Abstract. While the effects of single growth factors on endothelial cells (ECs) have been extensively studied, the importance of induction of growth factors such as PDGF-BB (platelet derived growth factor) in ECs and its impact on tumor cell functions are only partly understood. Human umbilical vein endothelial cells (HUVECs) were cultured under serum-free conditions and stimulated by 20 ng/ml VEGF (vascular endothelial growth factor) or 20 ng/ml bFGF (basic fibroblastic growth factor). As determined by real-time PCR, both VEGF and bFGF induced a significant (up to 4-fold) increase in PDGF-B RNA expression which was time- and dose-dependent (p<0.05). Similarly, conditioned medium (CM) from lung cancer cells (A549) which is known to contain multiple growth factors including VEGF and bFGF also induced PDGF-B RNA expression. Using ELISA assays, VEGF and bFGF significantly increased PDGF-BB protein secretion in HUVECs (p<0.01). By addition of BIBF 1000, a novel inhibitor of the VEGF and bFGF receptor kinases, the effect of VEGF on PDGF-B RNA induction was significantly antagonized (p<0.01). Furthermore, we studied the biological significance of EC-derived PDGF-BB on lung cancer cells. Interestingly, HUVEC-derived CM significantly stimulated migration of A549 cells (p<0.001) with a trend to further increased migration with the use of VEGF-stimulated (PDGF-BB rich) CM (p=0.2). Collectively, endothelial and lung cancer cells seem to interact via various paracrine pathways, e.g. by the reciprocal induction of VEGF and PDGF-BB. Thus, targeting key molecules would result in expression alterations of multiple factors and alter the biological functions of both stromal and tumor cells.

Introduction

The essential role of angiogenesis, the formation of new blood vessels from the preexisting vasculature, for the growth of solid tumors including non-small cell lung cancer (NSCLC) has been well documented (1). However, the induction of angiogenesis is a complex process that comprises a series of interlinked steps and is tightly regulated by a variety of angiogenic and anti-angiogenic factors (2). Furthermore, the new sprouts formed by endothelial cells (ECs) are highly susceptible to various apoptotic stimuli such as acidosis and hypoxia. With the attachment of perivascular cells such as pericytes and vascular smooth muscle cells (VSMCs), the ECs become less susceptible to apoptotic stimuli and more independent from the presence of survival factors (2,3). The stabilization effect of perivascular cells on ECs is believed to be mediated by direct cell-cell adhesions or in a paracrine way by secretion of various cytokines (3,4).

To date, vascular endothelial growth factor (VEGF) is still considered as one of the most potent angiogenic and EC survival factors, binding to two tyrosine kinase receptors (VEGFR-1 and VEGFR-2), which are found predominantly on the surface of vascular ECs (5). Various growth factors such as insulin-like growth factor-I (IGF-I) and PDGF-BB are able to induce VEGF in several cell types including cancer cells (6-8). By VEGF induction, the angiogenic process may be initiated. In addition, the platelet-derived growth factor (PDGF) has gained considerable interest in recent years. PDGF-BB, which seems to be the predominant isoform in most cell types including VSMCs, ECs and lung cancer cells (9,10), is a growth factor for various cell types including tumor cells and modulates angiogenesis through several effects including induction of VEGF, induction of VSMC migration, and regulating the blood flow secondary to constriction of VSMCs (11,12). The importance of PDGF-BB for migration of pericytes and VSMCs has been demonstrated in vivo where mice lacking PDGF-B also lack microvascular pericytes in various tissues with abnormal capillaries and formation of

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microaneurysms (13). As a result from these abnormalities, hemorrhages and edema occur when blood pressure increases.

While PDGF-BB is accepted as one important factor involved in tumor and stromal development, its regulating factors remain largely unknown. Only few data have been published regarding the induction of PDGF-BB by cytokines such as TGFβ-1 and interleukin-2 in various cell lines (14). For example, transforming growth factor beta-1 (TGFβ-1) was capable of inducing PDGF-B mRNA expression in colon cancer cells dependent on the differentiation grade of the cells (15). In contrast, TGFβ-1 induced PDGF-B mRNA levels in both normal human pancreas and in pancreatic adenocarcinoma cell lines (16). Recently, we demonstrated that treatment of pericytes and VSMCs with PDGF-BB led to VEGF induction, which in turn enhanced EC survival in a paracrine manner (3). In addition to the effect of PDGF on VEGF induction, we hypothesized that, in turn, VEGF may also have an effect on PDGF-BB expression.

