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PDGF-BB Increases Endothelial Migration and Cord Movements During Angiogenesis In Vitro

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Abstract To explore direct effects of platelet-derived growth factor (PDGF) on endothelial cells during angiogenesis in vitro, we have used cloned bovine aortic endothelial cells that spontaneously form cord structures. Recently we have shown that cells forming these endothelial cords express PDGF β-receptors and that PDGF-BB can contribute to cellular proliferation and cord formation. In this study we investigated whether PDGF-induced cellular migration might also contribute to endothelial repair and angiogenesis in vitro.

Ten individual endothelial cells in cords were tracked at an early stage of cord formation by video-timelapse microscopy. PDGF-BB (100 ng/ml) induced an increase in endothelial cell movement of 67 ± 15% as compared with diluent control. Interestingly, PDGF-BB also increased movements of entire cord structures, followed at branching points, by 53 ± 12% over diluent control. Taken together, these video-timelapse experiments suggested that the apparent movements of single endothelial cord cells might also be due to the motion of entire underlying cord structures in response to PDGF. To analyze the response of single endothelial cord cells we therefore examined whether PDGF-induced migration contributes to endothelial repair. Abrasions were applied with a razor blade to confluent monolayers of endothelial cells at an intermediate stage of cord formation. PDGF-BB concentration-dependently increased the distance to which cord-forming endothelial cells migrated into the abrasion. An increased number of elongated, i.e., probably migrating, endothelial cells was found in the abrasion in response to PDGF-BB. However, there was no effect of PDGF-BB on the total number of endothelial cells found in the abrasion. PDGF-AA affected neither the distance to which the cells migrated nor the number of elongated cells.

Actin and tubulin stainings revealed that these cytoskeletal structures were not appreciably altered by PDGF-BB. Furthermore, urokinase-type plasminogen activator transcripts were not modulated in response to PDGF-BB.

We conclude that in this model of angiogenesis in vitro PDGF-BB can elicit the movement of entire cord structures, possibly via u-PA-independent mechanisms. PDGF-BB also controls the migration of single cord-forming endothelial cells. Thus, PDGF-BB possibly contributes to endothelial repair and angiogenesis by direct effects on proliferation and composite movements of PDGF β-receptor-expressing endothelial cells and cords. J. Cell. Biochem. 64:403–413.

Key words: PDGF; PDGF receptor; cell migration; endothelial cell; endothelium; angiogenesis; in vitro; urokinase-type plasminogen activator

Growth-regulatory molecules such as fibroblast growth factor and vascular endothelial growth factor control endothelial migration and proliferation and play a pivotal role in vascular repair and angiogenesis [Folkman and Shing, 1992; Klagsbrun and Folkman, 1990; Risau, 1990; Battegay, 1995; Pepper et al., 1996a]. Similarly, platelet-derived growth factor-induced cellular events may be involved in atherosclerosis [Ross, 1993], vascular repair, and possibly angiogenesis [Risau et al., 1992; Pierce et al., 1992; Marx et al., 1994; Battegay et al., 1994, 1996; Battegay, 1995]. In these processes, PDGF can act as a mitogen and chemoattractant for several cell types such as fibroblasts, smooth muscle cells, inflammatory cells, and possibly endothelial cells.

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Although macrovascular endothelial cells generally do not express PDGF receptors in culture [Bar et al., 1989; Beitz et al., 1991], PDGF receptors are present on endothelial cells of various microvascular origins in vitro [Bar et al., 1989; Beitz et al., 1991; Marx et al., 1994; Risau et al., 1992] and in vivo [Plate et al., 1992; Holmgren et al., 1991]. Recently, we [Battegay et al., 1994] and others [Marx et al., 1994] have shown that PDGF β-receptor expression can be modulated during endothelial remodeling in vitro. Using cloned strains of bovine aortic endothelial cells that spontaneously form cords through consecutive stages of development [Cotta-Perreira et al., 1980; Iruela-Arispe et al., 1991] we found that PDGF-BB directly induced endothelial cell proliferation via PDGF β-receptors exclusively expressed on developing endothelial cords [Battegay et al., 1994]. Furthermore, PDGF-BB contributed to endothelial cord formation and morphogenesis [Battegay et al., 1994]. Thus, several studies suggest the possibility that PDGF-BB might affect endothelial proliferation directly via PDGF β-receptors [Bar et al., 1989; Battegay et al., 1994; Beitz et al., 1991; Smits et al., 1989; Reuterdahl et al., 1993; Holmgren et al., 1991]. PDGF-BB may thereby contribute to angiogenesis [Battegay et al., 1994]. However, it is not yet clear whether and how PDGF-elicited endothelial migration is involved in endothelial morphogenesis and remodeling.

