Polarized Secretion of a Platelet-derived Growth Factor–like Chemotactic Factor by Endothelial Cells In Vitro

Hans-Günter Zerwes and Werner Risau

Max-Planck-Institut für Entwicklungsbiologie, D-7400 Tübingen, Federal Republic of Germany

Abstract. Cultured bovine aortic endothelial cells secrete a potent migration-stimulating factor for vascular smooth muscle cells (SMCs) and adventitial fibroblasts. Vascular pericytes are 20-fold less responsive, and endothelial cells themselves do not respond at all. Checkerboard analysis of SMC migration in a microchemotaxis chamber assay shows that the factor is chemotactic. Chemotactic activity for SMCs and adventitial fibroblasts is specifically inhibited by antibodies against platelet-derived growth factor. Endothelial cells cultured on nitrocellulose filters secrete the platelet-derived growth factor–like factor almost exclusively into the basal compartment. We suggest that this factor plays an important role in the recruitment of vascular wall cells during the morphogenesis of blood vessels and pathological conditions, such as atherosclerosis.

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DIRECTED cell migration plays a major role during embryonic development. Neural crest cell migration (25), for example, is a key event of morphogenesis. The development of the vascular system similarly involves the migration of vascular sprouts into developing tissues and organs (10, 31). The migration of endothelial cells, which is an important event of angiogenesis, can be induced by soluble factors in vitro as well as in vivo (1, 10, 12, 32, 39).

The earliest blood vessels are derived from the so-called primordial capillary plexus, which differentiates from the blood islands in the splanchnopleuric mesoderm. Endothelial cells are the only constituent of these vessels. The question of how arteries and veins develop from these early vessels has been addressed by only few early morphological studies (11, 22). The development of adventitial cells of the capillaries in the tadpole tail has been analyzed by Clark and Clark (6). Their results, as well as the results from chimeric chick-quail embryos (24, 26), strongly suggest that vascular wall cells are derived from a different cell lineage than endothelial cells. The tracings of Clark and Clark (6) indeed indicate that cells may be attracted by the endothelium. From these observations, our working hypothesis emerged that endothelial cells themselves regulate the character of the vascular wall. Accordingly, aortic endothelial cells would interact with smooth muscle cells and capillary endothelial cells would interact with pericytes. Endothelial and smooth muscle cell (SMCs)[1] cocultures have provided evidence to support this hypothesis (16, 40). Endothelial cell surface heparan sulfate, for example, inhibits SMC proliferation (4, 5), whereas endothelial cell-derived growth factors stimulate it (15). DiCorleto and co-workers (8, 9) have shown that cultured endothelial cells produce, among other growth factors, a platelet-derived growth factor (PDGF)–like factor. Since PDGF itself has been shown to stimulate SMC and adventitial fibroblast (AF) chemotaxis (3, 18, 37), we have tested whether endothelial cell–derived factors are chemotactic for vascular wall cells. Here we show that a PDGF–like factor is the principal chemotactic factor secreted in a polarized manner by endothelial cells in vitro.

Materials and Methods

Cells

Bovine aortic endothelial cells (BAEC) were obtained by collagenase digestion of bovine thoracic aorta according to the method of Schwartz (36). Cells were cultured in DME supplemented with 10% FCS (Gibco, Grand Island, NY). BAEC-conditioned medium was collected according to the protocol of DiCorleto (8). When BAEC of passage 5, grown in T75 culture flasks (Falcon Labware, Oxnard, CA) had formed a confluent monolayer, the medium was replaced by serum-free DME. After 24 h, the medium was removed and discarded, and fresh serum-free DME was added. At 3-d intervals, the medium was collected, centrifuged to remove cellular debris, and concentrated 20-fold. Protein was determined according to Read and Northcote (30). Dilutions were prepared in serum-free DME. During the collection of BAEC-conditioned medium, the cells had a typical cobblestone-like appearance and showed no morphological signs of damage.

