Platelet-Derived Growth Factor-BB Controls Epithelial Tumor Phenotype by Differential Growth Factor Regulation in Stromal Cells

Wiltrud Lederle,*† Hans-Jürgen Stark,‡ Mihaela Skobe,§ Norbert E. Fusenig,† and Margareta M. Mueller*

From the Tumor and Microenvironment Group,* and the Divisions of Differentiation and Carcinogenesis† and Genetics of Skin Carcinogenesis,‡ German Cancer Research Center, Heidelberg, Germany; and the Department of Oncological Sciences,§ Mount Sinai School of Medicine, New York, New York

Platelet-derived growth factor (PDGF) stimulates tumor growth and progression by affecting tumor and stromal cells. In the HaCaT skin carcinogenesis model, transfection of immortal nontumorigenic and PDGF-receptor-negative HaCaT keratinocytes with PDGF-B induced formation of benign tumors. Here, we present potential mechanisms underlying this tumorigenic conversion. In vivo, persistent PDGF-B expression induced enhanced tumor cell proliferation but only transiently stimulated stromal cell proliferation and angiogenesis. In vitro and in vivo studies identified fibroblasts as PDGF target cells essential for mediating transient angiogenesis and persistent epithelial hyperproliferation. In fibroblast cultures, long-term PDGF-BB treatment caused an initial up-regulation of vascular endothelial growth factor (VEGF)-A, followed by a drastic VEGF down-regulation and myofibroblast differentiation. Accordingly, in HaCaT/PDGF-B transplants, initially enhanced VEGF expression by stromal fibroblasts was subsequently reduced, followed by down-regulation of angiogenesis, myofibroblast accumulation, and vessel maturation. The PDGF-induced, persistently increased expression of the hepatocyte growth factor by fibroblasts in vitro and in vivo was most probably responsible for enhanced epithelial cell proliferation and benign tumor formation. Thus, by paracrine stimulation of the stroma, PDGF-BB induced epithelial hyperproliferation, thereby promoting tumorigenicity, whereas the time-limited activation of the stroma followed by stromal maturation provides a possible explanation for the benign tumor phenotype. (Am J Pathol 2006, 169:1767–1783; DOI: 10.2353/ajpath.2006.060120)

Multiple research efforts have been focused on genetic alterations and functional abnormalities leading to cellular transformation. During recent decades, however, it has become evident that tumor cells strongly depend on a reactive stroma, with activated stromal cells playing an important role in tumor growth, invasion, and metastasis.1–6 In this context, carcinoma-associated or phenotypically altered stromal cells have been demonstrated to promote tumorigenic conversion of preneoplastic cells.3,7 In contrast, normal stromal cells were shown to inhibit the growth of carcinoma cells.8,9 The molecular mechanisms underlying these regulatory interactions between stromal and tumor compartment are only poorly understood, although growth factors are known to tightly control this complex interplay.

In this context the platelet-derived growth factor (PDGF) is a potent mitogen and chemotactant for mesenchymal cells, such as fibroblasts, and plays a critical role in wound healing and tumor development.10 PDGF acts as a dimer consisting of the polypeptide chains A, B, C, or D. The PDGF isoforms (PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC, and PDGF-DD) interact with two tyrosine kinase receptors. The PDGFR-α (α-receptor) binds all isoforms except PDGF-DD, whereas the PDGFR-β (β-receptor) only binds PDGF-BB and PDGF-DD with high affinity.11 The PDGFR-α plays an important role during early embryonic development and organogenesis,11,12 PDGFR-β

Supported by the German-Israeli Cooperation in Cancer Research between the German Cancer Research Center and the Ministry of Science (project number Ca89) and the European Union (grant QLK3-CT-2002-02136).

Accepted for publication July 20, 2006.

Address reprint requests to Margareta M. Mueller, Ph.D., Group: Tumor and Microenvironment (A101), German Cancer Research Center (Deutsches Krebsforschungszentrum), INF 280, 69120 Heidelberg, Germany. E-mail: ma.mueller@dkfz.de.
is widely expressed by mesenchymal cells and is found up-regulated in the granulation tissue during wound healing and chronic inflammation. The simultaneous overexpression of PDGF-B indicates a paracrine mechanism of action in these processes. PDGF-B is up-regulated in many tumor cell lines, promoting tumor growth and progression in an autocrine or paracrine manner depending on the presence of its receptors on tumor or stromal cells, respectively. Indeed, studies in different tumor models revealed a crucial role of the stroma in PDGF-mediated tumorigenesis. In this context, tumor-promoting functions of PDGF-B have been demonstrated by our group in an experimental model of human squamous cell carcinoma. Transfection of nontumorigenic PDGFR-deficient HaCaT keratinocytes with PDGF-B resulted in tumorigenic transformation giving rise to benign cystic tumors on subcutaneous injection. This clearly demonstrated a tumorigenic conversion of the preneoplastic keratinocytes by paracrine effects. However, the target cells of the paracrine PDGF action and the mechanisms driving this tumorigenic conversion remained unclear.

In the current study, we analyzed the paracrine interactions between the PDGF-B-transfected tumor cells and stromal cells using the matrix-inserted surface transplantation assay, which allows the detailed analysis of the kinetics of tumor stroma interactions. In addition, to allow a more detailed mechanistic analysis under defined experimental conditions, we assessed the contribution of specific stromal components by functional in vitro studies and verified the data again in the in vivo transplants to gain insight into the mechanisms by which PDGF modulates the stroma. We provide evidence that PDGF-BB exerts dual time-dependent effects on stromal fibroblasts. In surface PDGF-B transplants, we observed an initial stromal activation characterized by a strong recruitment of proliferating cells, eg, fibroblasts and inflammatory cells as well as a strong induction of angiogenesis. This was followed by down-regulation of angiogenesis and stromal cell activity coinciding with recruitment of pericytes to blood vessels.

Our data suggest that the PDGFR-negative endothelial cells were activated indirectly by PDGF-activated fibroblasts and their initially enhanced vascular endothelial growth factor (VEGF) expression. However, long-term stimulation of fibroblasts with PDGF-BB in vitro down-regulated VEGF expression and promoted their differentiation into myofibroblasts. Accordingly, in HaCaT/PDGF-B transplants in vivo, initially enhanced VEGF protein expression by stromal fibroblasts was subsequently reduced coinciding with enhanced pericyte recruitment. On the other hand, hepatocyte growth factor (HGF) was strongly overexpressed by cultured fibroblasts in response to short- and long-term PDGF-BB treatment. This was paralleled in the PDGF-B transplants in vivo by a persistent and strong HGF protein expression by stromal fibroblasts. Therefore, HGF was considered to promote epithelial proliferation. A similar initial stromal activation followed by down-regulation of angiogenesis with vessel maturation was previously determined as the characteristic stromal response in transplants of tumorigenic but benign HaCaT cells transfected with the H-ras oncogene. Thus the dual effects of PDGF-BB on the host stromal compartment could provide an explanation for the benign tumorigenic growth behavior of the PDGF-transfected cells observed on subcutaneous injection.

