PDGF-BB Modulates Endothelial Proliferation and Angiogenesis In Vitro via PDGF β-Receptors

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Abstract. To delineate potential angiogenic roles of platelet-derived growth factor (PDGF), we have investigated PDGF and its receptors on bovine aortic endothelial cells that exhibit spontaneous angiogenesis in vitro (angiogenic endothelial cells). Initiation of cord/tube formation by angiogenic endothelial cells required bovine or human serum. Neutralization of PDGF-BB in human serum with a monoclonal anti-PDGF-BB antibody reduced cord/tube formation by 37 ± 10%, whereas neutralizing anti-PDGF-AA and an IgG isotype-matched control antibody had no effect. DNA synthesis in response to PDGF-BB increased as the cords and tubes developed; furthermore, PDGF-BB induced the incorporation of BrdU in the nuclei of cells associated with these structures. PDGF β-receptor (PDGFR-β) mRNA increased concomitantly with cord/tube formation, and PDGFR-β were specifically localized by immunocytochemistry to developing and mature cords and tubes. However, PDGFR-β transcripts and protein were undetectable in nonangiogenic endothelial cells, and PDGF α-receptor mRNA was not expressed in either endothelial cell strain. In contrast to nonangiogenic endothelial cells, angiogenic endothelial cells did not express the PDGF B-chain, the required ligand for the PDGFR-β.

We conclude that (a) PDGF-BB can contribute to angiogenesis in vitro, (b) PDGFR-β are specific for cord/tube-forming endothelial cells and mediate endothelial proliferation and cord/tube formation, and (c) in angiogenic and nonangiogenic endothelial cells, the expression of PDGFR-β and PDGF B-chain is inversely correlated. We therefore suggest that paracrine PDGF might amplify angiogenesis via direct action on endothelially expressed PDGFR-β.

A NGIOGENESIS is critical to embryogenesis, wound repair, diabetic retinopathy, tumor growth, and other conditions. The formation of new vessels requires an increase in proliferation of endothelial cells, the expression of proteolytic enzymes by endothelial cells, the migration of endothelial cells toward an angiogenic stimulus, and the deposition and breakdown of extracellular matrix. The coordinated interplay of these processes leads ultimately to tubular morphogenesis, and, in the presence of pericytes, to capillary formation (for review see Risau, 1990; Ingber, 1991; Folkman and Shing, 1992; Montesano, 1992).

Molecules capable of inducing the formation of new vessels include acidic and basic FGF, TGF-α, TGF-β, tumor necrosis factor-α (TNF-α)[1], platelet-derived endothelial cell growth factor (PD-ECGF), angiogenin, angiotropin, vascular endothelial growth factor (VEGF), interleukin-8 (Koch et al., 1992), and low molecular weight substances (for review see: Risau, 1990; Folkman and Shing, 1990). Because endothelial proliferation is necessary for the formation of new vessels, angiogenic growth-regulatory molecules would be expected to induce mitogenesis in vascular endothelial cells. Indeed, FGF, VEGF, PD-ECGF, and TGF-α stimulate the proliferation of endothelial cells in vitro. However, angiogenin, TGF-β, and TNF-α have been shown to inhibit the proliferation of macrovascular endothelial cells (Folkman and Shing, 1992). The angiogenic properties of this latter group of polypeptides have been ascribed to the chemoattraction of other cells such as monocytes, which in turn might secrete endothelial mitogens. Alternatively, endothelial cells forming new capillary tubes might correspond to a distinct phenotype of endothelial cells with distinguishing responses to various growth-regulatory molecules.

The role of PDGF and its receptors in the formation of new vessels is unclear. The application of PDGF-BB to dermal wounds (Pierce et al., 1992) and to chick chorioallantoic membranes (Risau et al., 1992) promoted the appearance of new vessels. Similarly, transfection of the PDGF B-chain

1. *Abbreviations used in this paper*: PDGFR-α, platelet-derived growth factor α-receptor; PD-ECGF, platelet-derived endothelial cell growth factor; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

gene into human melanoma cells and subsequent growth of these cells in recipient animals demonstrated a substantial increase of vascularized connective tissue stroma in the resultant tumors (Forsberg et al., 1993). Hence, PDGF is a morphogen that can support the formation of a connective tissue stroma containing a functional vascular system. However, it is not clear whether PDGF elicits these effects by recruitment of inflammatory and/or connective tissue cells (Forsberg et al., 1993) or via a direct action on endothelial cells.

In general, macrovascular endothelial cells do not express PDGF receptors in culture (Bar et al., 1989; Raines et al., 1990); hence, PDGF does not induce a proliferative response in these cells. However, observations in macrovascular endothelial cells might not be relevant to angiogenesis because new vessels form from extant microvessels (Ausprunk and Folkman, 1977), the endothelial cells of which have been shown to express many phenotypic markers that distinguish them from macrovascular endothelial cells. In fact, some strains of microvascular endothelial cells (Bar et al., 1989; Smits et al., 1989; Beitz et al., 1991), capillaries in the rat brain (Smits et al., 1989), proliferating capillaries in glioblastomas (Hermanson et al., 1992; Plate et al., 1992) and in wounds (Reuterdahl et al., 1993) have been reported to express PDGF β-receptors.

Endothelial cells can also express the ligands of the PDGF receptors, the PDGF A- and B-chain genes, both in vivo and in vitro (for review see Raines et al., 1990). However, an earlier report indicated that c-sis (PDGF B-chain) was downregulated in endothelial cells differentiating into 3-dimensional tubular structures (Jaye et al., 1985).

In this study we have investigated the potential role of PDGF in angiogenesis. We used a model of angiogenesis in vitro in which endothelial cells form cords and tube-like structures; some strains of cloned bovine aortic endothelial cells assume a microvascular phenotype and organize into networks of cords and tubes (angiogenic EC), whereas other strains, derived by identical procedures, do not form cords/tubes and grow in monolayers or in monolayers with sprouts (nonangiogenic endothelial cells) (Cotta-Perreira et al., 1980; Iruela-Arispe et al., 1991; Iruela-Arispe and Sage, 1993). Both the differentiation of the angiogenic endothelial cells into cords and tubes and some changes in endothelial morphology and phenotype are highly reminiscent of endothelial processes associated with capillary formation in vivo. In contrast to some models of angiogenesis in vitro organization and differentiation of angiogenic endothelial cells into cords and tubes occurs spontaneously, i.e., in the absence of an imposed extracellular matrix substratum. Because most available matrix substrata contain contaminating and/or specifically bound growth-regulatory molecules (Vukicevic et al., 1992; our unpublished observations), this model seems especially suited to studies related to PDGF.

We addressed the following questions: (a) Does PDGF stimulate angiogenesis in vitro via endothelial cell proliferation and cord/tube formation? (b) Are PDGF receptors expressed on endothelial cells that form cords/tubes in vitro and on nonangiogenic EC? (c) Is PDGF expressed concomitantly with its receptors during cord/tube formation? Our results support a direct role for PDGF in angiogenesis in vitro through endothelial cell proliferation and cord/tube

formation by phenotypically distinct angiogenic endothelial cells.

Materials and Methods

Growth Regulatory Molecules and Antibodies

Human recombinant PDGF-AA and PDGF-BB were expressed in yeast saccharomyces cerevisiae E18-9 strain, purified and assessed for homogeneity as described (Herren et al., 1993a). Pfa 4 and Pfa 15 are mouse monoclonal antibodies against human recombinant PDGF-AA. Pfa 4 recognizes an epitope of PDGF-AA within the receptor-binding domain and neutralizes human PDGF-AA (Herren et al., 1993b), but does not neutralize human PDGF-BB. Pfa 15 was used for immunocytochemistry. Monoclonal antibody vsb8a was raised against the purified v-sis gene product and was provided by M. Brockhaus, F. Hoffmann-La Roche LTD, Basel; it neutralizes human recombinant PDGF-BB (1 ng/ml) at an IC50 of 14 μg/ml, but does neither neutralize human PDGF-AA (Herren et al., 1993b) nor human PDGF-AB or bovine PDGF-BB. Monoclonal antibody p17-8, raised against p17 virion core protein of human immunodeficiency virus type 1 (M. Brockhaus, F. Hoffmann-La Roche LTD), was used as an isotype-matched control antibody for neutralization experiments. Isotype matched nonimmune mouse IgG's (Biogenex, San Ramon, CA) were used for controls in immunocytochemistry experiments. A polyclonal rabbit antibody against the extracellular domain of the rat PDGF β-receptor (8529) (Herren et al., 1993a) or a nonimmune rabbit serum (Vector Labs., Burlingame, CA) was used for immunocytochemistry of bovine PDGF β-receptor. A monoclonal antibody against 5-bromo-2'deoxyuridine-5'monophosphate (BrdU) was purchased from Boehringer Mannheim Biochemicals (Rotkreuz, Switzerland). Biotinylated horse anti-mouse IgG and biotinylated goat anti-rabbit IgG were purchased from Vector Labs. Concentrations of PDGF isoforms in the human serum used for neutralization experiments were PDGF-AA: 21 ng/ml and PDGF-BB 13 ng/ml as measured with an isoform specific ELISA (H. Gallati, F. Hoffmann-La Roche LTD, Basel [Allam et al., 1993]).

