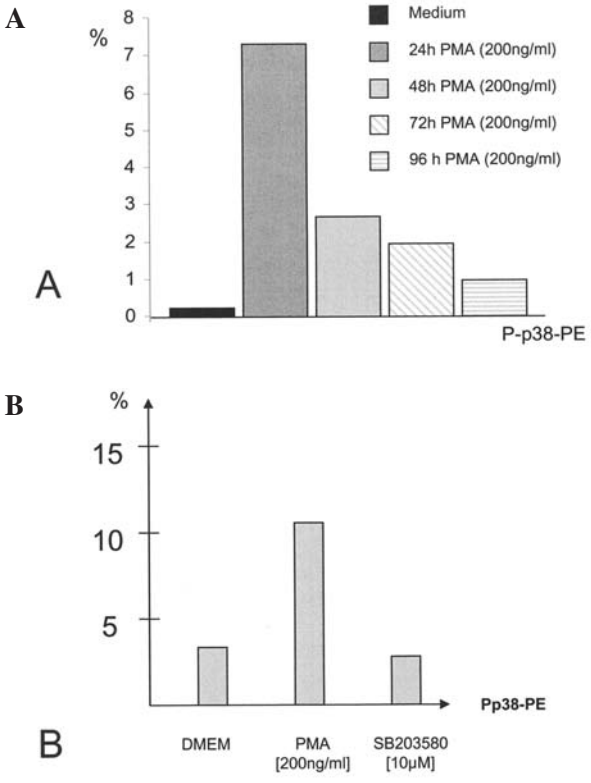

Figure 2. Activation of p38 by PMA. (A) Permanent cell lines of HNSCC were stimulated with 200 ng/ml PMA for 24, 48, 72 or 96 h, respectively. Activation of p38 was subsequently investigated by flow cytometric analyses of phosphorylated p38. Significant activation of p38 was determined within 24 h after PMA stimulation. With no additional stimulation, decreased levels of p38 phosphorylation were observed after 48, 72, or 96 h, respectively. (B) Incubation with the p38-specific inhibitor SB203580 (10 μM) for 24 h led to a decreased p38 MAPK activity.

PMA-mediated activation of p38 MAP kinase. To analyze the role of p38 in HNSCC we induced the phosphorylation of

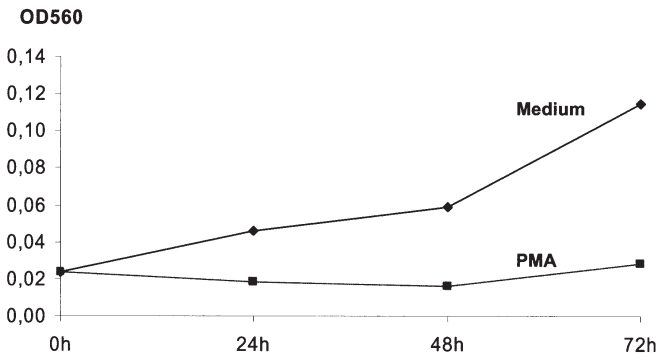

Figure 3. Decreased cell proliferation in response to PMA stimulation. Permanent cell lines of HNSCC were cultured in the absence and presence of PMA, and cell growth was analyzed for 72 h. The illustrated growth curves are the means of at least three independent measurements and indicate drastically decreased cell proliferation in response to PMA.

p38 in permanent HNSCC cell lines using phorbol 12-myristate 13-acetate (PMA) as described previously (16,17). Flow cytometric analyses revealed an increased phosphorylation of p38 in response to stimulation with 200 ng/ml PMA for 24 h. With no additional stimulation, decreased levels of p38 phosphorylation were observed after 48 h of incubation (Fig. 2A). Incubation with the specific p38 inhibitor SB203580 for 24 h resulted in inhibition of p38 activity as suspected (Fig. 2B)

Decreased cell proliferation in response to p38 activation. The influence of PMA on cell proliferation was investigated using MTT assay analyses. Therefore, cells of HNSCC were cultured in the absence and presence of PMA, and cell growth was analyzed for 72 h. Our data demonstrated a drastically decreased proliferation of cells of HNSCC in response to PMA stimulation (Fig. 3).

To analyze the condition of PMA-stimulated cells, we used the vital dye propidium iodide (PI) in conjunction with Annexin V-FITC staining to identify dead cells and cells which were in an early apoptotic stage (Annexin-V-FITC positive, PI negative), respectively.

In apoptotic cells, loss of the plasma membrane is one of the earliest features resulting in a translocation of the membrane phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the membrane, thereby exposing PS to the extracellular environment (18). Annexin V is a 36-kDa phospholipid-binding protein that has a high affinity for PS, and thus represents a sensitive tool for flow cytometric analysis of cells that are undergoing apoptosis (19,20).