PDGF-elicited migration has been demonstrated in many cell types. PDGF induces proliferation and migration of vascular smooth muscle cells [Pauly et al., 1995; Koyama et al., 1994a], monocytes, granulocytes, and fibroblasts [Siegbahn et al., 1990]. A fibroblastoid cell line transfected with PDGF α- or β-receptors migrated in response to PDGF [Ferns et al., 1990]. In porcine aortic endothelial cells transfected with PDGF β-receptors, PDGF-BB induced a strong, dose-dependent chemotactic response, which included a rapid reorganization of actin and the formation of membrane ruffles suggesting that most endothelial cells possess the downstream apparatus to migrate in response to PDGF [Westermark et al., 1990]. PDGF-induced cell migration has recently also been observed in specific strains of endothelial cells, namely in capillary endothelial cells from rat brain [Risau et al., 1992] and rabbit retinal endothelial cells [Koyama et al., 1994b]. Thus, limited data suggest that PDGF may also contribute to endothelial remodeling not only via endothelial proliferation but also via cellular migration.

Using the above-mentioned strains of cord-forming bovine endothelial cells (cfBAEC) as a model of angiogenesis in vitro [Cotta-Perreira et al., 1980; Iruela-Arispe et al., 1991; Battegay et al., 1994] we addressed three questions that emerge from the aforementioned studies: (1) Does PDGF-BB induce migration of endothelial cells during angiogenesis in vitro? (2) Does PDGF-BB elicit the movement of entire endothelial cords? (3) If so, does the PDGF-induced response require reassembly of cytoskeletal elements or protease expression?

MATERIALS AND METHODS

Cell Culture

Endothelial cells from adult bovine aortae were isolated, cloned, characterized, and maintained as previously described [Cotta-Perreira et al., 1980; Iruela-Arispe et al., 1991; Battegay et al., 1994]. After cloning, isolates that exhibited spontaneous organization of cord-like structures were further characterized for synthesis of von Willebrand factor and endocytosis of acetylated low density lipoproteins. Stock cultures were maintained in Dulbecco's modified Eagles's medium (DMEM) (Biochrom, Seromed, Berlin, FRG) containing 10% fetal calf serum (FCS) (Biochrom, Seromed). Cells were used between passages 5–14, plated at a density of 15,000 cells/cm^2 in DMEM containing 10% FCS, and cultured for the number of days specified. Prior to each experiment cells were rendered quiescent by incubation in serum-free DMEM for 48 h. At least two different isolates were used for each of the reported experiments.

Videolapse Microscopy

Cells were cultured in 35 mm tissue culture dishes for 7 days to an intermediate stage of cord development and then rendered quiescent. Video-timelapse microscopy was performed using a Nikon microscope with Nomarski optics. After a medium change to serum-free DMEM containing 8.8 mM sodium bicarbonate and 50 mM Hepes, cells were tracked for 24 h in the presence of diluent (10 mM acetic acid, 0.25% bovine serum albumin) and, after an additional medium change, for another 24 h in the presence of PDGF-BB (100 ng/ml, PDGF isoforms were a kind gift of Dr. M. Pech, F. Hoffman-

La Roche LTD, Basel, Switzerland [Herren et al., 1993]). Photographs were taken every 2 min over the two sequential 24-h periods. Ten single discernable endothelial cells in cords were tracked using the Image 1 program (Universal Imaging, West Chester, PA). Velocity of cells was measured during a 24-h period in the first, second, fourth, seventh, and fifteenth hour. Each hour was divided into three 20-min segments. The average moving speed for the ten cells was then calculated for each of these 20-min segments.

To track the movements of entire cord structures four intersecting or branching points of cords were followed at 200-min intervals during each of the 24-h periods; to measure the movements of branching points and to ensure reproducibility of results, lines along the axes of cords were drawn and the movement of line-intersections was tracked and quantified.

Abrasion Experiments

Cells were cultured in 6 well trays for 14 days and rendered quiescent. Abrasions were applied to the cultures with a razor blade. Thereafter, the cells were washed twice with serum-free DMEM. The cells were then treated with diluent (10 mM acetic acid, 0.25% bovine serum albumin), PDGF-AA, PDGF-BB, or 5% FCS for 24 h. Cells were subsequently fixed, stained with hematoxylin, and photographed at a magnification of ×32 with a Leitz Laborvert FS microscope (Wild, Heerbrugg, Switzerland). The maximal distance the cells had migrated, the total number of cells, and the number of elongated cells (defined as less than 50 µm wide and more than 125 µm long) within the abrasion were determined for every photograph over a length corresponding to 2.5 mm in the original culture.

Actin and Tubulin Staining

Cells were seeded on 6 well trays containing 20 × 20 mm sterile glass coverslips. The cells were grown for 14 days and made quiescent. Abrasions were applied with a razor blade and the cells washed once with PBS. Thereafter, the cells were incubated for 24 h in serum-free DMEM with or without PDGF-BB (100 ng/ml).

For actin staining the cells were rinsed briefly with PBS, incubated with 2% Triton X-100 containing 0.125% glutaraldehyde for 5 min, rinsed again with PBS, and fixed with 1% glutaraldehyde for 20 min. The coverslips were then washed 2 × 5 min with PBS before incubation for 2 × 10 min on ice with 0.5 mg/ml $NaBH_4$ to reduce aldehyde groups. The coverslips were rinsed briefly with PBS before incubation for 20 min with TRITC-phalloidin (Sigma, Buchs, Switzerland). Coverslips were mounted in Fluorsave (Calbiochem, La Jolla, CA) after a rinse in PBS.