Cell Culture

Endothelial cells from adult bovine aorta were isolated, cloned, characterized, and maintained as previously described (Cotta-Pereira et al., 1980; Iruela-Arispe et al., 1991); upon cloning, isolates that neither exhibited spontaneous organization of cord/tube-like structures (angiogenic EC), or that grew in monolayers with sprouts or in monolayers without sprouts (nonangiogenic EC), were established and characterized for synthesis of von Willebrand factor and endocytosis of acetylated low density lipoproteins. Stock cultures were maintained in DMEM (Biochrom Seromed, Berlin, FRG) containing 10% FCS (Gibco BRL, Life Technology, Basel, Switzerland). Endothelial cells used between passages 4–18 were plated at a density of 15,000 cells/cm^2 and were grown in DMEM containing 10% FCS for the days specified. To achieve cellular quiescence, cultures were incubated for 72 h in serum-free DMEM with one change of media after 24 h. For angiogenic and nonangiogenic endothelial cells, two different isolates each were used for any reported experiment.

Swiss 3T3 D1 cells were maintained as described (Seifert et al. 1989).

Determination of Cord/Tube Numbers

Cells were cultured in 6- or 24-well trays in media containing 10% FCS, changed to serum-free or serum-containing media when cellular subconfluence was reached, and treated as described in the specific experiments. Cells were fixed for 6 min in 100% methanol at −20°C and subsequently stained with hematoxylin to demonstrate the network of endothelial cords/tubes. Each culture was photographed with Polaroid 667 film (Polaroid Corporation, Cambridge, MA) at a magnification of 32× with a Leitz Laborvert FS microscope equipped with a Polaroid camera system (Wild, Heerbrugg, Switzerland). A grid of lines 5 mm apart was layered over the photograph and the numbers of intersections of cords/tubes crossing the lines was defined as the "number of cords/tubes". Crossings were then counted manually for each photograph. Each photograph corresponds to an area of 7.2 mm^2 and each datapoint corresponds to the average number of cords/tubes crossing the lines ± SEM of at least four photographs taken of 2 (6-well plates) or 4 (24-well plates) separate wells.

DNA Synthesis

[³H]Thymidine incorporation. Endothelial cells were plated in 24-well plates (Falcon, Becton Dickinson, Lincoln Park, NJ). Growth-regulatory molecules or serum was added directly to the cells maintained in serum-free DMEM. The cells were pulsed with 1 μCi/ml [³H]thymidine (Amersham, Zürich, Switzerland) added at the times and for the periods indicated in the figure legends. The cells were washed, and incorporation of [³H]thymidine into acid-insoluble material was determined as previously described (Battegay et al., 1990). Values are given as the mean ± SEM.

In situ localization of proliferating cells. Cells were plated on plastic Labtek™ slides (Nunc, Life Technology, Basel, Switzerland), cultured, made quiescent, and treated as described for [³H]thymidine incorporation at different stages of cord/tube formation. The thymidine analog BrdU (20 μM 5-bromo-2'-deoxyuridine and 20 μM deoxycytidine) (Sigma, Fluka-Chemie, Buchs, Switzerland) was added 12 h after stimulation of the cultures with growth-regulatory molecules or FCS, and the cells incubated between hours 12–24 for exactly 12 h with BrdU. Incorporated BrdU was detected by immunoperoxidase staining (Vectastain Elite, Vector Labs.) by a monoclonal antibody specific for BrdU (1 μg/ml) (Boehringer Mannheim Biochemicals) and a biotinylated horse anti–mouse IgG (10 μg/ml) (Vector Labs.). Labeled cells were visualized with 3-amino-9-ethylcarbacole (AEC) (Sigma). Cell nuclei were counterstained with hematoxylin.

RNA Isolation and Northern Blots

Cells were plated in 150-mm plastic dishes (Falcon), cultured for various periods of time, and made quiescent as described above. Total RNA was isolated by lysis of cells with acid guanidinium thiocyanate and subsequent extraction with phenol-chloroform (Chomczynski and Sacchi, 1987). 15 μg of total RNA/lane was separated in a 1% agarose/formaldehyde gel, and the RNA was transferred by vacuum transfer to Hybond™-N membranes (Amersham) (Sambrook et al., 1989). Filters were hybridized as previously described (Battegay et al., 1990) with cDNAs labeled with [³²P]deoxycytidine 5'-triphosphate (Amersham) using a random prime labeling kit (Boehringer Mannheim Biochemicals) to specific activities of 1–2 $\times$ 10⁹ cpm/μg. The following cDNA probes were used for hybridization: rat B-chain (Not I fragment, 745 bp) (Herren et al., 1993b), recombinant transfer vector pβ3 (Eco RI/Bam HI fragment, 1617 bp) (Herren et al., 1993a) containing mutagenized human PDGF β-receptor (Gronwald et al., 1988) and pα39.1 (Bam HI fragment, 1587 bp) containing PDGF α-receptor (Kelly et al., 1991), and chicken GAPDH (Pst I fragment, 1150 bp) (Dugaiczyk et al., 1983). Blots were hybridized ($\sim$20 $\times$ 10⁶ cpm) for 24 h, washed 2 $\times$ 15 min in 2$\times$ SSC/0.1%SDS and 2 $\times$ 15 min in 1$\times$ SSC/0.1%SDS at 65°C, and were subsequently exposed to XAR-2 film (Eastman Kodak Company, Rochester, NY).

Immunocytochemistry

Cells were plated on round glass coverslips that had been placed into 24-well trays, cultured for various periods of time, made quiescent, and treated with growth-regulatory molecules as described above. Cells were exposed to 25 ng/ml PDGF-BB, PDGF-AA, or diluent at 37°C for 30 min before fixation to elicit clustering of corresponding PDGF receptors. The cells were fixed in methanol and were washed twice in PBS: the coverslips were subsequently removed from the 24-well plates for further processing. Nonspecific binding was blocked by a 20-min incubation in normal goat serum (50% in PBS plus 0.1% BSA). Polyclonal rabbit anti-PDGFR-β (8529, 10 μg/ml), anti-PDGF-BB (vsb8a, 10 μg/ml), or anti-PDGF-AA (Pfa 15, 30 μg/ml) was added for 1 h at RT (22°C). Controls for primary antibodies included PBS, nonimmune rabbit IgG (10 μg/ml, Vector), and isotype matched nonimmune mouse IgG's (10 μg/ml or 30 μg/ml, respectively, Bio-genex). After two washes with PBS, a biotinylated goat anti–rabbit IgG (15 μg/ml, Vector) was applied for 30 min. The coverslips were washed with PBS and were incubated with streptavidin-FITC (20 μg/ml) (Jackson Immuno Research Labs. Inc., Milan Analytica AG, La Roche, Switzerland) for 20 min. After a final washing, the coverslips were mounted upside-down in mowiol (Osborn and Weber, 1982). Analysis was performed with a Zeiss axioskope (Zeiss, Oberkochen, FRG) equipped with HBO-50 epifluorescence, and photomicrographs were taken with a Zeiss MC-100 camera system.

Statistical Analysis

Values are given as the mean ± SEM. Summary statistics to test the global hypothesis and, if rejected, pairwise comparisons between means were evaluated with the Bernard-van Elteren test (generalization of the Friedman test) (Haux et al., 1984) using the data in an experiment as a block (PROC FREQ procedure, Cochran-Mantel-Haenszel statistics, SAS^R statistics package, Cary, NC). p-values are reported after adjustment for multiple comparisons with the Bonferroni-Holm method (Bauer, 1991). For global evaluation of the data described in Figs. 3 and 4, ANOVA's were performed on the logarithms of the counts using the factors "experiment," "drug" (Diluent, PDGF-AA, PDGF-BB), and "dose" (Fig. 3) or "day" (Fig. 4) and the interaction of drug with dose (Fig. 3) or drug with day (Fig. 4) (PROC GLM, SAS^R statistics package). For comparison of specific doses (Fig. 3) or days (Fig. 4) the ANOVA was repeated, and the Bonferroni-Holm adjustment was applied to the resultant p-values.