Materials and Methods

Cell Culture
HaCaT cells were cultured as described. HaCaT-PDGF-B transfectants and HaCaT control transfectants resulted from transfection of immortal nontumorigenic HaCaT cells with the vector pcDNA1 encoding a cDNA for PDGF-B as well as with the empty control vector as described. Two clones expressing similar PDGF-B levels were expanded. Both showed similar benign tumorigenic growth and gave similar results in all experiments. The transfectants were maintained in the presence of 400 μg/ml G418 (PAA, Cölbe, Germany). Human dermal fibroblasts were derived from explant cultures of adult human dermis and cultivated in Dulbecco’s modified Eagle’s medium (DMEM)/10% fetal calf serum (FCS) (Biochrom, Berlin, Germany). The isolation of human dermal microvascular endothelial cells was essentially performed as described, using MACS beads (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany). Endothelial cells were cultured in endothelial growth medium/10% FCS on collagen-coated culture dishes (PromoCell, Heidelberg, Germany). Cells were routinely tested for mycoplasma contamination as described and always found to be negative.

Surface Transplantation Assay
HaCaT-PDGF-B and control-transfectants (2 × 10^5 per chamber) were grown for 1 day on a type I rat collagen gel (3 mg/ml) mounted between two concentric Teflon rings (Renner, Dannstadt, Germany). Before transplantation, the chamber was covered with a silicon hat and then transplanted onto the dorsal muscle fascia of 6-week-old nude mice (Swiss/c nu/nu back crosses) as described. For 6 weeks, three transplants per week were dissected, embedded in Tissue-Tek (Miles Laboratories, Elkhart, IN), and frozen in liquid nitrogen for the preparation of cryostat sections. For labeling of proliferating cells, mice received tail vein injections of 5-bromodeoxyuridine (BrdU) and 2-deoxycytidine (65 mmol/L each) in 0.9% NaCl (100 μl) 1.5 hours before being sacrificed.

Antibodies and cDNAs
Primary antibodies used were: rat monoclonal antibody against mouse CD31 (BD PharMingen, Heidelberg, Germany), guinea pig pan-keratin antiserum (Progen, Heidelberg, Germany), sheep polyclonal antibody against BrdU (NatuTec, Frankfurt, Germany), rabbit collagen
type IV antibody (Novotec, Lyon, France), biotinylated mouse monoclonal antibody against α-smooth muscle actin (α-SMA) (Progen), rat monoclonal antibody against macrophages (ERMP-23; Acris, Bad Nauheim, Germany), rat monoclonal antibody against mouse neutrophil granulocytes (Serotec, Düsseldorf, Germany), rat affinity-purified polyclonal antibody sc-432 against PDGFR-

The staining procedure was performed as described.25 To acetone, rehydrated in PBS, and incubated in 2 mol/L BrdU staining, frozen sections were fixed 15 minutes in rehydrated in phosphate-buffered saline (PBS). For mRNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany). Reverse transcription was performed with 1 μg of mRNA (Omniscript reverse transcriptase; Qiagen) in a final volume of 40 μl. Three μl of the cDNA were amplified in a 30-μl mixture containing 0.2 mmol/L dNTPS (Sigma-Aldrich), 5 U of Taq-polymerase (Qiagen), and 0.15 μmol/L of each primer (PDGF-B forward: 5’-GAAGGACCTGGGTTCCCTG-3’; PDGF-B reverse: 5’-TTTCTCACCTGGACAGTGTC-3’, 50°C annealing temperature; VEGF-A forward: 5’-CATGACATTCTGG-CTTGGG-3’; PDGF-A reverse: 5’-GAGGCTCTCCCTCTCCCTGG-3’, 60°C annealing temperature; HGF forward: 5’-TCTCTCTGAGACCTTTAGAAATG-3’; HGF reverse: 5’-CTGATTCTTCTATCCATT-3’, 60°C annealing temperature; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1) forward: 5’-GTTGAAAGCTGGGCTCATTT-3’; GAPDH (2) reverse: 5’-GAGGGATCTCCTGCTCTGAAAGA-3’, 60°C annealing temperature; GAPDH (2) forward: 5’-GAGAAGGCTGGGCTCATTT-3’; GAPDH (2) reverse: 5’-CATGCGGACACGGAAGG-3’, 60°C annealing temperature; PDGF-α forward: 5’-AAGGAGCTATGTTATATTTATCTTT-3’; PDGF-α reverse: 5’-GTATAAGGGGTGCACAAAGGAAA-3’, 55°C annealing temperature; PDGF-B forward: 5’-CCTCTCATCAGCTTGGCAGAAAGA-3’, PDGF-B reverse: 5’-GCTCATGTCATGTAGCCACCG-3’, 60°C annealing temperature; VEGF-1 forward: 5’-TGAGGGTCTGTAATCTG-3’; VEGF-1 reverse: 5’-TATAGGGGTCCTGTAGAAAC-3’, 58°C annealing temperature; VEGF-2 forward: 5’-GAAAGCATCGAAGTCTCATGC-3’; VEGF-2 reverse: 5’-TCTCTCTGAGACCTTTAGAAATG-3’, 55°C annealing temperature; c-met forward: 5’-TGAAGCTAAATGCTGCAATA-3’; c-met reverse: 5’-CTCATCGGAGAAGCAGGAA-3’, 64°C annealing temperature). PCR amplification was as follows: 95°C for 5 minutes, 22 to 35 cycles of 95°C for 1 minute, 50 to 60°C for 1 minute depending on the primer set (see above), 72°C for 1 minute, and final extension at 72°C for 5 minutes. PCR products were analyzed in 2% agarose gels (Biozym Diagnostic, Hess. Oldendorf, Germany) with ethidium bromide (Sigma-Aldrich). For each primer set the number of amplification cycles was determined such that the product amplification was in the exponential phase. For each RT-Mix, GAPDH was co-amplified with the target gene to confirm equal amounts of starting cDNA.