For tubulin staining the cells were rinsed briefly with PBS, fixed for 20 min with 3% paraformaldehyde prewarmed to 37°C, washed with PBS and incubated with 0.1% Triton X-100 in PBS for 3.5 min. The coverslips were first incubated for 20 min with anti-α-tubulin monoclonal antibody (Clone DM-1; Sigma) at a 1:500 dilution and then for 20 min with Cy3 monoclonal IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at a 1:500 dilution. The coverslips were gently washed 2× at room temperature with PBS between each step for a total of 20 min. After briefly rinsing the coverslips with PBS, the cells were mounted in Fluorsave and viewed under an Axiophot microscope (Zeiss, Zürich, Switzerland).

Northern Blotting

Cells were grown for 14 days in 150 mm tissue culture dishes and made quiescent for 48 h prior to experiments. Twelve abrasions were applied per dish with a razor blade as described above and the cells washed once with PBS before stimulation with or without PDGF-BB (100 ng/ml) for 3, 6, and 24 h. Total RNA was isolated from dishes with and without abrasions [Chomczynski and Sacchi, 1987]. RNA (10 µg) were electrophoresed in a 1% agarose gel containing 2% formaldehyde and transferred overnight by capillary blotting onto nylon membranes (Hybond; Amersham, Zürich, Switzerland) in 10 × SSC. Membranes were baked at 80°C for 2 h. Hybridization was performed with ^{32}P-labelled cRNA probes for bovine or mouse u-PA and bovine PAI-1 as previously described [Pepper et al., 1993].

Statistical Analysis

All values are shown as mean ± SEM. Global hypothesis was tested using the StatView program (Abacus Concepts, Inc., Berkeley, CA) on a Macintosh personal computer with the Friedman test. If rejected, comparisons between means were evaluated with the Mann-Whitney U-test. Resulting *P* values were corrected for

multiple comparisons by the Bonferroni-Holm method.

RESULTS

The organization into cords by endothelial cells (cfBAEC) that were selected for this study progresses through consecutive stages: after the formation of a monolayer, "sprouting cells" emerge (1–3 days after plating), which then develop into wide cords (3–5 days). These subsequently remodel into thin and elongated cords (5–14 days) [Cotta-Perreira et al., 1980; Iruela-Arispe et al., 1991; Battegay et al., 1994].

PDGF-BB Increases the Movement of Single Endothelial Cells in Cords

To follow the movements of single endothelial cells and entire cords, video-timelapse microscopy was performed. cfBAEC were cultured to an early stage of cord formation (7 days), i.e., at a time where cords begin to emerge from the monolayer of endothelial cells [Battegay et al., 1994]. Subsequently, cells were rendered quiescent. Ten single, discernable endothelial cells in cords were tracked during three 20-min intervals of the first, second, fourth, seventh, and fifteenth hour for both of the 24-h treatments (diluent, PDGF-BB). In other words, 30 measurements were performed per hour and a total of 150 measurements made for each condition. The chosen cells were randomly distributed over the whole length of the cords.

Compilation of the data of all 20-min intervals and all cells demonstrated an average increase of 67 ± 15% (n = 3, $P = 0.0002$) in the moving speed of single endothelial cells in cords to PDGF-BB (100 ng/ml) as compared to diluent-treated control. Diluent-treated cells were moving at an average speed of 4.4 ± 0.2 μm/h and PDGF-BB-treated cells at 6.5 ± 0.4 μm/h. Moving speed slowly increased with time (Fig. 1) from 5.2 ± 1 μm/h (12 ± 20% over diluent control) for the first hour to 6.8 ± 1 μm/h (36 ± 57% over diluent control) for the fifteenth hour after addition of PDGF-BB. The respective speeds for diluent treatment are 4.8 ± 0.4 μm/h for the first hour and 3.7 ± 0.9 μm/h for the fifteenth hour. Thus, PDGF-BB-treated cells accelerated and diluent-treated cells decelerated with time, leading to an increased difference in speed with time (Fig. 1). Furthermore, cells often abruptly changed direction of movements. In 10% of all measurements cells did not move at all.

PDGF-BB Increases the Movement of Entire Cords

The apparent movement of single cells might also be due to the movement of the underlying cord structure. We therefore tracked the movement of entire cords. Four different branching points of cords, i.e., cord-intersections, were tracked at 200-min intervals during the two 24-h treatment periods (28 measurements for each condition) and the average speed during 24 h was determined. Figure 2 shows an example of the lines that were drawn along cord axes. Subsequently, the movement of the cord-intersections were followed (intersection points in Fig. 2). Superposition of two pictures that were taken 7 h apart reveals that "cords" (i.e., intersection points) moved more in response to PDGF-BB treatment (Fig. 2F) than when compared to diluent control (Fig. 2C). As can be seen from Figure 2, movement of cords not only consisted of lateral movement but also of elongation and shortening. Furthermore, changes in the angles between cord axes with PDGF-BB treatment seemed to exceed those of diluent control (Fig. 2).

PDGF-BB increased the movement of entire cords as defined by the movement of intersection points by 53 ± 12% over diluent-treated controls (n = 3, $P < 0.02$). The absolute speed of diluent and PDGF-BB treated cells was 13.2 ± 1.2 μm/h and 19.9 ± 1.2 μm/h, respectively. Thus, the apparent speed of cords seems to exceed the speed of single cells. While cords were moving continuously, single cells intermittently stopped migrating, thereby reducing the mean speed. These discordant types of movement suggest that single cells and cords can move independently from each other.