Results

The organization of endothelial cords and tube-like structures by angiogenic bovine aortic endothelial cells that were used in this study progresses through the following stages: (a) formation of an endothelial cell monolayer with the emergence of "sprouting cells" growing above or below the monolayer ($\sim$1–3 d after plating), (b) appearance of wide cords which develop from sprouting cells ($\sim$3–5 d), (c) remodeling of wide cords into thin and elongated cords ($\sim$5–14 d), (d) development of a lumen in some cords resulting in short tubes with the lumen often containing secreted material ($\sim$10–21 d) (Iruela-Arispe and Sage, 1993).
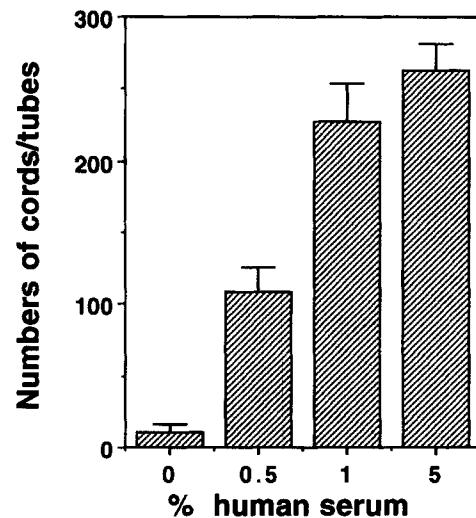

Figure 1. Serum is necessary for cord/tube formation in angiogenic endothelial cells. Cultures of bovine aortic endothelial cells forming cords and tubes (angiogenic endothelial cells) were established as described (Iruela-Arispe et al., 1991). Cells were plated (15,000 cells/cm²) in DMEM containing 10% FCS. After growth of the endothelial cells to subconfluence, the cells were changed to DMEM supplemented with different concentrations of human serum and grown for an additional 7 d. Cells were subsequently fixed and stained with hematoxylin to demonstrate the interconnecting network of endothelial cords/tubes. Each photograph corresponds to a surface area of 7.2 mm². Cords/tubes were counted for every photograph as described in Materials and Methods. Serum induced a significant increase of cord/tube numbers at all concentrations tested compared to serum free cultures ($p < 0.001$). The values correspond to quadruplicate counts ± SEM of a representative experiment ($n = 3$).

PDGF-BB Drives Cord/Tube Formation and Cellular Proliferation of Angiogenic EC

Originally we observed that cord/tube formation depended on the presence of serum in the culture media. In initial experiments, angiogenic endothelial cells were plated and cultured in serum-containing media until a subconfluent monolayer without cords/tubes formed (1–3 d). The cells were further cultured in serum-free media or media containing different concentrations of FCS or human serum. Cord/tube formation was subsequently assessed. Angiogenic endothelial cells survived in serum-free media but formed no or only a few abortive cords/tubes (Fig. 1). In contrast, numerous cords/tubes developed in the presence of serum. Cord/tube formation after 7 d of culture correlated dose-dependently with the concentration of fetal calf (data not shown) or human serum ($p < 0.001$ for pairwise comparison of each serum concentration with serum free culture) (Fig. 1). In parallel, cell numbers increased with serum concentration (data not shown). Thus, serum factors play a decisive role in driving cellular proliferation and concomitant cord/tube formation in this model of angiogenesis.

To assess whether PDGF present in serum might support cord/tube formation, we first analyzed whether cords/tubes still developed after neutralization of PDGF in serum. All three isoforms of PDGF (PDGF-AA, PDGF-AB and PDGF-BB) are released from human platelets and are therefore present in whole human serum (Hart et al., 1990). Because our anti-PDGF-BB antibodies neutralize human but not bovine PDGF-BB, we used human serum for the neutralization experiments. Angiogenic endothelial cells were cultured in media containing FCS until a subconfluent monolayer without cords/tubes was formed. Thereafter, the cultures were changed to serum-free media or to media containing 1% or 5% human serum, 1% or 5% human serum pretreated with a neutralizing monoclonal mouse antibody against PDGF-BB (vsb8a), against PDGF-AA (Pfa 4), or anti-HIV p17 protein (p17-8, IgG isotype-matched control), and were grown for an additional 7 d. In the presence of the neutralizing antibody against PDGF-BB the number of cords was reduced by 38
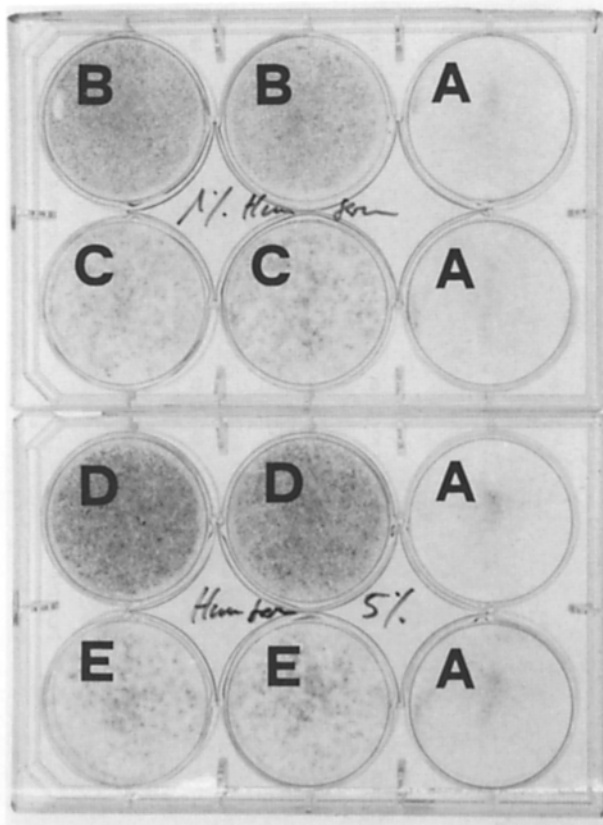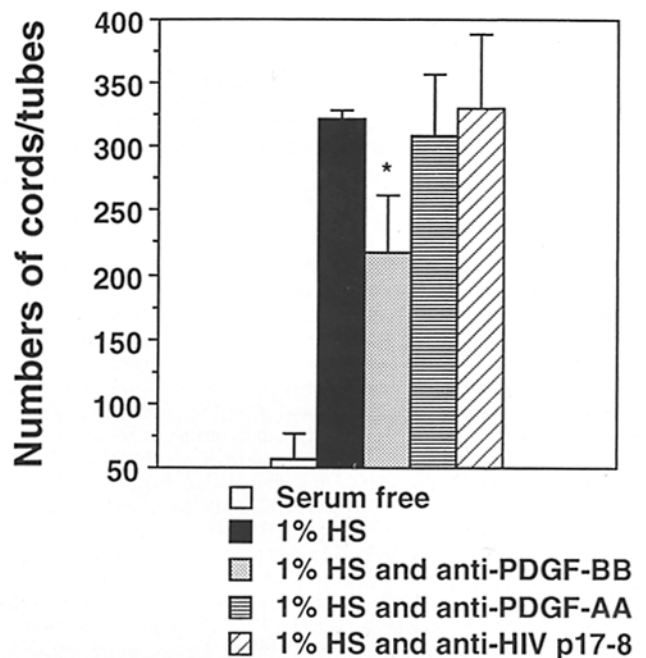

Figure 2. Neutralization of PDGF-BB in serum reduces cord/tube formation in angiogenic endothelial cells. Angiogenic endothelial cells were plated in 6-well trays, and treated as described in the legend to Fig. 1. Cultures were changed to media containing 1% or 5% human serum, 1% or 5% human serum pretreated with a neutralizing monoclonal mouse antibody against PDGF-BB (vsb8a, 50 μg/ml), PDGF-AA (PFA 4, 50 μg/ml), anti-HIV p17 protein (p17-8, IgG isotype-matched control, 50 μg/ml) and were grown for 7 d. Cords/tubes were subsequently processed as described in Fig. 1. Fig. 2 *a* shows a photograph of a representative experiment. The wells designated with *A*, show serum free controls; *B*, 1% human serum; *C*, 1% human serum and anti-PDGF-BB (50 μg/ml); *D*, 5% human serum; *E*, 5% human serum and anti-PDGF-BB (50 μg/ml). The anti-PDGF-BB, in contrast to the control antibodies, significantly (*) decreased serum-induced cord/tube numbers ($p < 0.01$). The values given in Fig. 2 *b* correspond to quadruplicate counts ± SEM of another experiment ($n = 3$).