Indirect Immunofluorescence

Frozen sections were fixed 5 minutes in 80% methanol at 4°C, followed by 2 minutes in acetone at −20°C and rehydrated in phosphate-buffered saline (PBS). For BrdU staining, frozen sections were fixed 15 minutes in acetone, rehydrated in PBS, and incubated in 2 mol/L HCl for 15 minutes (all steps at room temperature). The staining procedure was performed as described.25 Together with the secondary antibody, sections were incubated with 20 μg/ml Hoechst 33258/bisbenzimide (Sigma-Aldrich, Taufkirchen, Germany) for staining of the cell nuclei. Stained sections were examined and photographed with an Olympus AS-70 microscope fitted with epifluorescence.

In Situ Hybridization

In situ hybridization was essentially performed as described.26 In brief, 35S-labeled RNA probes for PDGF-B, VEGFR-1, and VEGFR-2 were prepared using T3, SP6, or T7 RNA polymerase (for anti-sense and sense, respectively) according to the manufacturer’s instructions (Roche Diagnostics, Mannheim, Germany). Cryostat sections were fixed in 4% paraformaldehyde, pretreated, hybridized, and washed at high stringency as described. For autoradiography, slides were coated with NTB2 film emulsion and exposed for 3 weeks. After development of the signals, the sections were counterstained with hematoxylin and eosin.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

mRNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany). Reverse transcription was performed with 1 μg of mRNA (OmniScript reverse transcriptase; Qiagen) in a final volume of 40 μl. Three μl of the cDNA were amplified in a 30-μl mixture containing 0.2 mmol/L dNTPS (Sigma-Aldrich), 5 U of Taq-polymerase (Qiagen), and 0.15 μmol/L of each primer (PDGF-B reverse: 5’-GAAGGACCTGGGTTCCCTG-3’; PDGF-B reverse: 5’-TTTCTCACCTGGACAGTGTC-3’, 50°C annealing temperature; VEGF-A forward: 5’-CATGACATTCTGG-CTTGGG-3’; PDGF-A reverse: 5’-GAGGCTCTCCCTCTCCCTG-3’, 60°C annealing temperature; HGF forward: 5’-TCTCTCTGAGACCTTTAGAAATG-3’; HGF reverse: 5’-CTGATTCTTCTATCCATT-3’, 60°C annealing temperature; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1) forward: 5’-GTTGAAAGCTGGGCTCATTT-3’; GAPDH (2) reverse: 5’-GAGGGATCTCCTGCTCTGAAAGA-3’, 60°C annealing temperature; GAPDH (2) forward: 5’-GAGAAGGCTGGGCTCATTT-3’; GAPDH (2) reverse: 5’-CATGCGGACACGGAAGG-3’, 60°C annealing temperature; PDGF-α forward: 5’-AAGGAGCTATGTTATATTTATCTTT-3’; PDGF-α reverse: 5’-GTATAAGGGGTGCACAAAGGAAA-3’, 55°C annealing temperature; PDGF-B forward: 5’-CCTCTCATCAGCTTGGCAGAAAGA-3’, PDGF-B reverse: 5’-GCTCATGTCATGTAGCCACCG-3’, 60°C annealing temperature; VEGF-1 forward: 5’-TGAGGGTCTGTAATCTG-3’; VEGF-1 reverse: 5’-TATAGGGGTCCTGTAGAAAC-3’, 58°C annealing temperature; VEGF-2 forward: 5’-GAAAGCATCGAAGTCTCATGC-3’; VEGF-2 reverse: 5’-TCTCTCTGAGACCTTTAGAAATG-3’, 55°C annealing temperature; c-met forward: 5’-TGAAGCTAAATGCTGCAATA-3’; c-met reverse: 5’-CTCATCGGAGAAGCAGGAA-3’, 64°C annealing temperature). PCR amplification was as follows: 95°C for 5 minutes, 22 to 35 cycles of 95°C for 1 minute, 50 to 60°C for 1 minute depending on the primer set (see above), 72°C for 1 minute, and final extension at 72°C for 5 minutes. PCR products were analyzed in 2% agarose gels (Biozym Diagnostic, Hess. Oldendorf, Germany) with ethidium bromide (Sigma-Aldrich). For each primer set the number of amplification cycles was determined such that the product amplification was in the exponential phase. For each RT-Mix, GAPDH was co-amplified with the target gene to confirm equal amounts of starting cDNA.

Stimulation of Cells with PDGF-BB

Short-term stimulation of fibroblasts was performed by seeding 2 × 10⁶ cells/cm² in DMEM/10% FCS. After 24 hours, the cells were shifted to DMEM/2% FCS and stimulated by a single application of 50 ng/ml of recombinant PDGF-BB (Beiersdorf AG, Hamburg, Germany). Eight, 24, 48, and 72 hours after stimulation, conditioned medium was harvested, cell number was determined, and the cells were subsequently lysed for RNA isolation. For long-term treatment, human dermal fibroblasts were seeded in DMEM/10% FCS (2 × 10⁶ cells/cm²), shifted to DMEM/2% FCS after 24 hours, and stimulated with different concentrations of PDGF-BB (10 and 50 ng/ml; Beiersdorf AG) for 16 days. Medium with growth factor was changed every 48 hours in parallel to collection of conditioned media, determination of the cell number, and subsequent cell lysis for RNA preparation. Conditioned media were analyzed for protein expression by enzyme-linked immunosorbent assay (ELISA).
Stimulation of human dermal microvascular endothelial cells with PDGF-BB was performed by seeding 9.55 × 10^6 cells/cm² on collagen-coated culture plates in endothelial growth medium/5% FCS followed by a single application of PDGF-BB (50 ng/ml). Conditioned medium was harvested in addition to determination of the cell number and cell lysis 10, 24, and 48 hours after stimulation. Conditioned media were analyzed for protein expression by ELISA.

For the induction of the α-SMA phenotype 2 × 10^4 cells/cm² of human dermal fibroblasts were seeded in DMEM/5% FCS and stimulated with increasing concentrations of PDGF-BB (10, 20, and 30 ng/ml) for 16 days. Thereafter, the cells were seeded on slides in definite cell numbers and further stimulated with the appropriate concentrations of PDGF-BB for another 48 hours. α-SMA-positive cells were determined by indirect immunofluorescence. The experiments were performed at least three times each with fibroblasts from two different explant cultures.

**Determination of Protein Secretion (ELISA)**

Secreted protein in conditioned media was measured by ELISAs for different cytokines (hPDGF-BB, hVEGF-A, hHGF) using Quantikine immunoassay kits from R&D Systems according to the manufacturer’s instructions. Samples were tested in duplicate. The data shown are mean values of at least three independent experiments.