Taken together, these experiments raise the possibility that both single cells and entire cord structures move in response to PDGF-BB and that the apparent movement of single cells within the cord measured by video-timelapse microscopy is due to the sum of the movements of the entire underlying cord structure as well as single endothelial cells.

PDGF-BB Elicits Migration of Cord-Forming Endothelial Cells Into an Abrasion

To quantitate the migratory response of single endothelial cells to PDGF and to test whether PDGF-elicited migration contributes to endothelial repair in vitro, a "wound" (abrasion) was

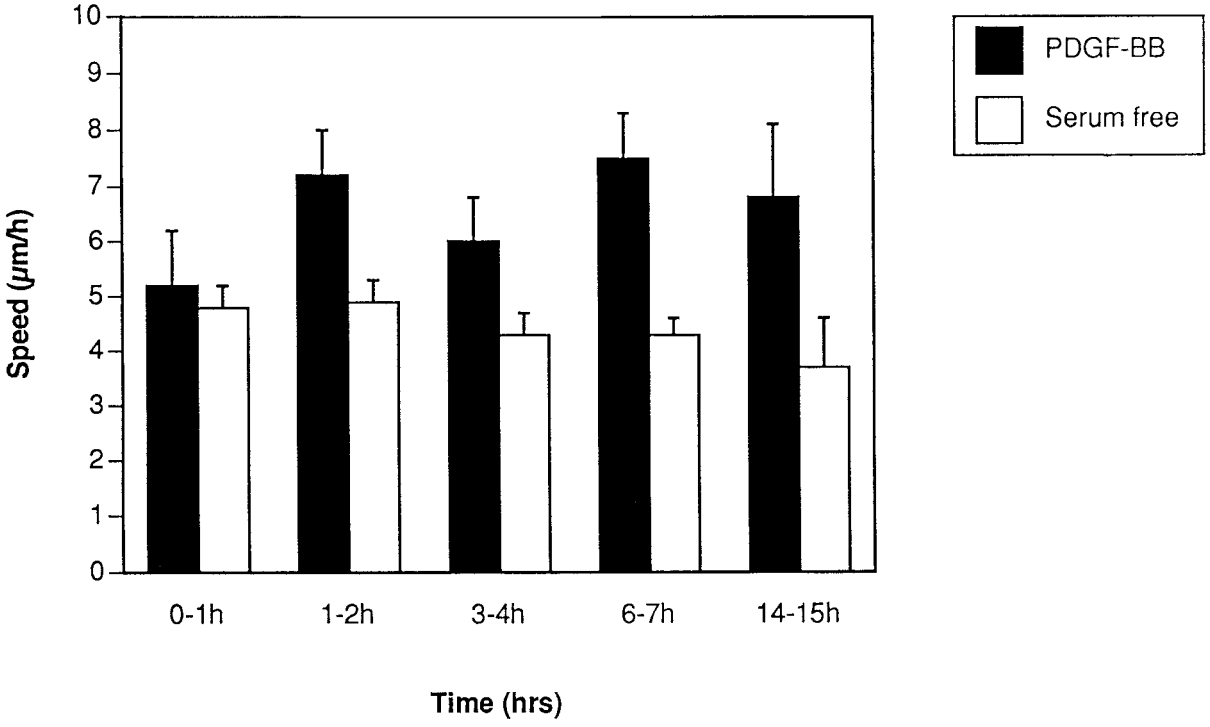

Fig. 1. PDGF-BB increases the speed of single cell movement in endothelial cords. Cells were grown for 7 days and then rendered quiescent in serum-free DMEM for 48 h. Single cells were tracked by video-timelapse microscopy in serum-free DMEM containing diluent for 24 h and with PDGF-BB (100 ng/ml) for another 24 h. Photographs were taken every 2 min. The speed of 10 single cells was determined for each of the three 20-min segments of the different 1-h intervals and then compiled. Values are shown as mean ± SEM.

applied with a razor blade to confluent endothelial cell monolayers containing fully established (14 days) cords. Subsequently, the cells were cultured for 24 h in the presence of diluent, PDGF-AA, PDGF-BB, or 5% FCS (Fig. 3A). Treatment of cells for 24 h did not lead to increased total cell number (data not shown). Thus, the number of cells counted within the abrasion represented migrating cells only.

The number of cells that had migrated into the abrasion, the distance from the edge of the abrasion to the border of cells migrating into the abrasion, and the number of elongated cells found within the abrasion were determined. Neither PDGF-AA nor PDGF-BB significantly increased the total number of cells migrating into the abrasion ($n = 5$, $P > 0.3$) (Fig. 4). However, PDGF-BB increased the distance to which the cells migrated in a concentration dependent manner (56 ± 6% over diluent controls, $n = 5$, $P < 0.005$) (Fig. 5). The average distance to which the cells migrated in response to PDGF-BB (100 ng/ml) was 744 ± 48 μm (average speed of 31 μm/h). PDGF-AA had no effect. For PDGF-AA (100 ng/ml) the average distance cells migrated was 467 ± 31 μm (average speed 19 μm/h). FCS (5%), used as a positive control, increased both the number of migrating cells (125 ± 0.04%, $n = 5$, $P < 0.005$) as well as the distance to which the cells migrated (73 ± 4% over diluent controls, $n = 5$, $P < 0.0005$). The average distance that cells migrated during 24 h was 475 ± 31 μm for diluent and 822 ± 31 μm for FCS (average speed was 20 μm/h for diluent and 34 μm/h for FCS).