± 4% in 1% human serum and 35.2 ± 7.5% in 5% human serum ($p < 0.01$, average reduction in all experiments 37 ± 10%, $n = 3$) (Fig. 2), compared to the cultures containing serum without antibody. The antibodies against PDGF-AA and the control antibody (anti-HIV p17) had no statistically significant effect on the numbers of cords/tubes (Fig. 2). Therefore, significant and substantial but not complete suppression of cord/tube formation occurred in serum pretreated with anti-PDGF-BB, in comparison to controls.

Cord/Tube Formation in Response to PDGF-BB Requires Additional Serum Factors

Next we tested whether PDGF-BB alone could drive cord/tube formation in the absence of additional serum factors. Angiogenic endothelial cells were plated in serum-containing media and changed to serum-free media after a subconfluent monolayer had formed. Thereafter either diluent, PDGF-AA, PDGF-BB, or FCS were added daily for 7 consecutive days. Neither PDGF-BB nor PDGF-AA in the absence of serum supported cord/tube formation. Hence, serum factors in addition to PDGF-BB are necessary to support continued cord/tube formation.

PDGF-BB Stimulates DNA Synthesis in Angiogenic But Not in Nonangiogenic EC

Because cord/tube formation results from both cellular migration and proliferation, we assessed DNA synthesis as a measure of cellular proliferation in response to PDGF-AA, PDGF-BB, and FCS in angiogenic endothelial cells. We examined the incorporation of [³H]thymidine into DNA in angiogenic endothelial cells kept in serum-containing media for 7 d and subsequently changed to serum-free media for 3 d to achieve cellular quiescence. At this stage, the cultures exhibited postconfluent monolayers with a profuse network of wide cords. PDGF-BB (100 ng/ml) stimulated [³H]thymidine incorporation into angiogenic endothelial cells by 65 ± 30% (mean ± SEM) above diluent control ($p < 0.001$) (Fig. 3). Half maximal stimulation of [³H]thymidine incorporation was observed between 1-10 ng/ml PDGF-BB and was statistically significant at concentrations of 1 ng/ml PDGF-BB ($p < 0.05$) and higher ($p < 0.001$) (Fig. 3). PDGF-AA did not increase [³H]thymidine incorporation in a statistically significant dose-dependent manner. FCS (10%) stimulated [³H]thymidine incorporation to 76 ± 19% above diluent control ($p < 0.001$), an increase similar to the one elicited by PDGF-BB. Because cells responding to PDGF-BB constituted a small minority of all cells (see below), the relative increase in DNA synthesis in response to PDGF-BB can be estimated to be very high in PDGF-responsive cells. For the same reason, variation from experiment to experiment was predicted to be relatively high.

No response to either PDGF-BB or PDGF-AA was observed in identically derived bovine aortic endothelial cells that did not form cords/tubes, e.g., that grew to confluent monolayers only or that formed monolayers with sprouts without further development into cords/tubes ("nonangiogenic" EC) (data not shown).

Although these results do not establish a rigorous mechanistic link between PDGF-BB-induced DNA synthesis and PDGF dependent cord/tube formation they nevertheless suggest that cord/tube formation by angiogenic endothelial cells
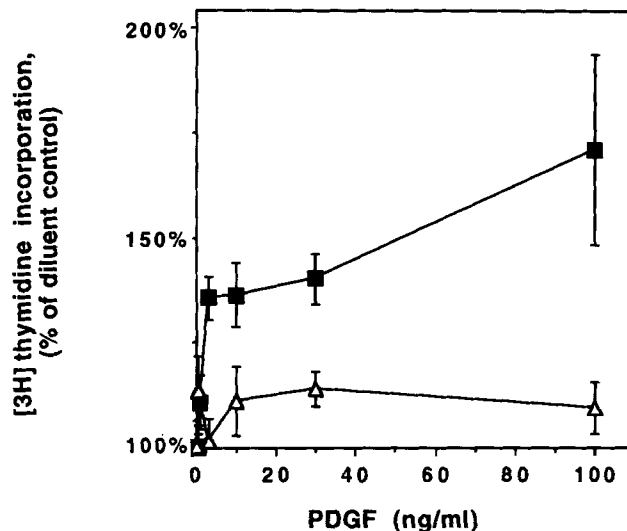

Figure 3. PDGF-BB, but not PDGF-AA, stimulates DNA synthesis in angiogenic endothelial cells. Angiogenic endothelial cells were plated in 24-well trays (15,000 cells/cm²) and were grown in DMEM containing 10% FCS for 7 d (intermediate stage of cord/tube formation in angiogenic endothelial cells and confluence in nonangiogenic EC). To achieve cellular quiescence, the cultures were subsequently incubated for a total of 72 h in DMEM without serum, with one change of media after 24 h. Human recombinant PDGF-AA (△), PDGF-BB (■), diluent (0.25% BSA in 10 mM acetic acid), or 10% FCS were added, and the cells were incubated with [³H]thymidine (1 μCi/ml) 20-24 h after the addition of growth-regulatory molecules. Incorporation of [³H]thymidine into acid-insoluble material was determined at the end of the indicated period. PDGF-BB ($p < 0.05$ at 1 ng/ml and $p < 0.001$ at concentrations of 3 ng/ml and higher), but not PDGF-AA, dose dependently increased DNA. Values of a representative experiment are given as the mean ± SEM ($n = 6$). [³H]thymidine incorporation for diluent controls was 70,205 ± 4,506 cpm (=100%) and 123,373 ± 1,640 cpm for 10% FCS.

in response to PDGF-BB is associated with cellular proliferation and not only due to a migratory response of the cells to PDGF-BB.

DNA Synthesis in Response to PDGF-BB Increases as Cords/Tubes Form

To assess DNA synthesis in response to PDGF at different stages of cord/tube formation, we cultured angiogenic endothelial cells for 1, 3, 7, and 14 d in serum-containing media, and then rendered the cells quiescent. Subsequent addition of PDGF-BB (100 ng/ml) increased [³H]thymidine incorporation 28 ± 16% at day 1, 39 ± 24% at day 3, 65 ± 30% at day 7, and 79 ± 21% at day 14 above respective diluent controls ($p < 0.001$) (Fig. 4). This response to PDGF-BB was dose-dependent at each timepoint (data not shown). In contrast to the response of the cells to PDGF-BB which monotonously increased with time in culture, i.e., in proportion to the number of cells outside the monolayer (sprouts and cords/tubes, see below), FCS (10%) induced its maximal response at day 1 (199 ± 94%). DNA synthesis in response to FCS decreased continuously as the cells differentiated into cords/tubes ($p < 0.005$). At day 14 the response to FCS had
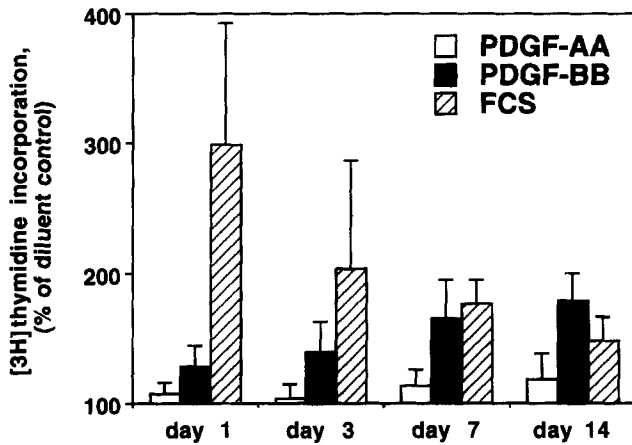
Figure 4. DNA synthesis in response to PDGF-BB increases as cords and tubes form. Cells were plated, grown, treated, and analyzed as described for Fig. 3. Angiogenic endothelial cells were maintained for the periods indicated on the x-axis. Angiogenic endothelial cells grow to a monolayer with sprouts between day 1 and 3, to an early stage of cord/tube formation between day 3 and 5, to an intermediate stage between day 5 and 14, and to a later stage between day 10 and 21. Human recombinant PDGF-AA (100 ng/ml), PDGF-BB (100 ng/ml), diluent (0.25% BSA in 10 mM acetic acid), or 10% FCS were used. PDGF-BB increased DNA synthesis above respective diluent controls ($p < 0.001$). DNA synthesis in response PDGF-BB increased above diluent controls ($p < 0.001$)

declined to 48 $\pm$ 19%, less than the response to PDGF-BB. PDGF-AA, which can only activate PDGF α-receptors (for review see Raines et al., 1990; Heldin, 1992), had no significant effect on [³H]thymidine incorporation at these stages of angiogenesis in vitro (Fig. 4).