The basal PDGF-BB expression of the HaCaT cell lines was measured in 48-hour conditioned media. ELISAs of long-term stimulated fibroblast cultures were analyzed in 48-hour conditioned media throughout the period of 16 days and calculated per 10^6 cells. ELISAs of stimulated endothelial cells were analyzed in 10-, 24-, and 48-hour media and calculated per 10^6 cells.

**Morphometric Analysis**

Quantification of BrdU incorporation, vessel density, and α-SMA-positive cells was performed using analySIS software (Soft Imaging Systems, Münster, Germany). For quantification of the mean blood vessel density, the in vivo proliferation rate and α-SMA-positive blood vessels in the surface transplants, three areas of 1.5 mm² per section of three different animals were analyzed per time point. Analysis was performed from two independent transplantation series. Quantification of α-SMA-positive fibroblasts on PDGF-BB stimulation in vitro was performed on 18 independent areas of 0.4 mm².

**Statistics**

Two-tailed Student's t-test was used for data analysis, with P < 0.05 considered to be statistically significant.

**Results**

**Enhanced Proliferation of PDGF-B-Transfected HaCaT Cells in Vivo in the Absence of PDGF Receptors**

In contrast to the very weak basal expression of the control transfectants and parental HaCaT cells, mRNA for the PDGF-B chain was strongly expressed by PDGF-B transfectants in vitro, as determined by RT-PCR (Figure 1A, top; one PDGF-B-transfected clone is depicted as example). Both PDGF-B-transfected HaCaT clones secreted 2 to 3 ng/ml PDGF-BB protein per 1 × 10^7 cells, whereas no PDGF-BB secretion was detectable in control-transfected and parental HaCaT cells. PDGFR-α and -β subunits were both absent at the RNA level in all HaCaT cell lines and in primary human keratinocytes whereas they were clearly present in human dermal fibroblasts (Figure 1A, bottom). In agreement with the lack of PDGFR expression on HaCaT cells, we observed no autocrine growth stimulatory effect of PDGF-BB, as previously demonstrated by comparable in vitro growth curves of parental HaCaT cells, control, and PDGF-B transfectants. PDGF-BB secreted by the transfectants caused increased proliferation of co-cultured fibroblasts, an effect that was abrogated by PDGF-BB blocking antibodies (data not shown).

To analyze the growth behavior of the tumor cells and the tumor stroma interactions in vivo, PDGF-B-transfected and control-transfected HaCaT cells were transplanted as intact monolayers on a native collagen type I gel onto the dorsal muscle fascia of nude mice. Samples were dissected weekly throughout a period of 6 weeks to analyze early steps in epithelial tumorigenesis. The continued expression of PDGF-B in vivo was confirmed by in situ hybridization on cryosections of tumor transplants, demonstrating a strong expression of PDGF-B mRNA in all vital cell layers of the stratified epithelia formed by the PDGF-B-transfectants (Figure 1, B and D), whereas it was lacking in control transfectants (Figure 1, C and E). Histological analysis revealed prominent hyperplasia of PDGF-B-expressing epithelia with a twofold to threefold increased number of PDGF-B-expressing cells compared with control transplants (Figure 1, B–G). This resulted most likely from an enhanced proliferation of the PDGF-B-transfected cells as confirmed by a twofold to fourfold increase in BrdU-positive epithelial cells as compared with control transfectants (Figure 1H). Because the HaCaT cells clearly lacked the PDGF receptors, we concluded that the PDGF-BB-mediated enhanced epithelial proliferation was induced indirectly by paracrine mediators from the stroma.

**Epithelial PDGF-BB Induces a Transient Stromal Cell Activation and Angiogenesis**

Transplants of PDGF-BB-expressing HaCaT cells strongly activated the stroma during the first 3 weeks after transplantation, leading to the rapid formation of a granulation tissue that replaced the collagen gel,
whereas the gel was mostly acellular in control transplants (Figure 1, F and G; and Figure 2, G and H). Stromal activation, however, was transient and was followed by a decline in cellular density after week 4, so that at week 6 the stroma beneath PDGF-B and control epithelia was comparable (Figure 1, D and E; and data not shown). The decrease in cellular density was not attributable to an increase in apoptosis, as analyzed by terminal dUTP nick-end labeling staining (data not shown). There were only a few apoptotic cells detectable in both control and PDGF transplants (data not shown).

The transient kinetics of PDGF-BB-mediated stromal cell activation became even more evident when stromal cell proliferation and angiogenesis were analyzed by immunofluorescence staining (Figure 2, A–D). A striking increase in the number of BrdU-positive, ie, proliferating, cells (Figure 2A, arrows) and in blood vessels (Figure 2A, arrowheads) was observed in the granulation tissue of 3-week-old PDGF-B transplants, as compared with the controls (Figure 2B). In contrast, blood vessel density and proliferation rate in the stroma of 6-week-old PDGF-B transplants were markedly reduced and comparable with control tissues (Figure 2H).
2, C and D). Quantification confirmed the steady increase in proliferation of stromal cells with a maximum of threefold greater than control levels in 3-week-old PDGF-B transplants, followed by a rapid decline to control levels (Figure 2E). Likewise, vascular density was two to three times higher in PDGF-B transplants during weeks 2 and 3 and was reduced to control levels at weeks 5 to 6 (Figure 2F). To determine endothelial cell activation that is a prerequisite for active angiogenesis, the expression of VEGFR-1 and -2 was assessed by in situ hybridization in the stromal compartment of the tumor transplants (Figure 2, G–J; data shown for VEGFR-2 and comparable for VEGFR-1). A strong up-regulation of both receptors in PDGF-B transplants was apparent during the first 3 weeks, coinciding with the kinetics of...
enhanced angiogenesis (Figure 2, G and H). After week 3, VEGFR expression declined, reaching nearly levels of the controls (Figure 2, I and J). These findings demonstrate that the stromal activation induced by epithelial-derived PDGF-BB is associated with an activation of endothelial cells at initial stages, whereas at later stages endothelial cell activation and angiogenesis are down-regulated to the control level despite an ongoing PDGF-BB expression in the epithelium.