Furthermore, PDGF-BB increased the number of elongated, i.e., possibly migrating or "activated," endothelial cells found within the abrasion (Figs. 3B and 6) as compared with serum-free control ($n = 5$, $P < 0.005$). The number of elongated cells per 2.5 mm of abrasion was 5 ± 0.9 for serum-free control. Surprisingly, FCS did not significantly increase the number of elongated cells found in the abrasions (number of elongated cells per 2.5 mm of abrasion was 8 ± 1.2). Thus, only PDGF-BB, but not FCS or PDGF-AA, increased the number of elongated endothelial cells within the abrasion.

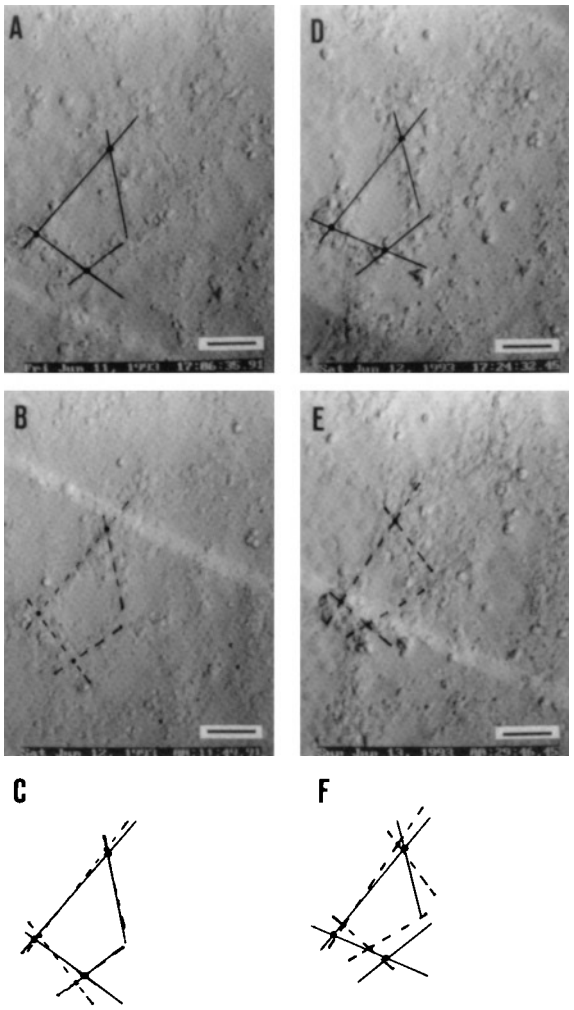

Fig. 2. PDGF-BB increases the movements of entire cords. Two sequential pictures 7 h apart (i.e., about twice the quantified unit of 200 min) were chosen for each treatment (diluent, PDGF-BB) to better visualize cord movements. Lines were drawn along cord axes and points of intersection were followed. **A:** Diluent at 180 min. **B:** Diluent at 580 min. **C:** Superposition of A and B. **D:** PDGF-BB at 180 min. **E:** PDGF-BB at 580 min. **F:** Superposition of D and E. A greater degree of cord movement, cord shortening, and change of angles between cords is seen in F when compared to C. Bar = 500 μm.