Bovine retinal capillary pericytes were isolated and cultured according to the method of Gitlin and D'Amore (17). The pericytes were identified by their typical morphology and by immunofluorescence using a monoclonal antibody (PC4) shown to be specific for SMCs and pericytes.

In all experiments, primary cultures of pericytes were used, contamination with other cell types (i.e., capillary endothelial cells, SMCs, and astrocyte-like cells) being less than 10% of the total cell number.

SMC cultures were established and grown as described by Ross (33). Cells of passages 1–6 were used in all experiments.

Bovine AFs were derived from explants of the adventitial layer of the bovine aorta and were cultured in DME + 10% FCS. In these experiments, AF's of passages 1–4 were used.

For nitrocellulose filter cultures BAECs (80,000 cells) were seeded into a 30 mm Millicell HA chamber (Millipore, Eschborn, FRG) and placed

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[1] *Abbreviations used in this paper:* AF, adventitial fibroblast; BAEC, bovine aortic endothelial cell; PDGF, platelet-derived growth factor; SMC, smooth muscle cell.

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into the wells of 6-well tissue culture plates (Costar, Data Packaging Corp., Cambridge, MA) containing DME + 10% FCS. After 3 d, during which the cells reached confluency, the medium was replaced by serum-free DME in both compartments of the assembly. After 24 h, the medium was discarded and fresh serum-free DME was added. The conditioned medium was collected at 3-h intervals and concentrated 75-fold.

Silver staining of BAEC cultures on nitrocellulose was performed as described. (14).

Chemotaxis Assay

We used the 48-well micro-chemotaxis chamber (Neuro Probe, Inc., Cabin John, MD) and polycarbonate filters (5 µm pore size, polyvinylpyvrolidine-free; Nuclepore, Tübingen, FRG) in all experiments. Cells were removed from the culture dish with trypsin/EDTA, centrifuged, and resuspended in serum-free DME. Trypsinization time was kept as short as possible (∼2 min). Longer incubation with trypsin resulted in decreased chemotactic response of the same cell type (data not shown).

Chemoattractant was added to the lower chamber and 16,000–20,000 cells in a volume of 40–50 µl were applied to the upper part of the assembly. The chamber was incubated for 5 h at 37°C in a humidified atmosphere containing 5% CO_2/95% air. The filters were removed from the assembly, fixed in methanol, and the cells on the upper side of the filters were wiped off. After staining with Mayer's Hemalum, the filters were embedded in Entellan (Merck, Darmstadt, FRG) between two glass slides. Nuclei of migrated cells were counted at a magnification of 160. Each value represents the mean number of nuclei (± SD) on 10 areas of 0.37 mm^2. Experiments were performed in triplicate.

Antibody

A polyclonal rabbit antibody directed against human PDGF (IgG fraction) (21) was a generous gift from Dr. C.-H. Heldin.

Results

Endothelial Cell-derived Factors Stimulate Cell Migration

Bovine endothelial cell cultures were established from calf aortas. Factors secreted into the serum-free medium by early passage endothelial cells were tested in a modified Boyden-Micro-chamber assay for their ability to stimulate cell migration. Bovine aortic SMCs, bovine aortic AFs, bovine retinal microvascular pericytes, and the endothelial cells themselves were used as target cells. Fig. 1 shows that SMCs and AFs actively migrated in response to the endothelial cell–derived factors in a dose-dependent manner, whereas microvascular pericytes were 20-fold less responsive. Endothelial cells did not respond. The decline of cell migration at higher concentration is usually attributed to the breakdown of the gradient between the two compartments and/or the down regulation of the specific receptors.

We also investigated the possibility that factors which promote cell adhesion might contribute to the migratory response—a phenomenon called haptotaxis (37). If we preincubated filters for 2 h with conditioned medium before performing the migration assay, we could not detect any change in the dose–response relationship (data not shown).