**Double-Paracrine Mechanism for PDGF-BB-Induced Angiogenesis**

The mechanisms of PDGF-BB-induced angiogenesis are still poorly understood. The PDGF receptors are expressed on a variety of mesenchymal cells such as bovine aortic endothelial cells and fibroblasts. Expression of the PDGFR subunits has also been detected on microvascular endothelial cells derived from adipocyte tissue. However, it remains unclear whether microvascular endothelial cells in the dermis express the PDGF receptors and can thus be directly stimulated by PDGF-BB. To address this question, normal human dermal microvascular endothelial cells were stimulated with PDGF-BB for 48 hours, and the expression of VEGFR-1 and -2 was analyzed by RT-PCR. In contrast to the observed VEGFR up-regulation in endothelial cells of early PDGF-B transplants in vivo (Figure 2, G–J), PDGF-BB failed to up-regulate the VEGF receptors on dermal microvascular endothelial cells in vitro (Figure 3A, top). Analysis of PDGFR expression revealed a complete lack of the mRNA for both receptor subunits on cultured dermal microvascular endothelial cells (Figure 3A, bottom), even after stimulation by PDGF-BB (data not shown). In addition and in contrast to the data obtained with microvascular endothelial cells from adipocyte tissue, we observed no mitogenic effect of PDGF-BB on these dermal endothelial cells (data not shown). To exclude the possibility that the endothelial cells had reached quiescence in vitro because of culture conditions and thus failed to express the PDGFR and to respond to PDGF, we analyzed the expression of the PDGFR-β subunit on CD31-positive endothelial cells in vivo by indirect immunofluorescence. This in vivo analysis confirmed the lack of the PDGFR-β subunit expression on CD31-positive vascular endothelial cells in the granulation tissue that was induced by the PDGF-B-expressing epithelial cells (Figure 3, B and C). The same lack of PDGFR-β co-staining in vivo was observed using additional endothelial cell markers such as endoglin (data not shown). We are aware that endothelial cells such as microvascular endothelial cells from fat tissue express the PDGFR and that the PDGFR-β subunit was shown to be crucial for the mitogenic response of endothelial cells to PDGF-BB. However, in vitro and in vivo data excluded a direct effect of PDGF-BB on dermal microvascular endothelial cells in our system and favored indirect paracrine mechanisms for PDGF-BB-mediated angiogenesis. Double-immunofluorescence staining with the macrophage-specific antibody ERMP-23 (Figure 3, D and E; arrows) and an antibody specific for neutrophil granulocytes (data not shown) demonstrated only minimal co-localization of PDGFR and macrophages. Our in vitro data on the expression of PDGF receptors in dermal fibroblasts suggested that the PDGFR-β-positive, not ERMP-23-labeled, cells were fibroblasts (Figure 3, D and E). This was confirmed by abundant co-staining of PDGFR-β with vimentin in vivo (data not shown).

To determine a potential contribution of PDGFR-positive fibroblasts to PDGF-mediated angiogenesis, human dermal fibroblasts were stimulated with PDGF-BB in vitro. Indeed, short-term PDGF-stimulation (50 ng/ml) induced a strong up-regulation of VEGF-A mRNA (Figure 3F) and protein, leading to a threefold increase in VEGF-A secretion after 72 hours compared with nonstimulated fibroblasts (Figure 3G). These results suggest paracrine mechanisms for the PDGF-BB-mediated angiogenesis, most probably via the up-regulation of VEGF-A in fibroblasts.

**PDGF-BB Enhances the Recruitment of α-SMA-Positive Mesenchymal Cells in Vivo and Up-Regulates the Expression of α-SMA in Cultured Fibroblasts**

Concomitantly with the stromal activation, expression of PDGF-BB by the transfected HaCaT cells exerted a remarkable effect on α-SMA-positive cells, ie, myofibroblasts in the tumor stroma in vivo. A large number of myofibroblasts accumulated within the granulation tissue of 3-week-old PDGF-B transplants, coincident with the infiltration of new blood vessels (Figure 4, A and B). This accumulation persisted during the whole observation period (data not shown). In addition, a frequent association of α-SMA-positive cells with blood vessels became obvious in later PDGF-B transplants (Figure 4, C and D; arrows). The recruitment of α-SMA-positive perivascular cells, presumably pericytes, was not only restricted to large vessels in the lower part of the granulation tissue, as seen in control transplants (Figure 4E, arrow), but was explicitly found also in newly formed small vessels in close vicinity to the epithelium (Figure 4, C and D; arrows). In contrast, small blood vessels in the granulation tissue of age-matched control transplants were mostly devoid of coverage by α-SMA-positive cells (Figure 4E, arrowheads). The onset of pericyte recruitment in PDGF-B transplants coincided with maximal blood vessel density and endothelial cell activation, as determined by VEGF-expression (Figure 4F and Figure 2, G–J), and was further enhanced in late transplants (Figure 4F).

The crucial contribution of PDGF-B to the accumulation of α-SMA-positive cells in the tumor transplants in vivo was confirmed by induction of α-SMA expression in fibroblasts in vitro. Long-term treatment (18-day) of cultured dermal fibroblasts with PDGF-BB (10 to 30 ng/ml) provoked a strong dose-dependent increase in the number of α-SMA-expressing cells, demonstrating a direct effect of PDGF-BB on fibroblast differentiation into α-SMA-positive myofibroblasts (Figure 4, G and H).
PDGF-BB Has Time-Dependent Dual Effects on VEGF-A Expression in Fibroblasts and a Persistent Stimulatory Effect on Their HGF Expression

Because fibroblasts were identified as mediators of the PDGF-induced angiogenesis by VEGF up-regulation, we addressed the question whether they could also be responsible for the late decrease in angiogenesis and the stromal maturation. Therefore, human dermal fibroblasts were stimulated for 16 days with increasing concentrations of PDGF-BB. The expression of a number of growth factors/cytokines that can influence the growth behavior of stromal and epithelial cells was analyzed by RT-PCR and ELISA: VEGF, HGF, FGF-7, aFGF, bFGF, transforming growth factor (TGF)-β1, GM-CSF, and the soluble form of VEGFR-1 (s-Flt). Although aFGF, bFGF, TGF-β1, and s-FLT were constitutively expressed at the mRNA level in treated and untreated fibroblasts, the amounts of secreted protein were below the detection limit of the ELISA, as was the case for GM-CSF (data not shown). Surprisingly, the epithelial growth factor FGF-7 was down-regulated in response to PDGF-BB throughout the whole stimulation period (data not shown). In contrast, we observed striking time-dependent alterations in the expression of VEGF. Whereas short-term treatment of human dermal fibroblasts in vitro (for 72 hours) enhanced the expression of VEGF-A (Figure 3, F and G), continued
The application of PDGF-BB had an opposite effect (Figure 5A), as determined by ELISA of fibroblast-conditioned media. Continuous treatment (every second day) with 10 ng/ml PDGF-BB resulted in ~20% reduced secretion of VEGF-A in fibroblasts from days 12 to 16 compared with untreated fibroblasts. Higher concentrations of PDGF-BB (50 ng/ml) further reduced the amounts of VEGF by ~50 to 60% below control level from day 6 to day 16 (Figure 4).