In the present study, we provide novel evidence that VEGF and lung cancer-derived conditioned medium (CM) induce PDGF-BB in ECs in a time- and dose-dependent manner, which could be blocked by inhibiting the VEGFR. In addition, EC-derived CM increased migration of A549 lung cancer cells which could be enhanced by the pre-stimulation of ECs with VEGF.

Materials and methods

Materials. Recombinant human VEGF-A, basic FGF and PDGF-BB were purchased from R&D Systems, Inc. (R&D Systems GmbH, Wiesbaden, Germany). The novel indolone derivative BIBF 1000, a small molecule tyrosine kinase inhibitor, was provided by Boehringer-Ingelheim (Boehringer Ingelheim Pharma, Ingelheim, Germany). Previous studies have demonstrated that BIBF 1000 effectively inhibits the VEGF receptors 1 through 3, PDGFRα and β and FGF receptors 1 and 3 at a dose of 1 μM (17).

Cell lines and culture conditions. A549 human lung cancer and human umbilical vein endothelial cells (HUVECs) were purchased from American Type Culture Collection (Manassas, VA) and were maintained in 10% and 15% FCS containing MEM, respectively (3,7). Cell media and substitutes were purchased from PAA Laboratories GmbH (Cölbe, Germany). All in vitro experiments were performed at 80-90% confluence unless described otherwise. Cells were used at passages 3-8 after their receipt from the supplier.

VEGFR expression. Total RNA was isolated from subconfluent HUVECs and A549 cells growing in standard medium using RNeasy (Qiagen, Hilden, Germany). From 1 μg of RNA, cDNA was synthesized for 1 h at 37°C, using random hexamers and Moloney murine leukemia virus reverse transcriptase (Promega, Heidelberg, Germany). The resulting cDNA was subjected to PCR with a thermal cycler (94°C for 1 min, 57°C for 1 min, and 72°C for 2 min for 28 cycles, followed by 41 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min) using the following primers: VEGFR-1: sense 5’-TGG GACAGTAGAAAAGGGCTT-3’, antisense 5’-GGTCCACTCTTACACGACAA-3’. VEGFR-2: sense 5’-CATCACATCCGACAGTAGAAAGGGCTT-3’, antisense 5’-GGTCCACTCTTACACGACAA-3’. VEGFR-3: sense 5’-ATGGACCGTCAAGGCTGAGA-3’, antisense 5’-GGCATGACTGTTGTCATGAG-3’.

Semi-automated analysis of PDGF-B gene expression by real-time quantitative reverse transcription PCR. After RNA extraction, cDNA was synthesized as described. PDGF-B RNA quantification was quantified using the HT7900 sequence detection system (TaqMan, Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The PCR reaction mixture contained 600 nM of each primer and 200 nM probe in a final volume of 22.5 μl. PCR conditions were at 50°C for 10 sec, 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The following primers were used: Sense 5’-CTGG AGAGCGTTGCTCTTG-3’, antisense 5’-CGCATCATGCG CGGC-3’, 6-FAM-labeled 5’-AGAAGGAGCCTGGGTGCCT CGTACCA-3’. As an internal control, 18S expression was determined for every sample (Sigma-Aldrich Chemie GmbH, Munich, Germany). Relative gene expression levels were calculated using standard curves generated by serial dilutions of a cDNA mixture. Expression levels for each sample were divided by the 18S expression level. Two independent analyses were performed for each sample and for each gene.

PDGF-B RNA induction by VEGF and bFGF. HUVECs were grown to 80% confluence in standard medium as described above, and medium was changed to 10% FCS-containing medium overnight. Cells were then incubated for various time-points in 0.5% FCS containing MEM (0.5% MEM) with 20 ng/ml VEGF and/or 20 ng/ml bFGF. In a different experiment, 80% confluent HUVECs were incubated for 24 h in 0.5% MEM, and then 20 ng/ml VEGF was added for incubation for various time-points. To determine the dose-response, we incubated HUVECs in 10% MEM overnight and then in 0.5% MEM containing increasing doses of VEGF for 24 h. Total RNA was extracted, and the PDGF-B mRNA expression was analyzed using real-time PCR.

