Abstract. Inhibition of angiogenesis by blocking angiogenic cytokines or their pathways has become a major target in experimental cancer therapies. This therapeutic approach requires a profound knowledge of growth factor profiles that contribute to tumor growth and progression. The respective knowledge is presently rather incomplete for head and neck squamous cell carcinomas (HNSCC). Therefore we studied the serum levels and expression of platelet-derived growth factor (PDGF) in HNSCC patients and in cell culture as well as the effect of a PDGF-receptor (PDGF-R) inhibition by Imatinib (Gleevec, STI571) on the secretion and expression activity of PDGF and vascular endothelial growth factor (VEGF) by postulating there is a correlation between the PDGF and VEGF networks. PDGF levels in patients with HNSCC, PDGF and VEGF secretion by HNSCC cells, were measured by ELISA, expression of PDGF and VEGF by RT-PCR. We found significantly increased PDGF levels in HNSCC patients' sera as well as in HNSCC cell lines. Treatment of the cell lines with Imatinib, a partially selective PDGF-R inhibitor, resulted in reduced secretion of PDGF and VEGF. This inhibiting effect was also reflected on the expression level of VEGF. In conclusion, the present study confirms the crucial role of PDGF in HNSCC growth and strongly suggests a correlation between the PDGF/PDGF-R and VEGF/VEGF-R pathway networks in HNSCC. Although further studies must be performed for a more complete understanding of this interaction, a targeting therapy for the inhibition of PDGF-R tyrosine phosphorylation by Imatinib may be a promising strategy for future tumor therapy by autocrine and paracrine inhibition of tumor growth and angiogenesis, presumably through simultaneous down-regulation of PDGF and VEGF.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is an aggressive epithelial malignancy known to be the most common neoplasm arising in the upper aerodigestive tract. Of all human cancers, HNSCC is the most distressing since the head and neck are sites of the most complex functional anatomy in the human body. In 2000, HNSCC was ranked as the eighth leading cause of cancer death worldwide. Approximately 481,100 new cases developed, and 320,000 persons succumbed to this disease (1). The overall five-year relative survival rate from HNSCC is generally less than 50% (2). Improved techniques in surgery, radiation and chemotherapy have increased the local control of HNSCC, but the poor five-year survival rate has remained unchanged for more than three decades (2). Chemotherapy is generally employed in advanced cases but the response rates average only 25-40% with overall survival being measured in months (3). Thus, it is imperative that new treatment modalities are developed to increase the long-term survival of patients with HNSCC.

Angiogenesis, the process leading to the formation of new blood vessels, plays a key role in the survival of cancer cells, in local tumor growth and in the development of distant metastasis (4). The intensity of angiogenesis has been shown to be increased in various human tumors, including HNSCC (5). The induction of tumor vascularization is regulated by a variety of angiogenic peptides released from tumor cells, macrophages and the extracellular matrix (6). These factors fulfill various functions, including endothelial cell migration, proliferation and capillary tubule formation (7). Many angiogenic factors have been identified including vascular endothelial growth factor (VEGF) (8). VEGF is a potent endothelial mitogen that activates the angiogenic switch in vivo and enhances vascular permeability (9). VEGF has been shown to increase tumor growth and angiogenesis in vivo in a nude mouse model (10). Enhanced expression of VEGF has been detected in a large variety of malignant human tumors including HNSCC (11-13). We previously demonstrated extended VEGF serum concentration in HNSCC patients compared with healthy controls (14). VEGF expression in HNSCC tumors strongly correlated with angiogenesis (15) and was inverseley correlated with apoptosis (16).