**Figure 4.** PDGF-BB enhances the recruitment of α-SMA-positive cells in vitro and induces the up-regulation of α-SMA in vitro. **A–E:** Immunofluorescence staining for α-SMA-positive mesenchymal cells (red) in frozen sections of 3- and 4-week-old transplants. Blood vessels stained with CD31 (green), nuclei with Hoechst/bisbenzimide (blue). Note the prominent recruitment of α-SMA-positive myofibroblasts in PDGF-B transplants at week 3 (A and B) and the frequent association of α-SMA-positive cells with blood vessels at week 4 (C and D, arrows). Age-matched control transplants are almost devoid of myofibroblasts (E). Recruitment of α-SMA-positive cells is restricted to blood vessels in the lower part of the granulation tissue (E, arrow). Blood vessels in the epithelial border zone are devoid of α-SMA staining (E, arrowheads). **F:** Quantification of α-SMA-positive blood vessels in relation to the total vessel number demonstrates a slight increase in PDGF-B transplants at week 3 and a dramatic increase at week 6. Bars are mean ± SD of two independent transplantation series. *P < 0.05. **G** and **H:** Long-term stimulation of human dermal fibroblasts with PDGF-BB for 18 days induced a dose-dependent increase in the expression of α-SMA (G), confirmed by quantification (H). Error bars represent the mean ± SD from three independent experiments (H). Scale bars = 200 μm (A, B, E, G), 50 μm (C, D).
5A). Decreased VEGF-A expression was not related to induction of cellular quiescence. Whereas the cell number of the untreated fibroblasts remained more or less constant because of the low FCS concentrations, a strong, dose-dependent increase in cell number was observed in the PDGF-stimulated fibroblasts throughout the 16 days (Figure 5B). Thus, despite ongoing fibroblast proliferation, PDGF-B-mediated up-regulation of VEGF during short-term treatment was followed by a down-regulation of VEGF expression in long-term treated fibroblasts. In contrast to its time-dependent dual effect on VEGF production by fibroblasts, PDGF-BB continuously enhanced the expression and secretion of HGF in these cells. Stimulation of fibroblasts with 10 ng/ml PDGF-BB provoked a 100-fold increase in HGF secretion after 10 days. Higher concentrations (50 ng/ml) resulted in more than 100-fold increased HGF protein levels (Figure 5C). This was in agreement with a strong and ongoing up-regulation of HGF mRNA throughout 16 days (Figure 5C). This dramatic up-regulation of HGF by stromal fibroblasts in response to PDGF provided a possible explanation for the enhanced epithelial proliferation we observed in the PDGF transplants, because the HaCaT cell lines all expressed the HGF receptor c-met (Figure 5D). Whereas the expression of various growth factors and cytokines is not influenced by PDGF-BB, it has time-dependent dual effects on VEGF-A production by fibroblasts with an initial up-regulation that is followed by a significant down-regulation. At the same time, it exerts a sustained stimulatory effect on HGF expression and secretion by fibroblasts.

**PDGF-BB Induces a Transient Increase in VEGF-A Expression and a Sustained Enhanced HGF Expression within the Tumor Stroma in Vivo**

To assess the effects of PDGF-BB expression on the tumor stroma in vivo, cryosections of PDGF and control transplants were analyzed for VEGF and HGF expression by immunostaining. In accordance with the effects
of PDGF-BB on fibroblasts in vitro, VEGF protein expression was markedly increased in the stroma of 2- and 3-week-old PDGF-BB transplants (Figure 6, A, B, and I), thereafter decreasing to the level of the controls (Figure 6, C, D, and J). Double-immunofluorescence analysis showed only a minimal overlap of the VEGF signal with
macrophages (Figure 6, A, C, I, and J) and no overlap with endothelial cells and neutrophil granulocytes (data not shown). In contrast, the VEGF antibody co-stained with the PDGFR antibody (Figure 6M), strongly indicating that PDGF-activated fibroblasts were the predominant producers of stromal VEGF. In further agreement with the in vitro data, HGF protein expression was increased early in the stroma of PDGF transplants and remained enhanced at late time points (Figure 6, E–H, K, and L). Again, PDGFR-positive stromal fibroblasts seemed to be main producers of HGF, as evidenced by a co-staining of HGF with PDGFR-β (Figure 6N) and the lack of co-staining with macrophages (Figure 6, E, F, and K), neutrophil granulocytes (Figure 6, G, H, and L), and endothelial cells (data not shown). These data confirm the crucial role of stromal fibroblasts as mediators of the PDGF-BB-induced transient angiogenesis and persistent epithelial proliferation.

Endostatin as Putative Regulator of VEGF Expression

Recent studies demonstrated that endostatin, the C-terminal fragment of collagen XVIII, down-regulates the expression of VEGF in a model of retinal vascularization. Endostatin is released from collagen XVIII by the action of proteases such as MMP-3 and MMP-9. Because we detected low levels of both MMPs in the PDGF transplants (data not shown), endostatin was a potent candidate for the observed time-dependent regulation of VEGF expression. Therefore, we analyzed the presence of endostatin in PDGF transplants by indirect immunofluorescence. Indeed, increased levels of endostatin were observed in PDGF transplants from weeks 2 to 4 compared with controls (Figure 7). Although endostatin was deposited in the stroma as well as in close proximity to CD31-positive blood vessels in PDGF transplants (Figure 7, A, C, and E), its presence in control transplants was mostly restricted to blood vessels (Figure 7, B, D, and F). The up-regulation of endostatin that we observed in PDGF transplants in vivo preceded the down-regulation of angiogenesis by 2 weeks and may provide a potential explanation for the down-regulation of VEGF and the resulting down-regulation of angiogenesis. Further studies to analyze this potential mechanism are currently under way.