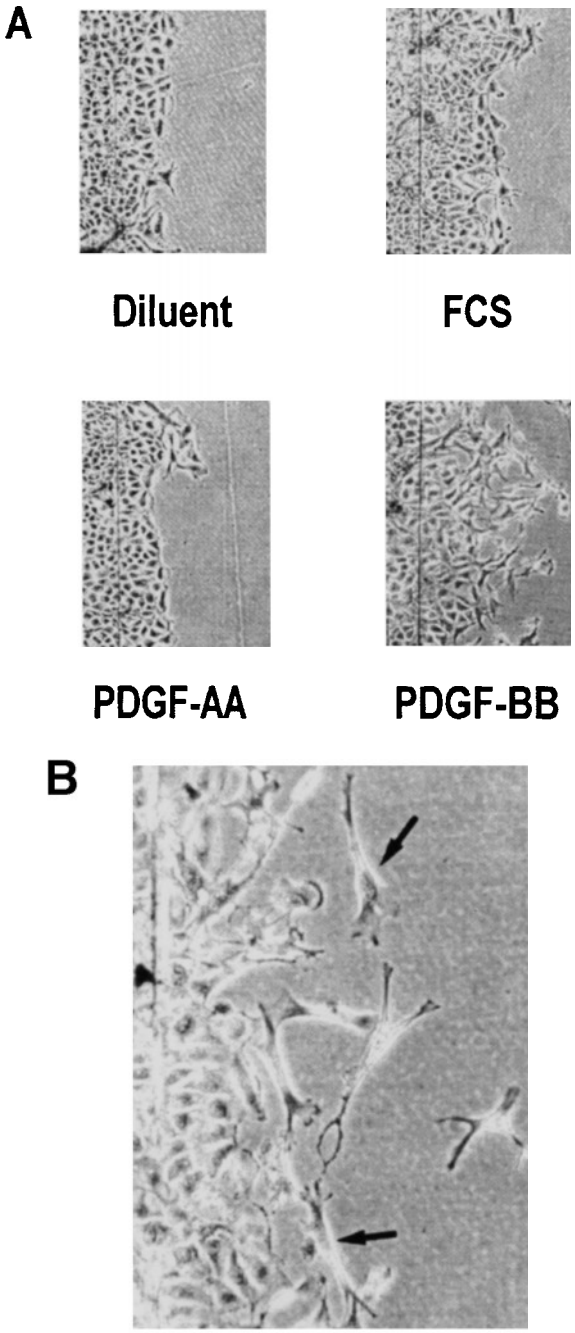

Fig. 3. PDGF-BB induces endothelial cell migration into abrasions. **A:** Cells were grown for 14 days and then rendered quiescent. After applying an abrasion with a razor blade, the cells were washed twice in serum-free DMEM and treated for 24 h with either diluent, FCS (5%), PDGF-AA, or PDGF-BB. Photographs were taken at a magnification of ×32. **B:** Typical elongated cfBAEC seen following treatment with PDGF-BB (designated by arrows). Magnification ×64.

PDGF-BB Does not Visibly Affect Cytoskeletal Structures in Cord-Forming Endothelial Cells

Movement of cells can be accompanied by visible reassembly of the cytoskeleton. In order to identify potential changes of cytoskeletal structures, actin and tubulin stainings were performed. cfBAEC were grown for 14 days on glass coverslips and abrasions were applied as described above. The cells were then incubated for 24 h with or without PDGF-BB (100 ng/ml).

Stainings for actin and tubulin revealed no difference in cytoskeletal structure between cultures with and without PDGF-BB. However, there were differences in the structure of actin filaments between cells situated in the monolayer and those cells situated in cords of the

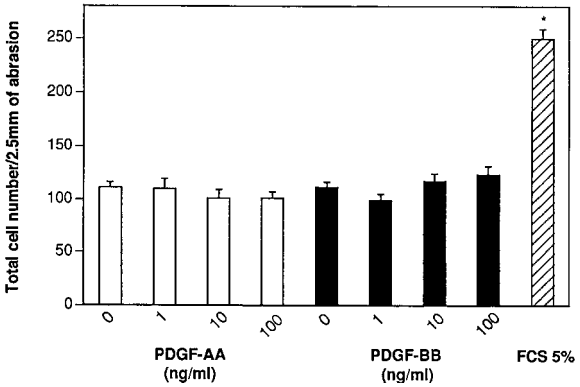

Fig. 4. PDGF-BB and PDGF-AA do not increase the total number of cells migrating into abrasions. Cells were treated as described for Figure 2 and counted on photographs over a length corresponding to 2.5 mm in the original culture. An increased number of cells in the abrasions was measured in response to FCS (250 ± 9, n = 5, *P = 0.0003).

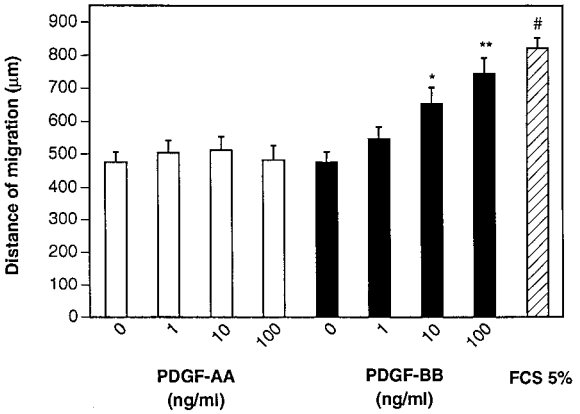

Fig. 5. PDGF-BB increases the distance to which endothelial cells migrate into abrasions in a dose-dependent manner. Distance from the abrasion to the leading edge of migrating cells was measured on photographs as described for Figure 2 (*P = 0.02; **P = 0.002; #P < 0.0001).

same culture. Cells in the monolayer exhibited actin fibers in an irregular pattern that converged at focal contacts. In contrast, actin fibers were straight and parallel to the longitudinal axes in cells situated in cords. In elongated cells within abrasions, mainly found upon PDGF-BB treatment (see above, Figs. 3B, 6), actin fibers were arranged in a regular pattern parallel to the cellular axes (data not shown).

Tubulin staining revealed that cells at the edge of abrasions reoriented their centrosomes. This reorientation seemed to be independent of PDGF-treatment and to occur as a consequence of the application of the wound (data not shown). There were no discernable differences in tubulin staining irrespective of cellular location (data not shown).