To confirm the results of [³H]thymidine incorporation and to determine the location of DNA-synthesizing cells, we performed BrdU-labeling at an intermediate and a late (data not shown) stage of cord/tube formation. Fig. 5 shows representative stainings of cords/tubes and adjacent areas in angiogenic cultures at an intermediate stage of cord/tube formation (day 7). BrdU-labeled nuclei were not distributed evenly but mostly localized to cords/tubes or cell clusters in monolayers (Fig. 5). Background DNA synthesis, i.e., the percentage of BrdU-incorporating nuclei under conditions of quiescence, was different in distinct areas of the cultures; 27 $\pm$ 11% in cords/tubes and 17 $\pm$ 7% in monolayers (compilation of counts from areas adjacent and far-removed from cords/tubes). PDGF-BB increased the number of labeled nuclei in all areas of the cultures both at an intermediate (Fig. 5 and below) and a late stage of cord/tube formation dose de-
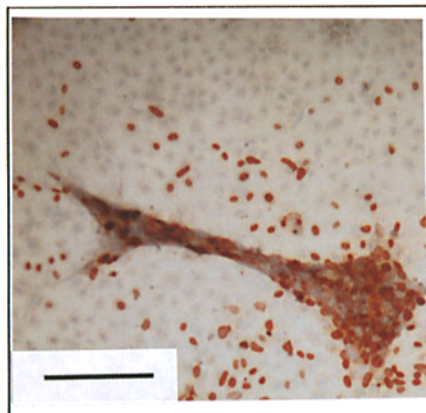
with time in culture whereas DNA synthesis in response to FCS decreased continuously as the cells differentiated into cords/tubes ($p < 0.005$). PDGF-AA had no significant effect. Values represent the mean $\pm$ SEM of all experiments ($n = 4$).
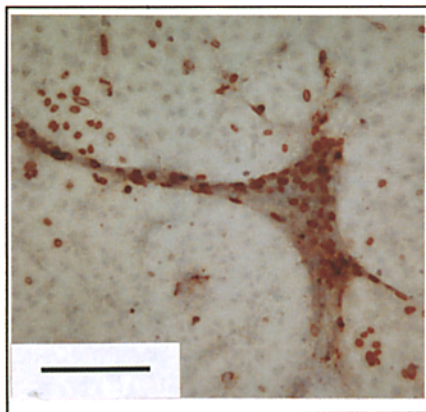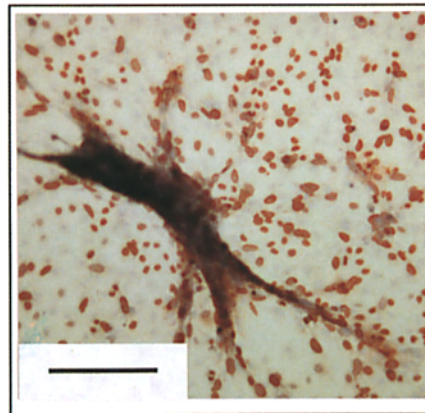

Diluent
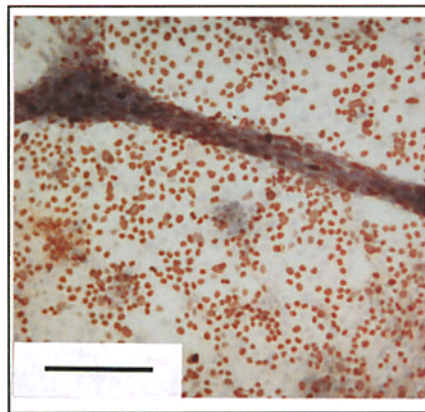
FCS

PDGF-AA

PDGF-BB

Figure 5. PDGF-BB increases DNA synthesis in areas of cord/tube formation. Cells were cultured on plastic Lab-tek slides and were treated at an intermediate stage of cord/tube formation (day 7) as described for Fig. 3. Human recombinant PDGF-AA (30 ng/ml), PDGF-BB (30 ng/ml), diluent (0.25% BSA in 10 mM acetic acid), or 10% FCS were used in the experiments. DNA synthesis was measured with incorporation of the nuclear analog BrdU for a labeling period of exactly 12 h between 12 to 24 h after stimulation of the cells. The incorporated analog was identified by an immunoperoxidase staining reaction. The scale bars correspond to 100 μm.

pendently (data not shown). Numerical evaluation of BrdU-labeled nuclei in the cords/tubes at an intermediate stage of cord/tube formation (7 days) demonstrated an increase of 69 ± 41% above diluent controls in cultures treated with PDGF-BB (30 ng/ml) ($p < 0.05$), 9 ± 9% with PDGF-AA (30 ng/ml) (not significant), and 102 ± 70% with FCS. In monolayers an increase of 124 ± 43% to PDGF-BB ($p < 0.01$), 20 ± 20% to PDGF-AA (not significant), and 384 ± 242% to FCS above diluent controls was observed. This staining procedure did not allow specific identification of cells in sprouts or surfacing cords. It is therefore possible that clusters of BrdU-incorporating cells in "monolayers" actually correspond to very early stages of developing cords. Labeled nuclei were found in clusters in both diluent controls and PDGF-BB-stimulated cells. However, because the number and size of such clusters within the monolayer seemed to be increased in PDGF-BB-stimulated cells (Fig. 5), we speculated that PDGF-BB might specifically affect endothelial cells in sprouts that are about to develop into cords/tubes and endothelial cells within cords/tubes, but not endothelial cells in the monolayer directly attached to the plastic dish. To substantiate this hypothesis we analyzed PDGF β-receptor expression in detail.

PDGF β-Receptors Are Exclusively Localized on Sprouts and Cords/Tubes of Angiogenic Endothelial Cells, and Their Expression Increases with Cord/Tube Formation

The current model of PDGF ligand/receptor interactions stipulates distinctive receptor specificities of the PDGF A-chain and B-chain. The PDGF A-chain can only elicit its effects through the PDGF α-receptor, whereas the PDGF B-chain elicits its effects through both the PDGF α- and β-receptor (Seifert et al., 1989; for review see Raines et al., 1990; Heldin, 1992; Herren et al., 1993). This model, together with the effects of PDGF-BB and the lack of a response to PDGF-AA in angiogenic endothelial cells, predicts

the presence of PDGF β-receptors and the absence of PDGF α-receptors in angiogenic EC.

To evaluate the presence of PDGF receptors, we measured both PDGF β-receptor and α-receptor mRNA expression by Northern blot analysis. As shown in Fig. 6, PDGF β-receptor transcripts were expressed at low levels in angiogenic endothelial cells. Moreover, PDGF β-receptor mRNA expression increased with time (Fig. 6), possibly indicative of an association of the receptors with the appearance of cords/tubes. In nonangiogenic endothelial cells, however, PDGF β-receptor mRNA was undetectable (Fig. 6). The PDGF α-receptor gene was expressed in neither angiogenic or nonangiogenic endothelial cells but present in Swiss 3T3 cells used as controls (data not shown).