Effect of BIBF 1000 on VEGF-induced PDGF-B RNA expression. After incubation of HUVECs in 10% MEM overnight, the medium was changed to 0.5% MEM with 1 μM BIBF 1000 or diluent. After 1 h, 20 ng/ml VEGF was added, and cells were incubated for 24 h. After RNA extraction, PDGF-B expression was determined using real-time-PCR.

Effect of BIBF 1000 on VEGF signaling. Seventy-percent confluent HUVECs were incubated in 10% MEM overnight. Subsequently, medium was changed to 0.5% FCS containing medium with/without 1 μM BIBF 1000 or diluent. After 1 h, 20 ng/ml VEGF was added for an additional 15 min. Cells were rinsed in PBS and protein was extracted from cell lysates by standard techniques. Western blot analysis was performed as described (7) using the polyclonal rabbit anti-phospho-p44/p42 MAPK antibody (Cell Signaling Technology, Beverly, MA) and the anti-B-actin antibody (Sigma).

Induction of PDGF-BB protein by VEGF and bFGF. HUVECs were cultured in standard medium up to 80-90% confluence. Then, medium was changed to 0.5% MEM containing 50 ng/ml VEGF and/or 50 ng/ml bFGF, and cells were incubated for
56 h. After confirmation of cell viability >95%, the medium was collected, centrifuged, filtered through a 0.22-μm membrane and stored at -20°C (3). PDGF-BB protein expression was analyzed using ELISA assays (Quantikine, R&D Systems) following the instructions provided by the manufacturer (3).

**Induction of PDGF-B RNA expression by lung cancer-derived conditioned medium.** CM from two human lung cancer cell lines (A549 and HTB-56) and HUVECs were prepared as described (3), and stored at -20˚C until use. Subsequently, subconfluent HUVECs were incubated in 10% FCS-containing medium overnight and then in lung cancer-derived CM for 4 h and 24 h. RNA was extracted, and PDGF-B expression was determined by real-time PCR.

**Effect of HUVEC-derived CM on A549 monolayer cell growth.** Subconfluent HUVECs were cultured in 2% FCS containing MEM with/without 20 ng/ml VEGF. After 48 h, the CM was collected. Human A549 lung cancer cells (4000 cells/well) were plated in 96-well plates. Following cell attachment, medium was changed to 2% MEM or HUVEC-derived CM. After 48 h, 3-[4,5-Dimethyl-thiazol-2-yl]2,5 diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) was added to a final concentration of 0.5 mg/ml, and cells were incubated for 90 min. Medium and MTT were removed, dimethyl sulfoxide was added for 1 min, and absorption was read at 570 nm.

**Migration of A549 cells treated with HUVEC-derived CM in vitro.** Uncoated migration chambers (Becton Dickinson GmbH, Heidelberg, Germany) were seeded with 40,000 A549 cells in 2% FCS-containing medium. The bottom wells were filled with HUVEC-derived CM or 2% MEM supplemented with or without 20 ng/ml PDGF-BB. After 6 h, the assay was stopped, non-migrated cells were removed, and migrated cells were fixed and stained using 0.2% crystal-violet (Merck, Darmstadt, Germany). Migrated cells were counted in five distinct areas at 100-fold magnification (18).

**Statistical analysis.** Experiments were performed at least in triplicate. All results are expressed as mean ± standard error (SE). Data were analyzed using two-sided Student’s t-test. Values of p<0.05 were considered statistically significant.

**Results**

**Time- and dose-dependent effects of VEGF and bFGF on PDGF-B expression.** Subconfluent HUVECs were treated with VEGF in 0.5% FCS containing medium for various times. Control cells were harvested at each time point to exclude distortions from changes in culture conditions or cell confluence. VEGF induced PDGF-B RNA expression after 4 h and, significantly, after 24 h of incubation (p=0.08 and p=0.03, respectively, Fig. 1A). In a different approach, HUVECs were more thoroughly deprived of the effect of FCS by culturing them in 0.5% MEM overnight and then in 0.5% MEM with 20 ng/ml bFGF and/or VEGF for 24 h. After extraction of total RNA, the PDGF-B expression was determined. There was a significant increase in PDGF-B expression with the addition of bFGF (p<0.05; bars, mean ± SE). However, there was no significant additive effect of both VEGF and bFGF additions.