The platelet-derived growth factor (PDGF) is a potent mitogen for many different cell types. PDGF plays a central
role in regulating cell proliferation, chemotaxis, and survival in normal cells as well as in various disease states such as cancer, atherosclerosis, and fibrotic disease (17). PDGF is composed of A, B, C, and D polypeptide chains that form the homodimers PDGF-AA, BB, CC, and DD and the heterodimer PDGF-AB. Its biological activities are linked to two tyrosine homodimers PDGF-AA, BB, CC, and DD and the heterodimer composed of A, B, C, and D polypeptide chains that form the in normal cells as well as in various disease states such as prostate, ovarian, and non-small cell lung cancer (reviewed in ref. 17). Previous studies have demonstrated that PDGFs and their receptors are not only involved in human cancers through a paracrine but also an autocrine stimulation of tumor cell growth (19). In addition to its directly mitogenic effect on tumor cells, PDGF also nourishes the tumor proliferation by stimulating the angiogenetic process directly and indirectly (20,21). PDGF enhances migration and proliferation of endothelial cells, smooth muscle cells and pericytes (19,22).

The recognised importance of tumor-induced angiogenesis has stimulated the development of agents that are able to interfere with the molecules involved in this process (4). In recent years, different compounds that inhibit PDGF-receptor kinases have been developed. Imatinib (Gleevec, also known as STI571; Novartis Pharma, Basel, Switzerland) is a protein-tyrosine kinase inhibitor of the 2-phenylamino-pyrimidine class that was developed initially for its selectivity against the BCR-ABL fusion protein present in nearly all patients with chronic myeloid leukemia (23). In addition, it was found that Imatinib also inhibits the kinase activity of the PDGF-R α-[and -(ß)] and c-Kit-receptor (24). Imatinib is in clinical use for the treatment of BCR-ABL positive chronic myeloid leukaemia and c-Kit-positive gastrointestinal stromal tumors. In an animal model for dermatofibrosarcoma protuberans, Imatinib was shown to inhibit tumor growth by blocking PDGF-R and induction of apoptosis (25).

To date, the reports on the activity of PDGF in HNSCC as well as the effect of a targeted therapy directed at inhibiting PDGF-R activity in HNSCC are rare.

In the present study, we sought a) to incipiently certify the overexpression of PDGF in HSNCC and b) to determine the effects of an inhibition of the PDGF-R on the expression and secretion of PDGF in head and neck cancer cell lines and whether this affected the expression and secretion of VEGF in vitro by hypothesising there is crosslinking between the PDGF/PDGF-R and VEGF/VEGF-R pathway systems (shown in Fig. 2).

Materials and methods

Characterization of PDGF in patients with primary head and neck squamous cell carcinoma and in head and neck cancer cells. For an introductive analysis of serum levels of PDGF (-AB), we investigated the sera from patients of the Department of Otorhinolaryngology, Head and Neck Surgery, University Hospital Mannheim with histologically new diagnosed head and neck squamous cell carcinomas (n=88, 14 females, 74 males, mean age 53) as well as from healthy normal controls (n=46). The peripheral venous blood samples were processed 24 h after collection. The serum was separated from the cellular components by centrifugation at 1000 g for 15 min and aliquoted. The sera samples were collected in sterile cryotubes and stored at -80˚C until usage. Then, the PDGF concentrations in the sera were determined by using the quantitative immunoassay technique of ELISA as described below. Furthermore, we performed RT-PCR for PDGF-A/-B to confirm the expression of PDGF in HNSCC using 4 different UM-SCC cell lines which were well-described human HNSCC cell lines (RT-PCR and cell cultures performed as described below). All studies were approved by the Ethics Committee of the Faculty of Medicine Mannheim, University of Heidelberg, Germany. Informed consent was obtained from all participants.

Cell culture. The 4 different UM-SCC cell lines were well-described human HNSCC cell lines obtained from T. Carey (The University of Michigan, Ann Arbor, MI, USA). Cell cultures were carried out in Falcon petri dishes at 37˚C in a 5% CO2 fully humidified atmosphere using Dulbecco’s modified minimum essential medium (DMEM)(Fisher Scientific Co., Pittsburgh, PA, USA) supplemented with 10% fetal calf serum (FCS) and antibiotics [Life Technologies, Inc. (Gibco BRL), Gaintshurs, MD, USA], Imatinib (Gleevec) was kindly provided by Novartis Pharma, Basel, Switzerland. It was stored at room temperature and diluted in sterile water as necessary at the time of use. The HNSCC cells were incubated with different concentrations of lnatinib (0.1; 0.2; 0.4; 0.6; 0.8; 1.0 μmol/l) for 24 and 72 h, then the supernatants were collected in sterile test tubes and stored at -20˚C until usage. Cells were harvested for PCR analysis at -80˚C.