To localize PDGF-responsive cells and to document the presence of PDGF receptors and their activation by PDGF, we performed immunocytochemical staining of PDGF β- and α-receptors. Angiogenic endothelial cells at different stages of cord/tube formation were incubated with a polyclonal rabbit antibody against the ectodomain of the PDGF β-receptor. We found diffuse staining of PDGF β-receptors on cords/tubes and sprouts, but no staining in monolayers in angiogenic and nonangiogenic cultures (data not shown). To better visualize PDGF β-receptors and to demonstrate their activation we preincubated the cells with PDGF-BB 30 min before fixation. As reported previously (Tingström et al., 1992), this approach increased the sensitivity of the immunocytochemical staining and demonstrated clustering PDGF β-receptors on sprouting cells and cords/tubes, but not in monolayers of angiogenic (Fig. 7 a) and nonangiogenic endothelial cells (data not shown). As predicted from the PDGF receptor subunit model (Seifert et al., 1989), no clustering of PDGF β-receptors could be elicited by preincubating the cells with PDGF-AA (data not shown). However, an identical staining pattern to the one shown in Fig. 7 a was obtained when angiogenic cells were preincubated for 30 min with human PDGF-BB and subsequently stained with an anti-human PDGF-BB antibody (vsb8a), thus ascer-
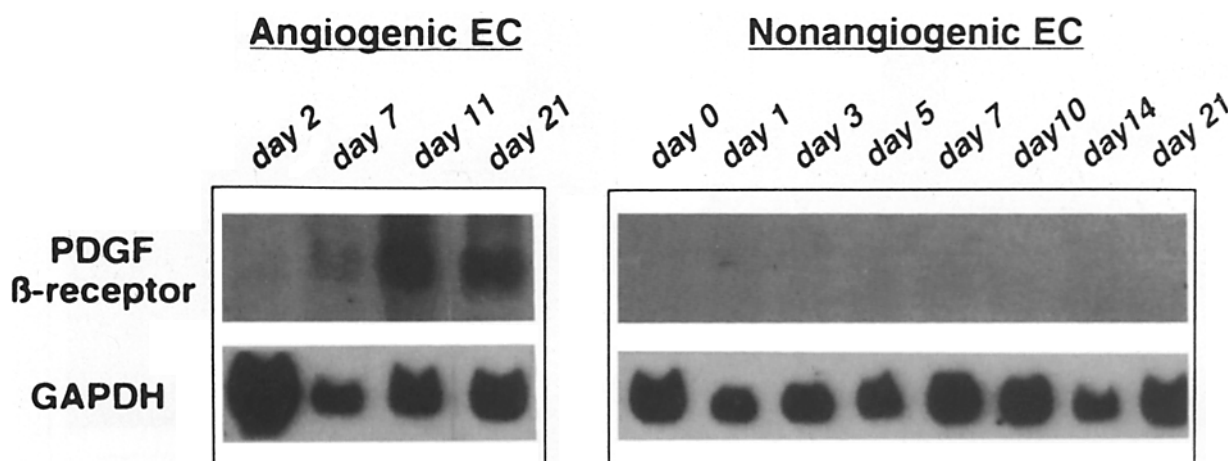

Figure 6. PDGF β-receptor mRNA is found exclusively in angiogenic endothelial cells and increases concomitantly with cord/tube formation. Total RNA was isolated from endothelial cells by homogenization and lysis with acid guanidinium thiocyanate followed by phenol-chloroform extraction. 15 μg of total RNA/lane was separated in a 1% agarose/formaldehyde gel and transferred to a nylon membrane. Filters were hybridized with [^{32}P]dCTP-labeled cDNA probes for human PDGF β-receptor and for GAPDH. Angiogenic endothelial cells are shown in the left and nonangiogenic in the right panel.
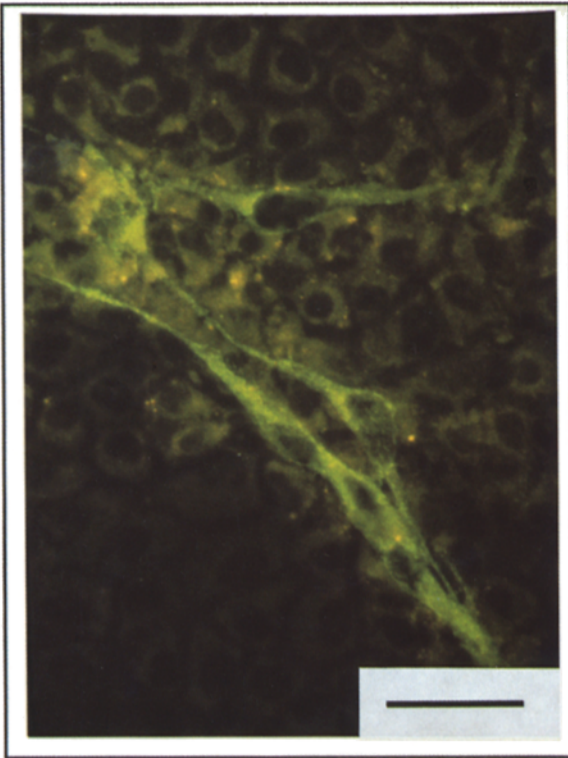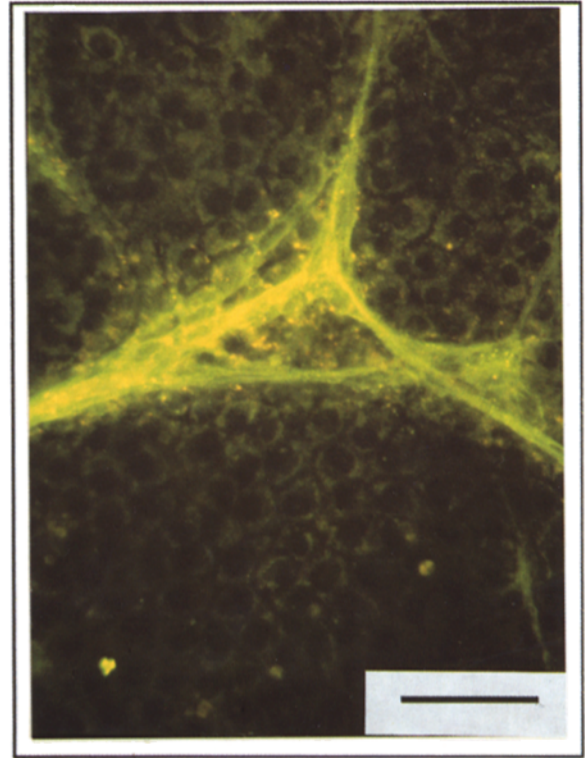
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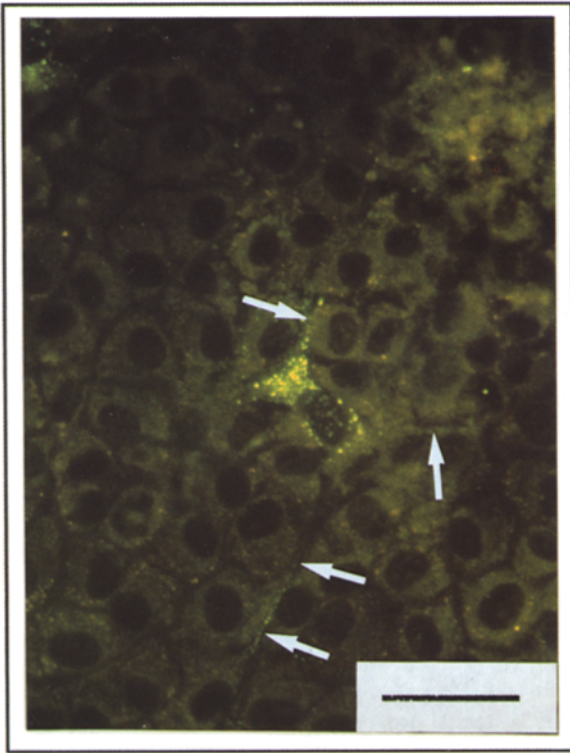
day 1
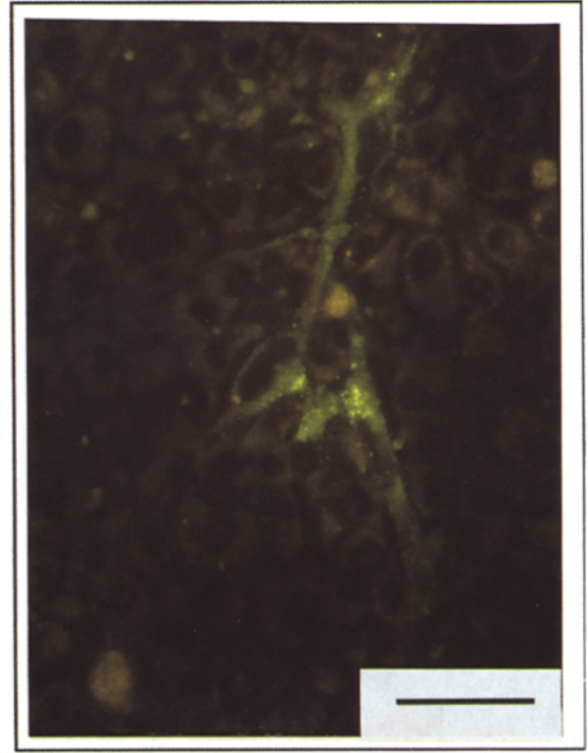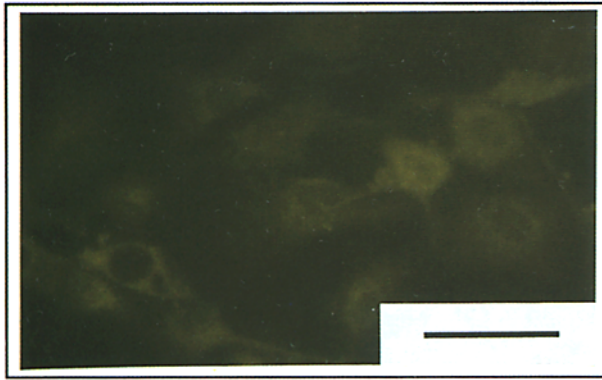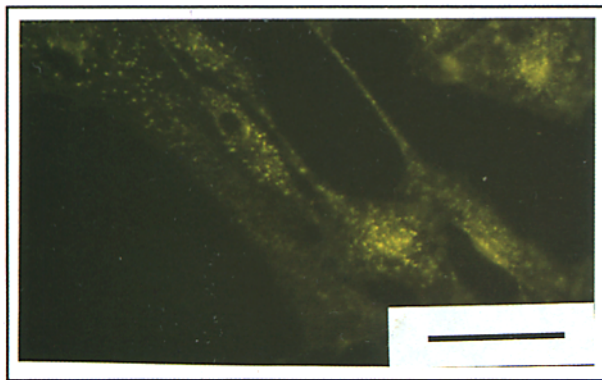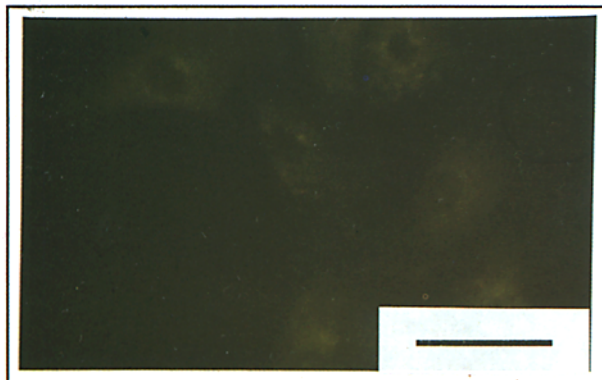
day 3