Flow cytometric analyses of permanent HNSCC cell lines revealed no increase in apoptosis or cell death in response to PMA even after 48 h of incubation (Fig. 4). These data indicate that PMA has an inhibitory effect on HNSCC cell growth with no effect on cell vitality, and therefore suggest an activating function in other regulatory routes.

Increased cytokine secretion upon p38 activation. IL-6 and IL-8 are prominent cytokines in HNSCC and are known to be involved in oncogenic processes such as anti-apoptosis, angiogenesis and metastasis (21). Therefore we analyzed the influence of PMA-mediated activation of p38 on IL-6 and IL-8 secretion in HNSCC. Permanent cell lines of HNSCC

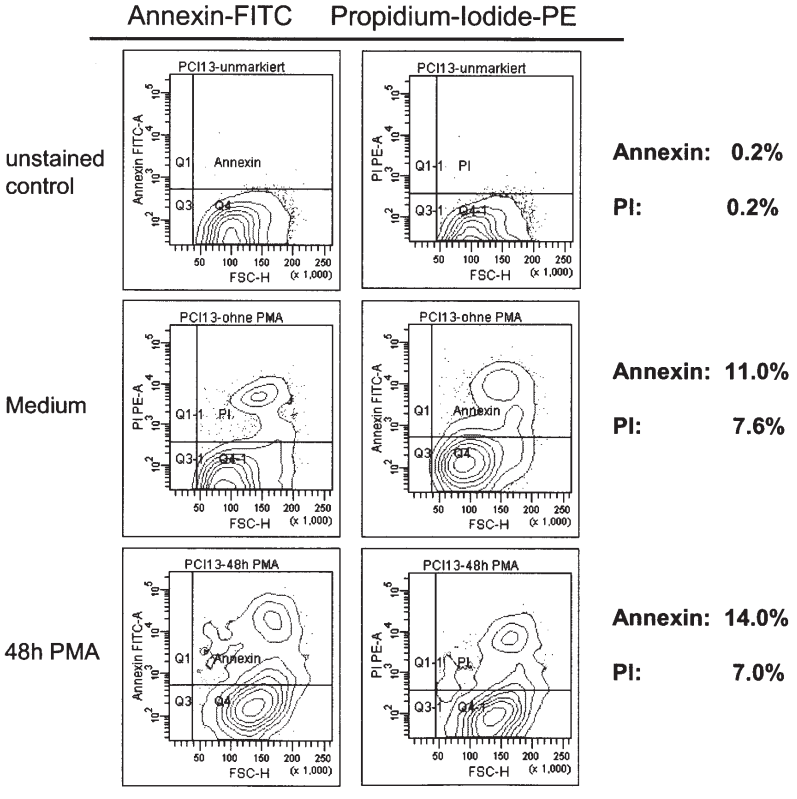

Figure 4. Vitality of HNSCC in response to PMA. The vital dye propidium iodide (PI) was used in conjunction with Annexin V-FITC staining to identify dead cells or cells which were in an early apoptotic stage (Annexin-V-FITC positive, PI negative), respectively. Flow cytometric analyses revealed no increase in apoptosis or cell death in response to PMA.