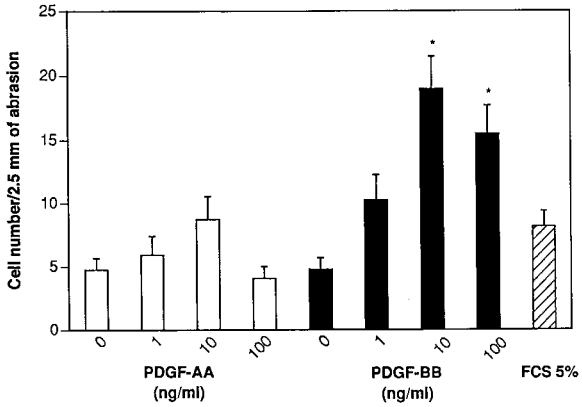

Fig. 6. PDGF-BB, but not PDGF-AA, increases the number of elongated cells in abrasions. Elongated cells (less than 50 μm wide and more than 125 μm long) were counted over a length corresponding to 2.5 mm in the original culture. Treatment with FCS (5%) did not yield an increased number of elongated cells while PDGF-BB did (*P < 0.01).

These experiments suggest that the cellular context of cfBAEC may affect the phenotype of actin but not of tubulin filaments. They further suggest that PDGF-BB has no major discernable effect on the appearance of cytoskeletal structures and that PDGF-BB-induced cord movement is not associated with actin reassembly and centrosome reorientation.

PDGF-BB Does Not Modulate Expression of u-PA and PAI-1 in Cord-Forming Endothelial Cells

Proteases are involved in extracellular matrix degradation during cell migration and angiogenesis. One of the proteases involved in tissue remodeling is urokinase-type plasminogen activator (u-PA) [Menashi et al., 1993; Pepper et al., 1996b]. We therefore assessed expression of u-PA and a corresponding inhibitor, PAI-1, by Northern blotting in cfBAEC with fully established cords. Dishes with and without abrasions were incubated with or without PDGF-BB (50 ng/ml) for 3, 6, and 24 h. u-PA mRNA was detectable at low levels, and neither wounding nor addition of PDGF-BB appeared to affect its expression (data not shown). PAI-1 transcripts were not detectable under any of the above-mentioned conditions. Because the fraction of cells migrating in response to PDGF-BB into abrasions is extremely small, modulation of u-PA mRNA would not be expected to be detectable by Northern blotting. However, the fraction of cells that express PDGF β-receptors in cfBAEC with fully established cords is substantial [Battegay et al., 1994]. Therefore, the lack of modulation of u-PA mRNA

in response to PDGF-BB suggests that the movement of entire cords does not require an increase in u-PA expression.

DISCUSSION

The role of PDGF in angiogenesis and its mode of action, direct or indirect, are not yet fully established. PDGF-BB induced an angiogenic response in chick chorioallantoic membrane [Risau et al., 1992], in wounds [Reuterdahl et al., 1993], in soft tissue appendages [Khouri et al., 1994], as well as during the formation of vascular anastomoses after ischemia of hind limbs in rats [Brown et al., 1995]. Melanoma cells transfected with PDGF-B chain and subsequently injected into nude mice were surrounded by a highly vascularized connective tissue stroma [Forsberg et al., 1993]. All of these in vivo studies suggest that PDGF is associated with angiogenesis as a prominent element of tissue remodeling. Furthermore, PDGF-BB induced new tissue de novo in an in vivo growth chamber, presumably by recruiting highly proliferative mesenchymal progenitor cells, smooth muscle cells, and perhaps endothelial cells [Khouri et al., 1994]. Further in vivo studies have substantiated the possibility that PDGF might induce the migration of endothelial cells [Risau et al., 1992; Reuterdahl et al., 1993; Khouri et al., 1994; Brown et al., 1995], a prerequisite for angiogenesis [Risau, 1990; Folkman and Shing, 1992]. Nevertheless, these studies do not allow us to distinguish between the direct effects of PDGF on relevant endothelial cells (direct angiogenesis) and the recruitment or stimulation of inflammatory and connective tissue cells, which in turn release angiogenic factors (indirect angiogenesis).

The potential direct role of PDGF in angiogenesis has been addressed in various models in vitro. For example, PDGF-BB stimulated capillary tube formation in cocultured microvascular fragments and myofibroblasts isolated from rat epididymal tissues [Sato et al., 1993]. In this system, PDGF-BB was unable to directly stimulate capillary growth in the absence of myofibroblasts [Sato et al., 1993]. Recent studies suggest that PDGF can also directly affect endothelial behaviour and angiogenesis. PDGF receptors can be modulated during endothelial morphogenesis and remodeling in vitro [Battegay et al., 1994; Marx et al., 1994]. PDGF receptor expression observed in microvascular endothelial cells was downregulated when endothelial cells formed cords and tubes and became quiescent in a gel composed of extracellular matrices [Marx et al., 1994]. Recently, we found that PDGF-BB increased proliferation and formation of cord structures in cloned bovine endothelial cells that spontaneously form cords in the presence of serum [Battegay et al., 1994]. In these experiments, PDGF-BB contributed to cord formation via direct interaction with PDGF β-receptors which were expressed on forming cords to a much greater extent than on cells of the underlying monolayer [Battegay et al., 1994]. In both models [Battegay et al., 1994; Marx et al., 1994], cellular quiescence appears to be associated with a down-regulation of PDGF β-receptors. These studies [Battegay et al., 1994; Marx et al., 1994] have thus far not allowed us to determine the factors that regulate the expression of PDGF β-receptors on endothelial cells. Yet it is reasonable to suppose that endothelial morphogenesis and angiogenesis can be affected at one time or another by the activation of PDGF β-receptors in response to PDGF-BB.