day 7

day 14

Figure 7.

Figure 7. PDGF β-receptor protein is expressed on endothelial cells cords/tubes and clusters upon activation with PDGF-BB. Angiogenic and nonangiogenic endothelial cells were plated, cultured, and stimulated as described in the legend for Fig. 3. Clustering of the PDGF β-receptor was elicited by treatment of the cells with PDGF-BB (25 ng/ml) at 37°C for 30 min, before processing of the slides. Immunofluorescence was performed with a polyclonal anti-PDGF β-receptor (Herren et al., 1993). Fig. 7 a shows PDGF β-receptor expression at different stages of cord/tube formation, and

taining the validity of the staining with the antibody against the PDGF β-receptor. Staining without primary antibodies or with nonimmune rabbit IgG or with isotype matched nonimmune mouse IgG's did not yield any signal (data not shown).

It is notable that early in cord/tube formation, PDGF β-receptors were expressed exclusively on sprouts and their cellular projections (Fig. 7 a, panel I). Conversely, staining of nonangiogenic endothelial cells did not reveal PDGF β-receptors, and interestingly, sprouts in these cultures lacked PDGF β-receptors. Furthermore, PDGF β-receptor staining increased continuously as cords/tubes formed (Fig. 7 a, panels I–IV).

PDGF α-receptor protein was investigated by preincubation of the angiogenic endothelial cells with human PDGF-AA, followed by staining with anti-human PDGF-AA antibody (Pfa 15). No staining of PDGFR-α protein was found in angiogenic or nonangiogenic endothelial cells. However, in Swiss 3T3 cells which express both PDGF α- and β-receptors and which were used as controls, specific staining of PDGF α-receptors was demonstrated with the same staining procedure (Fig. 7 b). Thus, neither angiogenic nor nonangiogenic endothelial cells expressed PDGFR-α mRNA and protein, in agreement with the lack of effects of PDGF-AA on these cells.

Angiogenic Endothelial Cells Express No PDGF B-chain, in Contrast to Nonangiogenic EC

Expression of the PDGF β-receptor raised the question whether proliferation of angiogenic endothelial cells might be stimulated in an autocrine manner by PDGF-BB. We therefore probed Northern blots with a human PDGF B-chain cDNA. However, angiogenic endothelial cells did not express the PDGF B-chain at day 7 (Fig. 8), or at any other stage of cord/tube formation (data not shown). In contrast, nonangiogenic endothelial cells expressed PDGF B-chain transcripts constitutively (Fig. 8); the human PDGF B-chain cDNA used in these experiments therefore hybridized to the bovine PDGF B-chain.

Discussion

Endothelial cells comprise the inner lining of the vasculature, an extensive and in most instances continuous monolayer. Normally these endothelial cells have a very low rate of proliferative activity (Folkman and Shing, 1992). However, in angiogenesis microvascular endothelial cells (Ausprunk and Folkman, 1977) respond to angiogenic stimuli by

7 a, panel day 1 shows an enlargement of a sprout with a "traction cell" (see text). Arrows point to the elongated cellular projections of the cell that exhibit PDGF receptors. Scale bars correspond to 30 μm in panels for day 1 and 3 and to 50 μm in panels for day 7 and 14. Fig. 7 b shows controls for the analysis of PDGF α-receptors by immunocytochemistry in Swiss 3T3 cells; cells were cultured in media containing 10% FCS and changed to serum free media 48 h before the experiment. Cells were prestimulated with either diluent (panel I), PDGF-AA (50 ng/ml, panel II), or PDGF-BB (50 ng/ml, panel III) for 30 min at 37°C. After fixation cells were stained with anti-PDGF-AA (PFA 15). Scale bars correspond to 30 μm.
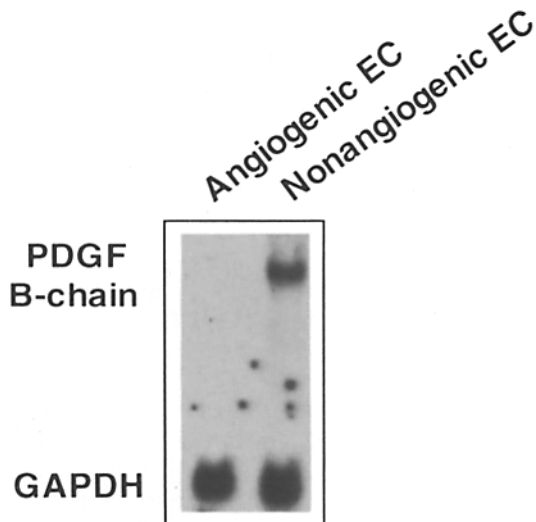
Figure 8. Angiogenic endothelial cells do not express the PDGF B-chain, the ligand for the PDGF β-receptor, in contrast to non-angiogenic endothelial cells. Total RNA was isolated from endothelial cells at day 7, fractionated, and transferred to a nylon membrane as described in the legend to Fig. 6. Filters were hybridized with cDNA probes for human PDGF B-chain and GAPDH.

increased proliferation, by expression of proteolytic enzymes, and by synthesis of specific extracellular matrix components (Risau, 1990; Ingber, 1991; Folkman and Shing, 1992; Montesano, 1992). These processes are necessary for endothelial cells to form new endothelial tubes, and, in conjunction with pericytes, to remodel new capillary vessels. Endothelial cells originating from microvessels in some respects bear little resemblance to the continuous monolayer of quiescent endothelial cells that line the macrovasculature and that do not express PDGF receptors (Raines et al., 1990). In this study we have provided evidence that PDGF-BB can elicit endothelial proliferation and the formation of endothelial cords/tubes via direct action on PDGF β-receptors that are exclusively expressed on a specific phenotype of endothelial cells, that we have termed "angiogenic endothelial cell."

PDGF-BB Could Play a Direct Role in Angiogenesis

The potential roles of PDGF in angiogenesis are not well delineated. Specifically, it is unclear whether PDGF can act directly on angiogenic endothelial cells, as reported for a structurally related protein, VEGF (Risau, 1990; Folkman and Shing, 1992). In vivo models of angiogenesis cannot determine whether PDGF induces neovascularization via direct action upon endothelial cells or indirectly via recruitment of other cells (Montesano, 1992). For example, PDGF and other proteins such as TGF-β are chemotactic for monocytes and other cells which, in turn, might release angiogenic molecules (Wiseman et al., 1988). Recently, it has been shown that PDGF can control angiogenesis in vitro indirectly through the action of other cells (Sato et al., 1993). Sato et al., using an angiogenesis system in which myofibroblasts and microvascular endothelial cells were cocultured, found that PDGF induced the secretion of an endothelial cell growth factor by myofibroblasts, which in turn stimulated the

formation of cord-like structures by endothelial cells (Sato et al., 1993).