Discussion

PDGF, as potent stimulator of mesenchymal cells, enhances tumor growth by autocrine and paracrine mechanisms. Autocrine effects of PDGF have been described for tumors of mesenchymal origin, eg, glioma or sarcoma. In contrast, PDGF promotes breast carcinogenesis in a paracrine manner by inducing a pronounced desmoplastic response in the tumor-surrounding stroma. In skin carcinogenesis, we could previously demonstrate tumor-initiating functions of PDGF by transfection of nontumorigenic HaCaT keratinocytes with PDGF-B, which resulted in the growth of benign tumors on subcutaneous injection. The lack of PDGFR expression in these keratinocytes indicated paracrine mechanisms that target the tumor stroma as the driving force for
the tumorigenic growth behavior. However, the mecha-
nistic basis of these paracrine effects remained unclear.
To elucidate these interactions, PDGF-B-transfected ker-
atinoocytes and control transfectants were transplanted as
xenografts on nude mice, using the matrix-inserted sur-
face transplantation assay, a model ideally suited for the
analysis of early tumor-stroma interactions.6,20
On transplantation, PDGF-B-transfected HaCaT cells
formed a stratified epithelium with increased proliferation
rate compared with controls, leading to pronounced ep-
ithelial hyperplasia at later time points. The PDGF-B
transfectants lacked both PDGFR subunits and showed
similar in vitro growth behavior as the controls (data of
growth curves19), excluding a direct autocrine growth stimulatory effect of PDGF-BB on the epithelial cells.
Because the PDGF-B transfectants also did not show any
alterations in growth factor and receptor expression com-
pared with the parental cells (eg, TGF-β1, TGF-α, EGF,
GM-CSF, bFGF, GM-CSF-receptor-α and -β, FGF-7-re-
ceptor), the enhanced proliferation rate was considered
to be an indirect, potentially stroma-mediated effect of
PDGF-BB and interpreted as a driving force for the ep-
ithelial hyperplasia and the benign tumor formation.
We therefore analyzed PDGF-BB-mediated stromal
alterations.
Interestingly, the stroma was only transiently activated,
resulting in increased cellular density and proliferation
that persisted for 3 weeks after transplantation and was
subsequently down-regulated to control levels. With com-
parable kinetics, PDGF-BB induced an early and strong
angiogenic response and endothelial cell activation with
VEGFR up-regulation during the first 3 weeks, followed by
down-regulation of the VEGFR and a subsequent reduc-
tion in blood vessel density to control levels. This down-
regulation of stromal activation and angiogenesis oc-
curred despite the persistent PDGF-B expression by the
hyperplastic epithelium, suggesting dual time-dependent
effects of the growth factor. To verify this assumption, we
complemented the in vivo analysis by functional in vitro
studies on specific stromal cells.
The mechanisms of PDGF-BB-mediated angiogenesis
are still controversially discussed, depending on the
presence of the PDGF receptors on endothelial cells.
Whereas porcine aortic endothelial cells expressed very
low or undetectable levels of both PDGFR subunits,41,42
the expression of the PDGFR-β subunit was demon-
strated on tumor endothelia43 as well as on activated
bovine aortic endothelial cells.27 For aortic endothelial
cells, Battegay and colleagues27 demonstrated that the
number of endothelial cords/tubes was dependent on the
serum-concentration and that the PDGFR-β expression
increased with cord/tube formation. In contrast, micro-
vascular endothelial cells derived from adipocyte tissue
expressed both PDGFR subunits in two-dimensional cul-
tures under low-serum condition30,31 and both PDGF re-
cipients and the responsiveness toward PDGF were
down-regulated in three-dimensional cultures during in vitr
angiogenesis.30 To analyze in our model whether
angiogenesis of the early transplants was directly or in-
directly induced by PDGF-BB, we stimulated human der-
mal microvascular endothelial cells with PDGF-BB in vitro.
Stimulation was performed with 10% FCS to obtain near
physiological conditions and to mimic conditions re-
quired for the induction of an angiogenic phenotype.27
Surprisingly, and in clear contrast to the results obtained
by Beitz and Marx and their colleagues,30,31 the endo-
thelial cells did not respond to PDGF-BB, neither by
up-regulation of the VEGF receptors nor by increase in
cell number. Their unresponsiveness could be explained
by the lack of the PDGFR subunits, excluding direct stimulatory effects of PDGF-BB. These rather unexpected
in vitro results were further supported by the absence
of PDGFR-β staining on CD31-positive dermal microvascu-
lar endothelial cells in the tumor transplants in vivo. The
same lack of PDGFR-β co-staining in vivo was observed
using additional endothelial cell markers such as endog-
lin (data not shown). The PDGFR-β subunit has been
demonstrated to be the main receptor mediating the
mitogenic and angiogenic response toward PDGF-BB.30
Therefore, the lack of PDGFR-β expression in microvas-
cular dermal endothelial cells in vivo and in vitro sug-
gested an indirect paracrine mechanism of the PDGF-
induced stromal activation and angiogenesis in vivo.
Further analysis to identify potential PDGF target cells
excluded neutrophil granulocytes because they were
negative for PDGFR-β staining (data not shown). Double
immunostaining with a macrophage-specific antibody
demonstrated that only a minor part of the PDGFR-posi-
tive cells were macrophages, suggesting their potential,
although minor, contribution to the stromal activation.
The major PDGFR-β-positive cells were identified as stromal
fibroblasts because of their vimentin expression in vivo
and the PDGFR expression on isolated cultured fibro-
blasts in vitro. To analyze the potential contribution of
fibroblasts to PDGF-induced angiogenesis, fibroblast
cultures were stimulated with PDGF-BB for 72 hours.
Interestingly, VEGF-A expression was clearly enhanced
in these short-term treated cultures. The in vivo relevance
of this up-regulation was substantiated by a strong VEGF
protein staining in the granulation tissue of early PDGF
transplants. Double immunostaining revealed the com-
plete overlap of signals for VEGF and the PDGFR-β.
Moreover, no overlap between the macrophage-specific
and the VEGF antibodies was detected in the upper
granulation tissue. Therefore, we considered stromal fi-
broblasts as the PDGF-B targets and the main VEGF
producers in the stroma. Taken together, these findings
strongly favor a double-paracrine mechanism for PDGF-
BB-mediated angiogenesis during the first 3 weeks after
transplantation via the activation of fibroblasts that results
in an enhanced release of VEGF-A, which in turn activi-
vates endothelial cells (Figure 8, left).
In a second phase starting from week 4, the granula-
tion tissue exhibited a drastic reduction in cellular prolif-
eration, VEGFR-expression, and vascular density. This
stromal normalization coincided with a persistent accu-
mulation of free myofibroblasts and a prominent associ-
ation of α-SMA-positive cells, most probably pericytes,
with blood vessels. PDGF-BB that is also released from
endothelial cells has been well documented to enhance
the recruitment of α-SMA-positive pericytes.44–46 This
renders endothelial cells quiescent, ensuring vessel sta-
and thereby promoting blood vessel maturation. In our studies, we observed a marked down-regulation of VEGFR in endothelial cells in vivo, which was previously demonstrated to be characteristic for the induction of endothelial quiescence. To analyze the mechanisms of the late normalizing effects of PDGF in the surface transplants, we performed long-term stimulation assays of human dermal fibroblast cultures with PDGF-BB for 16 days. In these cultures, PDGF-BB promoted differentiation to a myofibroblast phenotype, as evidenced by a strong increase in the number of \( \alpha \)-SMA-expressing cells. Comparable effects of PDGF-BB have been demonstrated by the induction of \( \alpha \)-SMA in cultured hepatic stellate cells, peribiliary fibrogenic cells, and the differentiation of fibrocytes into myofibroblasts in the CAM assay. In this context, the persistent accumulation of free myofibroblasts observed in late PDGF-B transplants strengthens the assumption that myofibroblasts could be progenitors of pericytes, as suggested by Chambers and colleagues. Additionally, this differentiation process and the enhanced association of \( \alpha \)-SMA-positive cells with blood vessels could explain the decrease in the number of free cells in the late PDGF transplants that was not attributable to an increase in apoptosis (data not shown). Moreover, the analysis of growth factors/cytokines in long-term stimulated fibroblasts in vitro provided an additional potential mechanism for the down-regulation of angiogenesis in late PDGF transplants in vivo. Whereas the expression of most growth factors was not changed by PDGF treatment, the secretion of the proangiogenic factor VEGF-A was markedly decreased in long-term PDGF-treated fibroblasts compared with the untreated controls. This effect was confirmed in vivo by the strong reduction in VEGF protein staining observed in the stroma of late PDGF transplants, providing in vivo evidence for the time-dependent changes in VEGF expression by fibroblasts attributable to long-term PDGF-BB treatment (Figure 8, right). Further immunofluorescence analysis demonstrated increased levels of endostatin in the stroma of PDGF transplants from week 2 to week 4 preceding the down-regulation of angiogenesis. Endostatin is cleaved from collagen XVIII by the action of matrix metalloproteinases, whose presence could also be demonstrated in low amounts in the stroma of PDGF transplants (W. Lederle, S. Vosseler, N.E. Fusenig, and M.M. Mueller, unpublished results). Besides its anti-proliferative effects on endothelial cells, endostatin has previously been shown to down-regulate angiogenesis.
VEGF expression in a mouse model of retinal vascularization. In accordance with these findings, we suggest that endostatin could be the putative mediator of VEGF down-regulation in late PDGF transplants. Therefore, both paracrine effects of PDGF, the expression of endostatin, and the subsequent down-regulation of VEGF provide additional explanation for down-regulation of angiogenesis. The key role of VEGF for maintaining stromal activation and angiogenesis has been previously demonstrated by our group by blockade of VEGF signaling in the stroma of highly malignant HaCaT-ras transplants. Interestingly, stromal normalization induced by VEGFR-blockade inhibited tumor invasion and resulted in a pre-malignant tumor phenotype in surface transplants, exhibiting a similar phenotype to the PDGF transplants with respect to vessel maturation and reduced expression of stromal proteases. In transplants with VEGFR blockade, MMP-9 and MMP-13 were almost completely down-regulated. In PDGF transplants, we found a very low de novo expression of stromal MMP-9 and MMP-3, whereas stromal MMP-13 was completely missing (W. Lederle, S. Vosseler, N.E. Fusenig, and M.M. Mueller, unpublished results). However, these low MMP levels seem to be sufficient to release endostatin from collagen XVIII, thereby mediating the VEGF down-regulation and the induction of vessel maturation. We conclude that the PDGF-BB-mediated late stromal normalizing effects that we observed in the PDGF-expressing transplants might explain the persistent benign tumor phenotype despite the ongoing enhanced epithelial proliferation (Figure 8). Because the keratinocytes were devoid of PDGF receptors, the high proliferative activity of the epithelial tumor cells also had to be ascribed to indirect paracrine stromal effects. As a probable cause for this epithelial hyperproliferation, we identified a PDGF-induced enhanced expression and secretion of HGF by dermal fibroblasts in vitro and a strong staining for HGF in early and late PDGF transplants in vivo. Because macrophages, neutrophil granulocytes, and endothelial cells (data not shown for the endothelial cells) were negative for HGF staining, whereas the PDGFR-β antibody co-stained with the HGF antibody, we concluded that stromal HGF was predominantly produced by PDGF-activated fibroblasts. Strong stimulatory effects of HGF on the proliferation of HaCaT cells have been demonstrated in vitro, and all HaCaT cell lines used in this study express the c-met receptor. This suggests another double-paracrine effect of PDGF-BB by HGF overexpression in stromal fibroblasts that promotes epithelial proliferation, resulting in a hyperplastic epithelium. Although we could not demonstrate PDGF-induced alterations in the expression of aFGF, bFGF, GM-CSF, TGF-β1, and s-Flt, we cannot completely exclude the involvement of additional factors that might support early stromal activation as well as epithelial proliferation and induce the late down-regulation of angiogenesis and stromal maturation.