ELISA for VEGF/PDGF (-AB). VEGF concentrations were determined by ELISA technique (R&D Systems, Wiesbaden, Germany). The system used a solid-phase monoclonal antibody and an enzyme-linked polyclonal antibody raised against recombinant VEGF165. The specificity of anti-human VEGF antibodies used in the ELISA kit were examined by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting (14,15). According to the manufacturer’s directions, each ELISA assay measured 100 μl of supernatant. All analyses and calibrations were carried out in duplicate. The calibrations on each microtiter plate included recombinant human VEGF standards provided in the kit. Optical density was determined using a micro-plate reader at a wavelength of 450 nm. Wavelength correction was set to 540 nm and concentrations were reported as pg/ml. The coefficient of variation of inter-assay determinations reported by the manufacturer varied from 6.2% to 8.8% when VEGF concentrations ranged between 50 and 1000 pg/ml. The PDGF (-AB) -ELISA technique (R&D Systems, Wiesbaden, Germany) was conducted in the same manner as that for VEGF-ELISA. The solid-phase monoclonal antibody was in that case a monoclonal antibody specific for PDGF-BB, the enzyme-linked polyclonal antibody was specific for PDGF-AA.

RT-PCR. A multiplex approach was used for the determination of levels of the gene transcription of VEGF. The RT-PCR step was conducted with a commercial Multiplex-PCR Kit
RT-MPCR provides a method to detect multiple gene expression by amplifying all the genes under the same conditions, the kit used detects the expression of human GAPDH, VEGF189, 165, 121 and VEGF receptors flt-1 and flk-1 genes. Reverse transcription and first strand synthesis was performed using 1-2 μg of mRNA and 50 μM Oligo (dT) as recommended by the manufacturer. The RT process was performed by incubating the RT mixture at 37˚C for 60 min and terminated by heating RT mixture at 95˚C for 10 min and then chilling on ice. PCR was then conducted with Taq DNA polymerase using 5 μl cDNA derived from the first strand synthesis plus 5 μl of hVEGF1G MPCR primers, the cDNA amplification was performed with 32 cycles of 94, 58-60˚C. The resulting DNA was run on a 2% agarose gel and stained with ethidium bromide, and the image was captured with a Gel-Doc (Bio-Rad) imager. The image was analyzed, and samples were normalized to the expression of GAPDH. The product yields fragments 750 (GAPDH), 554 (flt-1), 408 (flk-1), 306 (VEGF189), 234 (VEGF165) and 104 (VEGF121) bp long. For the RT-PCR of PDGF-A, mRNA expression was found to be significantly higher in the HNSCC patient group than those in the control group (mean HNSCC group 5945.28 pg/ml; mean control group 1708.52 pg/ml; p=<0.001; shown in Fig. 3). On the contrary, we could not find any significant correlation to the clinicopathological stage of the tumor (tumor localization, histological grading, T-stage and lymph node status, staging). Serum levels of PDGF (-AB) were expressed as a mean ±SD. All data were subjected to the Kruskal-Wallis test (Chi-square approximation). A p-value ≤0.05 was considered statistically significant.

**Results**

Characterization of PDGF in patients with primary head and neck squamous cell carcinoma and in head and neck cancer cells. Initially we investigated the serum levels of circulating PDGF (-AB) in HNSCC patients and the correlation with the clinicopathological status of the tumor (tumor localization, histological grading, T-stage and lymph node status, staging) by using the quantitative immunoassay technique of ELISA. The serum levels of PDGF (-AB) were found to be significantly higher in the HNSCC patient group than those in the control group (mean HNSCC group 5945.28 pg/ml; mean control group 1708.52 pg/ml; p=<0.001; shown in Fig. 3). On the contrary, we could not find any significant correlation to the clinicopathological stage of the tumor (tumor localization p=0.4038, histological grading p=0.1064, lymph node status p=0.4770, staging p=0.7663; data not shown). RT-PCR for PDGF-A mRNA exhibited PDGF-A expression in all 4 HNSCC cell lines (Fig. 4). Among the head and neck cancer cell lines, a relatively intense signal of the band was noted in UM-SCC 14C cells. This cell line was chosen for further study. RT-PCR for PDGF-B mRNA showed no expression of PDGF-B in all HNSCC cell lines (data not shown).