PDGF-BB can also affect endothelial migration; rabbit retinal endothelial cells [Koyama et al., 1994b] and rat brain capillary endothelial cells [Risau et al., 1992] migrated in response to PDGF. In our study, using video-timelapse microscopy, the speed of endothelial cell movement was increased upon addition of PDGF-BB. Video-timelapse microscopy experiments did not allow us to fully assess the migratory response of single endothelial cells to PDGF. However, PDGF-BB concentration-dependently increased the distance to which the cells migrated into abrasions applied to confluent monolayers of endothelial cells. Thus, PDGF-BB elicited the migration of single endothelial cells and thereby contributed to endothelial repair. PDGF-AA had no such effect. These observations are in line with our previous results. cf-BAEC expressed PDGF β-receptors but not PDGF α-receptors [Battegay et al., 1994], which are required for cells to respond to PDGF-AA. Furthermore, only a fraction of cells, i.e., those in cords [Battegay et al., 1994] expressed PDGF β-receptors, and it is only these cells that can respond to PDGF-BB by migrating into the abrasion. Because PDGF β-receptor expressing cells represent only a fraction of all cells in the culture, total cell numbers found in an abrasion were not appreciably increased in response to PDGF-BB, whereas the distance to which they migrated was substantially increased.

Migrating cells have previously been shown to disassemble their actin cytoskeleton [Westermark et al., 1990; Ferns et al., 1990] and to reorient their centrosomes [Ettenson and Gotlieb, 1992, 1993]. In line with these studies, we observed centrosome reorientation in cells at the edges of abrasions. However, this reorientation seemed not to be influenced by PDGF-BB. In contrast to PDGF-receptor transfected fibroblasts [Ferns et al., 1990] and endothelial cells [Westermark et al., 1990], no actin breakdown or membrane ruffling was observed in response to stimuli in cfBAEC, suggesting that ruffling is relatively specific for certain cell types [Ferns et al., 1990]. Migrating endothelial cells had their actin bundles arranged along the cell axis and demonstrated no dense peripheral band in line with a previous description in porcine endothelial cells [Ettenson and Gotlieb, 1992]. In contrast, resting cells within the monolayer showed central fibers that were randomly distributed and a dense peripheral band.

In addition to the increase of single endothelial cell movement, whole cord structures increased their moving speed. Hence, the movements of single cells within cords are composed of movements of the cells themselves and the movements of the underlying cords. Similar to an observation in areas of shear-stressed endothelium in vivo [Gottlieb et al., 1991] actin bundles within cords seemed to extend beyond single endothelial cells, thus creating the impression of single microfilament bundles extending throughout the cord. Possibly, the strong interconnecting attachments of endothelial cells organized in cords allow them to respond passively or actively to traction [Vernon et al., 1992]. Interestingly, cellular traction might be involved in angiogenesis by guiding cells through extracellular matrix and by forming pathways for cellular migration [Vernon and Sage, 1995]. The addition of cytochalasin D, i.e., the blocking of the development of an actin cytoskeleton, abrogates the ability of cord-forming endothelial cells to form cords and to exert tension on the supporting extracellular matrix [Vernon et al., 1992].

While centrosome reorientation could be observed in single migrating cells, no changes in tubulin structure were seen in endothelial cords. This suggests that cord movement is not associated with major changes in actin or tubulin structure. Hypothetically, cord movements could be due to a contractile response of the cells to PDGF-BB that does not require actin reassembly. Taken together, these results suggest that active and passive cord movements play a role in endothelial morphogenesis. However, the potential role of the movement of entire endothelial structures in angiogenesis needs to be examined further.

Proteases involved in extracellular matrix degradation are important in endothelial cell migration and in angiogenesis. u-PA is secreted by endothelial cells and locally activates plasmin which in turn degrades components of the extracellular matrix [Menashi et al., 1993; Pepper et al., 1996b]. In our experiments we found a low level expression of u-PA and no PAI-1 expression. Similarly, endothelial cells in vivo do not appear to produce significant amounts of PAI-1 [Christ et al., 1993; Pepper et al., 1996b]. u-PA was expressed in endothelial cells forming cords during development of corpus luteum and disappeared after endothelial cells completed neovascularization [Bacharach et al., 1992]. Although modulation of u-PA expression in a small fraction of cells migrating in response to PDGF-BB into an abrasion is unlikely to be detectable by Northern blotting, neither PDGF-BB nor multiple wounding of endothelial monolayers containing cords appeared to modulate u-PA expression. As hypothesized above, the movements of entire cords to PDGF-BB might correspond to a contractile response that neither requires cytoskeletal reassembly nor u-PA expression. Thus, endothelial cord movements may require mechanisms which are different from those required for simple endothelial migration.

We conclude that PDGF-BB can induce movement of entire cords and migration of single endothelial cells in vitro. Possibly, cord-movement and single endothelial cell migration correspond to two mechanistically different processes in response to PDGF-BB. PDGF-BB may thus contribute to angiogenesis and endothelial repair by directly activating PDGF β-receptor-expressing endothelial cells.

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