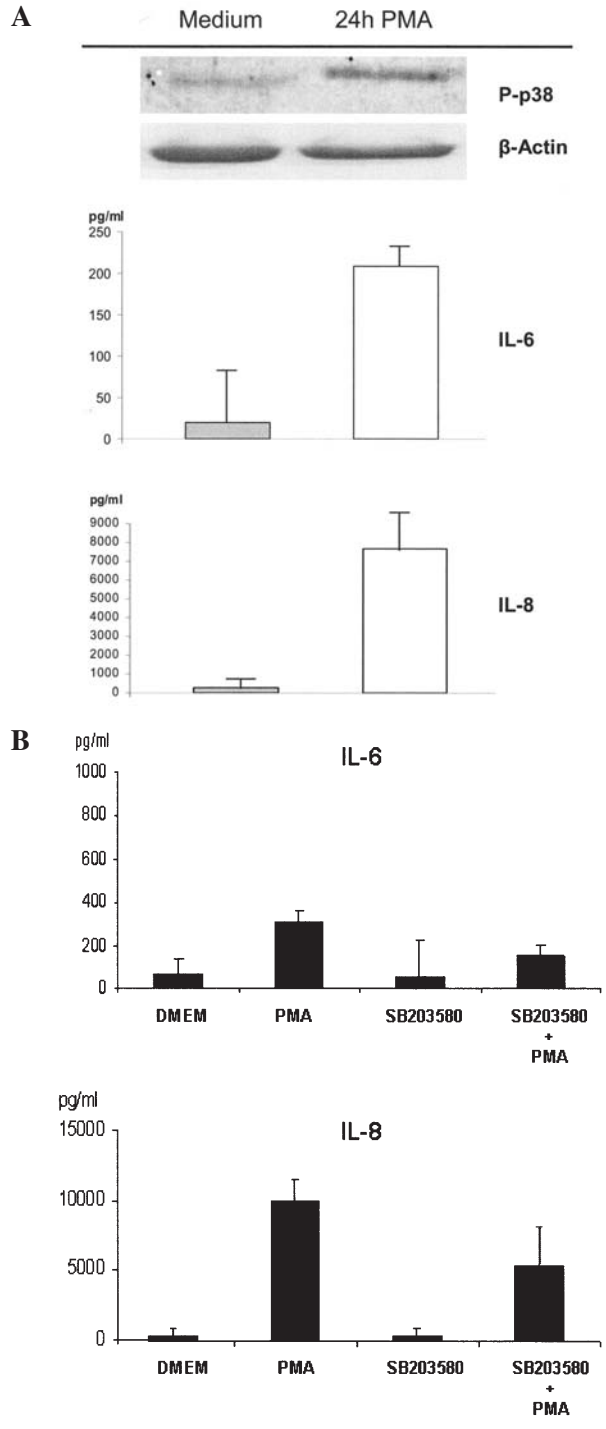

Figure 5. Secretion of cytokines IL-6 and IL-8 in response to PMA and the specific p38 inhibitor SB203580. HNSCC cell lines were stimulated with 200 ng/ml PMA in the presence (10 μM) and absence of SB203580 for 24 h. Culture supernatants were subjected to cytokine analyses. (A) Strongly increased secretion levels of IL-6 and IL-8 were found in response to PMA stimulation, whereas the increased phosphorylation status of p38 MAPK in response to PMA is indicated by Western blot analyses. (B) Inhibition of p38 MAPK using the specific inhibitor SB203580 strongly diminished the secretion of cytokines IL-6 and IL-8 despite stimulation with PMA.

were stimulated for 24 h with 200 ng/ml PMA and the specific inhibitor SB203580 of p38 MAPK (10 μM), respectively. Culture supernatants were subjected to cytokine analyses. Our data demonstrated strongly increased secretion levels of cytokines IL-6 and IL-8 in response to 24 h of PMA stimulation (Fig. 5). Our data clearly showed that incubation with the specific p38 inhibitor SB203580 led to a strongly decreased expression of IL-6 and IL-8 despite PMA stimulation (Fig. 5B). These data indicate the role of p38 MAPK in the regulation of PMA-induced expression of IL-6 and IL-8 in HNSCC.

Discussion

Alterations in immune, inflammatory as well as angiogenetic 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 the immune system. Therefore, a better understanding of the secretion and regulation pathways of immune suppressive and proangiogenic cytokines in HNSCC is essential to increase the clinical perspective of this tumor type as well as to develop immunomodulatory strategies against HNSCC.

Previous data suggest a partial Th2 cytokine bias in HNSCC patients and a more aberrant expression of cytokine expression in the plasma of patients with a more advanced disease. These patients reveal increased levels of cytokines IL-4, IL-6 and IL-10 which are able to promote antibody responses in general (22). It has been suggested that those individuals who are genetically predisposed to produce high levels of IL-6 display a reduced capacity to reach the extreme limits of human life, whereas individuals producing high levels of IL-10 are significantly increased among centenarians (23). 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 (23).

Angiogenesis has been linked to increased metastasis formation and decreased survival of patients with HNSCC (24,25). In HNSCC, angiogenesis is significantly triggered by vascular endothelial factor (VEGF) as well as IL-8 (26,27).

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 (28-30). Stimulation of primary HNSCC cultures with exogenous IL-1 resulted in significantly increased levels of various cytokines such as IL-4, IL-6 and granulocyte macrophage colony stimulating factor (GM-CSF) which strongly suggests that cells of HNSCC secrete cytokines not only to inhibit but to trigger local immune cells such as dendritic cells for its own purpose (11). Our results show a strong expression of activated p38 MAP kinase in solid HNSCC tumors compared to permanent cell lines and show increased secretion levels of IL-6 and IL-8 after activation of p38. Interleukin-6 is a multifunctional regulator of immune response and hematopoiesis. It has been recently reported that expression of IL-6 correlates with prognosis in various cancer patients. Interleukin-6 directly influences proliferation and invasion potential of head and neck cancer cells. These results suggest a direct influence of IL-6 on cell proliferation and its invasion potential as the first step of tumor metastasis (31). Expression of the MAPK extracellular signal-regulated kinase (ERK1/2) is associated with NF-κB-

and AP-1-related IL-8 and VEGF secretion in HNSCC (26,27). Our data showed a strong influence of p38 MAPK on cytokine-regulating pathways in HNSCC. Interleukin-1 is known to be constitutively expressed in HNSCC and participates in NF-κB- and AP-1-mediated IL-8 secretion in HNSCC (32). In human vascular smooth muscle cells this AP-1-induced IL-8 expression was dependent on p38 activation (33). Further investigations will have to be carried out to elucidate the role and contribution of p38 MAPK in the development of the immunosuppressive HNSCC micro-environment.

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