**Effect of BIBF 1000 on VEGF-mediated PDGF-B induction.** BIBF 1000 was used as an inhibitor of VEGF receptors in this context.
study. Pre-incubation of HUVECs with BIBF 1000 abrogated the effect of VEGF on PDGF-B RNA expression (p<0.01, Fig. 3A). Cell viability was not obviously impaired during incubation with BIBF 1000. To confirm the blocking effect of BIBF 1000 on VEGF signaling, Western blot analysis was performed. While VEGF induced Erk1/2 phosphorylation in HUVECs, addition of BIBF 1000 blocked this effect (Fig. 3B).

**Effect of lung cancer-derived conditioned medium on PDGF-B RNA induction.** Several lung cancer cell lines are known to produce VEGF including A549 cells which were used in the following experiment (19). HUVECs were incubated with CM derived from A549 lung cancer cells which led to a significant increase in PDGF-B RNA expression (Fig. 4).

**Effect of HUVEC-derived conditioned medium on A549 lung cancer cells in vitro.** To further study the biological significance of the previous findings, HUVECs were incubated with or without VEGF for 48 h to induce PDGF-BB secretion in these cells. Subsequently, A549 lung cancer cells were seeded into migration chambers. The bottom wells were filled with HUVEC-derived CM or 2% MEM supplemented with or without 20 ng/ml PDGF-BB. After 6 h, treatment with VEGF-stimulated CM resulted in a significant higher migration rate of A549 compared to the effect observed with normal unstimulated CM (*p<0.05; **p=0.2; bars, mean ± SE).

**Figure 2.** Induction of PDGF-BB protein by VEGF. Eighty percent confluent HUVECs were cultured for 56 h in 0.5% MEM containing 50 ng/ml VEGF and/or 50 ng/ml bFGF. After confirmation of cell viability >95%, the medium was collected and analyzed using ELISA assays. Secretion of PDGF-BB protein was significantly stimulated by the addition of either VEGF or bFGF to the medium (*p<0.01 versus time-point 0; bars, mean ± SE).

**Figure 3.** Blockade of VEGF-mediated PDGF-B induction by BIBF 1000. A, after incubation of HUVECs in 10% MEM overnight, the medium was changed to 0.5% MEM with 1 μM BIBF 1000 or diluent. After 1 h, 20 ng/ml VEGF was added, and cells were incubated for 24 h. Addition of BIBF 1000 significantly inhibited PDGF-B RNA induction by VEGF (*p<0.01; bars, mean ± SE). B, 60-70% confluent HUVECs were incubated with 1 μM BIBF 1000 or diluent. After 1 h, VEGF was added for 15 min. Protein was extracted and Western blot analysis performed. Activation of the MAP kinase pathway was markedly impaired with the addition of BIBF 1000.

**Figure 4.** Induction of PDGF-B by lung cancer-derived conditioned medium. Conditioned medium (CM) was collected after culturing A549 lung cancer cells in 0.5% MEM overnight and then for 4 and 24 h in 0.5% MEM or CM. Lung cancer-derived CM induced the expression of PDGF-B RNA dependent on the incubation time, which was significant after incubation for 24 h (*p<0.05; bars, mean ± SE).

**Figure 5.** Stimulation of A549 lung cancer cell migration by HUVEC-derived conditioned medium. Conditioned medium (CM) from HUVECs was collected after incubation with or without VEGF for 48 h to induce PDGF-BB secretion in these cells. Subsequently, A549 lung cancer cells were seeded into migration chambers. The bottom wells were filled with HUVEC-derived CM or 2% MEM supplemented with or without 20 ng/ml PDGF-BB. After 6 h, treatment with VEGF-stimulated CM resulted in a significant higher migration rate of A549 compared to the effect observed with normal unstimulated CM (*p<0.05; **p=0.2; bars, mean ± SE).
Discussion