Obviously, any direct effect of PDGF would require the expression of PDGF receptor(s) on neovascular endothelial cells. Although most macrovascular endothelial cells do not express PDGF receptors (Raines et al., 1990), several groups have reported the presence of PDGF β-receptors on various strains of microvascular endothelial cells (Bar et al., 1989; Smits et al., 1989; Beitz et al., 1991). Effects of PDGF-BB have also been identified on endothelium-dependent vascular relaxation of rat aortic rings, i.e., macrovessels (Cunningham et al., 1992). In vivo, PDGF β-receptors were demonstrated on endothelial cells of capillaries in the rat brain (Smits et al., 1989), in glioblastomas (Hermanson et al., 1992; Plate et al., 1992), and in wounds (Reuterdahl et al., 1993). These observations suggest potential direct effects of PDGF-BB on endothelial cells undergoing angiogenesis.

In this study we have demonstrated that PDGF-BB can also directly induce endothelial cell proliferation and cord/tube formation during angiogenesis in vitro via PDGF β-receptors. We used cloned strains of bovine aortic endothelial cells that spontaneously form cords/tubes without exogenous extracellular matrix through consecutive stages of differentiation (angiogenic EC) (Cotta-Pereira et al., 1980; Iruela-Arispe et al., 1991). Nevertheless, formation of these cords/tubes is not strictly spontaneous, because serum is necessary for endothelial cells to proliferate and undergo morphogenesis. Here we report that PDGF is one of the serum elements that can amplify cord and tube formation via endothelial cell proliferation. Additionally, PDGF-BB-induced endothelial cell migration might contribute to angiogenesis in vitro. Cellular proliferation and cord/tube development can be inhibited substantially by neutralization of PDGF-BB but not PDGF-AA in serum. However, identically derived bovine endothelial cells that do not differentiate into cords/tubes (nonangiogenic endothelial cells) and that form monolayers or monolayers with some sprouting cells, do not respond to PDGF.

In angiogenic endothelial cells, cord and tube formation could not be abrogated completely by neutralizing PDGF-BB. Residual amounts of PDGF, for example as a result of incomplete neutralization by the antibody, or PDGF-like molecules, not detected by our neutralizing antibody, might have exerted their action via the PDGF β-receptor and thus have upheld residual cord/tube formation after neutralization of PDGF-BB. Alternatively, it is possible that PDGF-BB is not an absolute requirement for the morphogenesis of cords/tubes. Furthermore, PDGF-BB alone in serum-free media is not sufficient to drive cellular proliferation and cord/tube formation suggesting that additional serum constituents are required in conjunction with PDGF-BB to drive angiogenesis. However, the identity of serum factor(s) that expedite PDGF-stimulated angiogenesis and that support PDGF-free angiogenesis in vitro remain unclear.

PDGF β-Receptors Define a Phenotype of Endothelial Cells Undergoing Angiogenesis In Vitro

As expected the mitogenic effect of FCS decreased continuously as angiogenic endothelial cells achieved greater cellular density and differentiated into cords and tubes (Fig. 4). In contrast, earliest expression of the PDGF β-receptor protein emerged after cellular confluence had been reached and

was found exclusively on sprouting cells and later on cords and tubes in angiogenic cultures. PDGF β-receptor mRNA (Fig. 6) and protein expression (Fig. 7 *a*) increased in parallel with cord/tube formation and correspondingly also the mitogenic response to PDGF-BB (Fig. 4). Thus, PDGF β-receptors were exclusively expressed and activated on the minority of cells that emerged outside the cellular monolayer, i.e., in the cells that were actively engaged in the formation of cords and tubes. These observations point to phenotypically distinct cellular populations within angiogenic cultures that are associated with differences in cell shape, spatial organization, the nature of the substrate, and the extracellular matrix in the microenvironment; accordingly, angiogenic endothelial cells in sprouts or cords/tubes specifically express collagen type I, SPARC, and decorin, macromolecules that are not or that are only minimally produced by confluent monolayers of nonangiogenic endothelial cells (Iruela-Arispe et al., 1991; Järväläinen et al., 1992). Moreover, in our angiogenesis model, cellular shape differs in the endothelial cells of sprouts and cord/tubes, compared with endothelial cells residing in the cellular monolayer of the same cultures. Angiogenic endothelial cells that emerge, i.e., sprout, outside the confluent endothelial monolayer assume a trapezoid shape with elongated cellular projections that wrap around extracellular matrix components after leaving the cellular monolayer of the same cultures ([Vernon, R. B, personal communication] and Fig. 7 *a*, panel *I*). The generation of traction by these cells contributes to the reorganization of extracellular matrices in tracks, which serve as a scaffold for developing interconnecting cellular projections and the creation of a cellular network (Vernon et al., 1992). These sprouts or traction cells express PDGF β-receptors but, interestingly, morphologically similar cells in nonangiogenic endothelial cells that form sprouts but no interconnecting cellular projections and no cords/tubes do not express PDGF β-receptors. Also, endothelial cells in mature cords/tubes express PDGF β-receptors and are more elongated and rounded whereas endothelial cells in the monolayer are more polygonal and flat. Hypothetically, control of growth by shape and microenvironment may translate into distinct responses of endothelial cells to growth factors. For example, the angiogenic response of endothelial cells to FGF can be controlled by modulation of the composition of the extracellular matrix and the resulting changes in cell shape (Ingber, 1991). Hence, the expression of PDGF β-receptors, and thus the response to PDGF-BB, might depend on the specific microenvironment and the cellular shape of angiogenic endothelial cells.

Conversely, angiogenic cells that express PDGF β-receptors might contribute to a specific microenvironment that is favorable for angiogenesis. PDGF-BB could act directly on angiogenic endothelial cells via PDGF β-receptors to change cell shape, to modulate the generation and degradation of extracellular matrix, to regulate the generation of traction forces, to alter the proteolytic potential, and to modify the response of angiogenic endothelial cells to other growth-regulatory molecules.

Although neovascularizations arise from microvessels (Ausprunk and Folkman, 1977), macrovascular endothelial cells can also unfold the developmental program that allows formation of cords, tubes, branches, and capillary-like networks; treatment of macrovascular endothelial cells with PMA (Montesano and Orci, 1987) or clonal isolation of bovine aortic endothelial cells (Iruela-Arispe et al., 1991) induced conversion to an "angiogenic" phenotype. Hypothetically a set of angiogenic genes might be activated that control the emergence of an angiogenic versus a nonangiogenic phenotype of endothelial cells. Our study suggests that expression of the PDGF β-receptor might be coupled to a more committed state of differentiation toward an angiogenic endothelial phenotype. Hence, in contrast to other angiogenic molecules, PDGF-BB might conceivably not control the switch to an angiogenic phenotype of endothelial cells, i.e., the initiation of angiogenesis, but rather processes associated with cells already committed to active neovascularization.

The appearance of PDGF β-receptors on endothelial cells engaged in cord and tube formation is indicative of multiple additional alterations toward a potentially distinct angiogenic phenotype of endothelial cells. TGF-β, which is angiogenic in vivo, has been shown to promote the proliferation of angiogenic, but not nonangiogenic endothelial cells (Iruela-Arispe et al., 1993) or macrovascular endothelial cells (Heimark et al., 1986; Müller et al., 1987). Similarly, TNF, although generally growth-inhibitory for endothelial cells, has been shown to promote microvessel formation at low concentrations (Fajardo et al., 1992). We propose that the response to many growth-regulatory molecules may be determined by the specific phenotype of the target endothelial cell. Additional research to better understand and define distinct phenotypes of endothelial cells, such as angiogenic and nonangiogenic endothelial cells, is clearly needed.

Angiogenic Endothelial Cells Are Not the Source of PDGF-BB

Expression of the PDGF β-receptor in angiogenic endothelial cells led us to question whether these cells might stimulate their proliferation in an autocrine manner by concomitant expression of the PDGF β-receptor and the PDGF B-chain. However, angiogenic endothelial cells did not constitutively express the PDGF-B-chain, a result in keeping with a previous report on human umbilical vein endothelial cells that differentiated into three-dimensional tubular structures (Jaye et al., 1985). Macrophages, platelets, tumor cells, and many other cell types can release PDGF-BB (Raines et al., 1990) and might therefore serve as a paracrine source of PDGF-BB and other growth-regulatory molecules in angiogenesis. In contrast, nonangiogenic endothelial cells, as previously shown by others (Collins et al., 1985; Jaye et al., 1985), express PDGF B-chain transcripts. The PDGF β-receptor and its ligand, the PDGF B-chain, are therefore expressed by angiogenic and nonangiogenic endothelial cells in a mutually exclusive manner. Regulatory mechanisms that are as yet unknown might be involved in precluding concomitant expression of these two interrelated genes and thus an autocrine loop.

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