In summary, our findings suggest that continuously expressed PDGF-BB exerts dual time-dependent effects on stromal fibroblasts resulting in an early stimulatory and a late normalizing effects of PDGF on the tumor stroma (Figure 8). Initially, PDGF-BB expression by epithelial cells enhances the expression of VEGF and HGF in fibroblasts, stimulating angiogenesis and the growth of the c-met-positive PDGF-transfected epithelial cells. Subsequently, continuous PDGF-BB secretion by a hyperplastic epithelium inhibits VEGF secretion by fibroblasts, possibly via up-regulation of endostatin in the stroma, induces their differentiation into α-SMA-positive myofibroblasts, and promotes pericyte recruitment. These effects could explain the down-regulation of endothelial cell activation and the induction of blood vessel maturation, leading to stromal normalization in late transplants. In contrast, HGF remains strongly up-regulated, further promoting epithelial proliferation (Figure 8). We hypothesize that these late normalizing effects on the tumor stroma of PDGF-BB determine the benign tumor phenotype of the highly proliferating tumor cells. These findings bring further insight into the complex interplay between tumor and stromal cells and emphasize the importance of the stroma in controlling the tumor phenotype.

Acknowledgments

We thank H. Steinbauer and R. Beck for excellent technical assistance.

References

13. ReuterdaHL C, Sundberg C, Rubin K, Funa K, Gerdin B: Tissue localization of beta receptors for platelet-derived growth factor and plate...
49. Benjamin LE, Hemo I, Keshet E: A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. Development 1998, 125:1591–1598