Effect of Imatinib on HNSCC cell lines. To determine the effect of Imatinib on UM-SCC 14C cells, we added different concentrations of Imatinib (0.1-1.0 μmol/l). To quantitate PDGF as well as VEGF secretion to the supernatant of HNSCC cell lines treated with medium (control) or medium containing Imatinib, ELISA was performed after 48 and 72 h.
PDGF levels were significantly decreased by the Imatinib treatment at all concentrations with a maximal reduction at 0.6 μmol/l for 24 h (66.533 pg/ml; 24 h negative control 145.072 pg/ml) and at 0.2 μmol/l for 72 h (328.405 pg/ml; 72 h negative control 568.461 pg/ml). These results are shown in Fig. 5.

VEGF levels were also significantly decreased by the Imatinib treatment at all concentrations with a maximal reduction at 0.6 μmol/l for 24 h (966.612 pg/ml; 24 h negative control 2068.00 pg/ml) and at 0.4 μmol/l for 72 h (5103.42 pg/ml; 72 h negative control 8691.63 pg/ml). These results are shown in Fig. 6. Endogenous PDGF-A/PDGF-B as well as VEGF expression in UM-SCC 14C cells treated with medium (control) or medium containing Imatinib for 24 and 72 h was measured by RT-PCR. The expression of PDGF-A was stable and not influenced by the Imatinib treatment after 24 and 72 h, the expression of PDGF-B remained absent at all times. On the contrary, we observed a reduction of VEGF expression after 24 h as well as after 72 h. In agreement with the results of secretion measurements, we noted a maximal decrease of expression at an Imatinib concentration of 0.6 μmol/l for 24 h and at 0.2/0.4 μmol/l for 72 h. Representative results are shown in Fig. 7.

**Discussion**

The inhibition of angiogenesis of malignant tumors has become an important strategy to be considered in novel approaches to cancer therapy. It is widely accepted that an increase in the tumor cell population must be preceded by an increase in microvessels supplying the neoplasm (4,6). It was demonstrated that the supernatant from HNSCC cell lines induced an angiogenic response in vivo (26). Dray et al demonstrated a strong correlation between high microvessel counts and recurrent or metastatic disease in HNSCC. Their data also suggested that microvessel counts were associated not only with an increased incidence of early recurrence or
metastasis, but also with more aggressive forms of recurrent or metastatic disease (27).

One of the major angiogenic stimulators is the VEGF, a directly acting endothelial cell mitogen. A significant body of evidence is accumulating that favours the notion that VEGF plays an important role in the development of solid tumors, such as head and neck cancer (9). VEGF expression has been detected in a large number of malignant human tumors including HNSCC (11-13).

PDGF is another crucial growth factor for tumor cell growth, angiogenesis and survival of the tumor (17). PDGF was shown to be a potent angiogenetic factor in vitro and in vivo (20,21,28,29). As VEGF and PDGF are recognised contributors to the angiogenetic process and as recent studies hinted at an interaction between these mitogens (30-33), we suggested that these factors may be co-regulated and this prompted us to investigate whether there is a correlation between the PDGF/PDGF-R axis and the activity of VEGF in HNSCC cells.

As reports on the expression of PDGF in human malignancies are uncommon, we carried out a preliminary analysis on the state of PDGF in patients with HNSCC and in HNSCC cell lines. Our results demonstrated elevated serum levels of PDGF (-AB) in the majority of patients with primary head and neck cancer compared to healthy controls, although there was no correlation with the clinicopathological stage of the

Figure 5. PDGF (-AB) concentrations in supernatant of UMSCC cells after 24 h and 72 h incubation with different concentrations of Imatinib (0.1-1.0 μmol/l; ELISA).

Figure 6. VEGF concentrations in supernatant of UMSCC cells after 24 h and 72 h incubation with different concentrations of Imatinib (0.1-1.0 μmol/l; ELISA).