In the present study, we provide evidence for an intense paracrine signaling between ECs, VSMCs and tumor cells. In particular, we explored factors that regulate the expression of PDGF-BB. With the exception of a few studies that have demonstrated the induction of PDGF-BB by various inflammatory cytokines such as TGFβ-1 and IL-2 (14,16), the regulation of PDGF-BB remains to be explored. Earlier, we demonstrated that VEGF is induced by tumor-cell derived PDGF-BB in various cell types effecting EC apoptosis (3). Hence, we postulated an opposite effect of VEGF on PDGF-BB induction in ECs in forms of a mutual induction loop. In HUVECs, VEGF caused a time- and dose-dependent increase in PDGF-B RNA and PDGF-BB protein expression. In addition, we also investigated the effect of bFGF on PDGF-BB induction. Comparable to the effect of VEGF, bFGF also induced PDGF-B gene and protein expression. However, there was no additive effect when HUVECs were stimulated with both VEGF and bFGF. Collectively, both VEGF and bFGF seem to contribute to PDGF-BB expression in ECs. Interestingly, similar results were detected with incubation of HUVECs in high concentrations of serum that is known to contain several growth factors including bFGF and VEGF (20,21). These data are congruent to a previous study showing an increase in PDGF-A mRNA by serum due to activation of transcription (22). However, even small variations of growth factor concentrations may be of importance. Abramsson and associates provided evidence that not just the presence of PDGF-BB but rather an extracellular gradient adjacent to these growth factors in the lung cancer cell-derived CM, the presence of antagonistic factors may be a possible explanation for these findings, as well. However, as a result of PDGF-BB inductions of PDGF-B RNA expression, however, the overall induction of PDGF-B was less than with either VEGF or bFGF alone as previously demonstrated. Besides a lower concentration of these growth factors in the lung cancer cell-derived CM, the presence of antagonistic factors may be a possible explanation for these findings, as well. However, as a result of PDGF-BB induction by lung cancer cell-derived growth factors, ECs may exert further effects to stromal cells such as stimulation of migration of perivascular cells (25). These data support the current hypothesis of a modulating effect of cancer cells on ECs. In fact, the effect of tumor cell-derived CM on ECs has been extensively studied largely demonstrating that malignant cells produce a host of growth factors that induce EC growth, inhibit EC apoptosis, and favour vascular permeability (26-28). Further, several groups defined the effect of cancer cell-derived VEGF on expression changes of endogenous VEGF and VEGFRs in EC monoculture (27). Collectively, tumor cells modulate ECs' functions by secretion of various growth factors thereby orchestrating angiogenesis.

Additionally, ECs may conversely also modulate A549 lung cancer functions, e.g. by induction of PDGF-BB. To test this hypothesis, we cultured lung cancer cells in HUVEC-derived CM. While there was no significant effect on lung cancer cell growth, HUVEC-derived CM significantly stimulated migration of A549 lung cancer cells. When HUVECs were pre-stimulated with VEGF, this stimulated CM further increased lung cancer cell migration. As demonstrated using ELISA assays, VEGF-stimulated CM contained larger amounts of PDGF-BB. A direct effect of VEGF on A549 cells could be excluded by demonstrating the lack of VEGF expression on these cells. However, the difference of stimulated CM versus unstimulated CM was not significant, presumably because of the already marked effect of the unstimulated CM. Also, additional factors are likely to be involved. These novel data further support the hypothesis of a mutual interaction of cancer cells and ECs.

While the effect of ECs on cancer cells has not been described in more detail, some in vitro studies investigated the communication between bone cells and ECs demonstrating that there seems to be a reciprocal regulation between ECs and osteoblast-like cell lines. However, at least in one study, a direct cell-cell contact was necessary for an effect of ECs on osteoblast-like cells (29). Collectively, ECs may be important contributing members of the intricate communication pathways that link tumor and stromal cells via diffusible signaling molecules, and perhaps by direct cell contact-mediated mechanisms as well. In this regard, both PDGF-BB and VEGF are important growth factors in regulating angiogenesis and seem to be involved in an intense paracrine loop with reciprocal induction. Still, blocking receptors for both VEGF and PDGF-BB may be more effective in vivo than inhibition of a single receptor alone possibly by more efficiently interfering with the intercellular crosstalk (30). However, future developments of better therapeutic strategies will critically depend on a more thorough look into the intensive signaling loops involving tumor and stromal cells. Targeting key molecules may disrupt this signaling crosstalk which may result in alterations of biological functions of both stromal and tumor cells and potentially improve the therapeutic response.

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References