Figure 7. RT-PCR after 24 and 72 h of incubation with different concentrations of Imatinib (0.1-1.0 μmol/l). Expression of PDGF-A was not influenced by Imatinib; Expression of PDGF-B remained absent (data not shown). Expression of VEGF was reduced with a maximal decrease contrary to the maximal decrease of secretion at 0.6 μmol/l for 24 h and at 0.2/0.4 μmol/l for 72 h.
tumor, suggesting there is no interrelation between the role of PDGF and the tumor measurements. Our findings are in line with previous studies that reported on elevated PDGF-AB serum levels in patients suffering from HNSCC as well as the expression of PDGF-AB protein in native tumor tissue and its maintained secretion in the derived HNSCC cultures (34-36). Likewise, other investigations found no correlation between PDGF expression levels in tumor derived cells and clinicopathological factors (36,37).

The elevation of PDGF in HNSCC is also represented on mRNA level, where we showed PDGF-A expression in all 4 HNSCC cell lines. In contrast, we did not observe an expression of PDGF-B in any of the investigated UM-SCC cell lines. Chang et al found that the expression of PDGF was much higher and more frequent in head and neck tumor tissue compared to paired normal tissue (38). Li et al reported on up-regulated expression of PDGF in oral squamous cell carcinomas (39). However, neither study specified between the PDGF-A or -B chain. Aeberson et al described the expression of PDGF-B in oropharyngeal cancers and proved by immunoreactivity that the presence of PDGF-B was confined to the tumor cells themselves, whereas PDGF-A was often expressed to a marked degree by vascular endothelial cells in the stroma (37). However, Worden et al described in accordance with our findings, an expression of PDGF-A in HNSCC tissue (40). Collectively, these observations and our findings support the notion that HNSCC cells provide complex expression patterns of PDGF. The evaluation of the expression of PDGF chains and their respective receptors in different human malignancies provided distinct findings for expression patterns in various tumor entities (reviewed in ref. 17). However, the knowledge of the expression patterns of PDGF chains and their respective receptors in HNSCC remains incomplete.

Imatinib (Gleevec) is a protein-tyrosine kinase inhibitor that was also found to inhibit the kinase activity of the PDGF-R [-α and -β](24), and it was shown that Imatinib inhibits tumor growth by blocking PDGF-R and the induction of apoptosis (25). In this present study, we hypothesized that Imatinib may inhibit HNSCC cell growth, angiogenesis and survival of the tumor via the inhibition of an PDGF/PDGF-R autocrine growth pathway as well as inhibition of a VEGF paracrine loop. Our obtained results showed that an inhibition of PDGF-R in HNSCC by Imatinib leads to a reduced secretion of PDGF (-AB) protein. These results also suggest that HNSCC cells exhibit an autocrine loop in the pathway of the PDGF/PDGF-R system. However, these results could not be shown on the mRNA level, whereas, the inhibition of PDGF-R in HNSCC cells caused the reduction of secretion as well as the expression of VEGF. To our knowledge, this is the first study demonstrating these effects in HNSCC cell lines. Comparable results were recently published for other tumor entities, it was reported that a PDGF-R inhibition by Imatinib inhibited the growth of neuroblastoma and leads to the inhibition of VEGF expression (41). In PDGF-R expressing immortalized ovarian cancer cells, PDGF potently induced VEGF secretion, while Imatinib reduced PDGF stimulated VEGF production to basal state (42), another study demonstrated decreased PDGF-R and VEGF expression after the use of Imatinib in non-small cell lung cancer (NSCLC) xenografts (43).

In summary, the present study confirmed the crucial role of PDGF in HNSCC growth and strongly suggests a correlation between the PDGF/PDGF-R and VEGF/VEGF-R pathway networks in HNSCC. Although further studies must be undertaken for a more complete understanding of this interaction, a targeting therapy for the inhibition of PDGF-R tyrosine phosphorylation by Imatinib may be a promising strategy for future tumor therapy by autocrine and paracrine inhibition of tumor growth and angiogenesis, presumably through simultaneous PDGF and VEGF down-regulation.

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