Endothelial Cell-derived Factors are Chemotactic for SMCs

Chemokinesis can be distinguished from true chemotaxis by varying the amounts of factor in the upper and lower compartments of the Boyden chamber (checkerboard analysis). By using this approach, we could demonstrate that the endothelial cell–derived factors are chemotactic for SMCs (Fig. 2).

A PDGF-like Factor is the Principal Chemotactic Factor

A polyclonal antibody against PDGF, which inhibits its mitogenic activity (21) was used to evaluate whether PDGF-like activity could be responsible, solely or in part, for the chemotactic activity. Fig. 3 shows that SMC (*a*) and AF (*b*) chemotaxis is inhibited by antibodies against PDGF. Normal rabbit antibodies have no effect on cell migration. At a concentration of ∼10 µg IgG/ml of PDGF antibodies, a 50% inhibition of migration as compared to control IgG is observed. Greater than 75% inhibition occurs at high concentrations of antibody (above 40 µg/ml). Inhibition of migra-

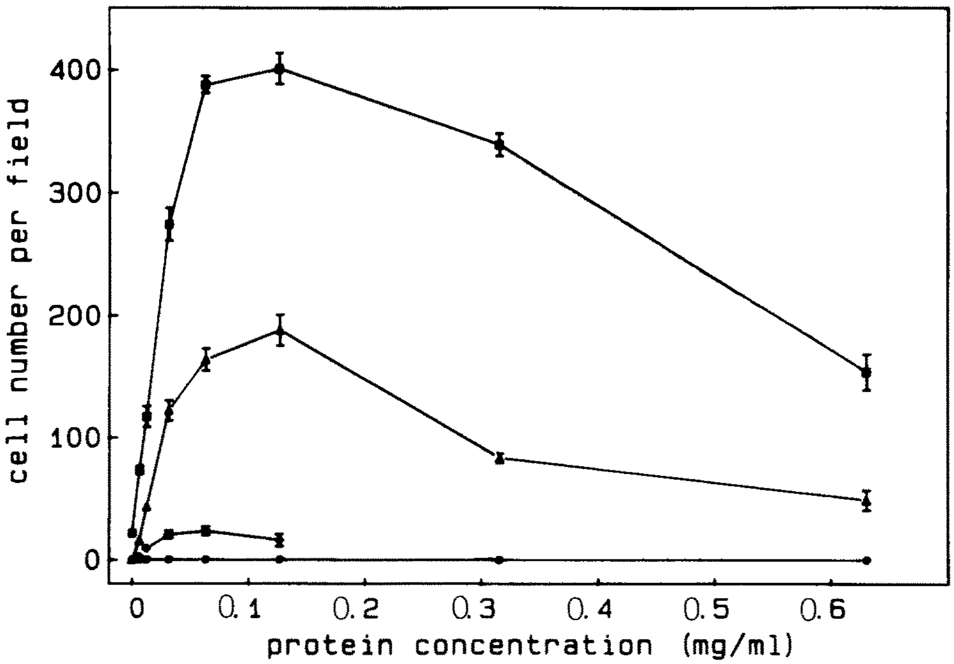

Figure 1. Migration of different cell types: SMCs (■), BAECs (●), AFs (▲), and pericytes (♦). The number of cells migrated in response to increasing concentration of BAEC-conditioned medium was determined.

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tion is similar for SMC and AF. Purified human PDGF (gift from B. Westermark, Uppsala, Sweden) also stimulates SMC chemotaxis in a dose-dependent manner which is inhibitable by anti-PDGF antibodies (our unpublished results).

Endothelial Cells Secrete the PDGF-like Chemotactic Factor in a Polarized Manner

Endothelial cells cultured on nitrocellulose membranes offer the advantage to analyze conditioned medium from the api-

Protein concentration in lower chamber (μg/ml)	Protein concentration in upper chamber (μg/ml)			
	0	5.5	10	55
0	0	10 ± 2	105 ± 8	35 ± 5
5.5	113 ± 15	134 ± 13	107 ± 6	55 ± 5
10	158 ± 15	143 ± 11	145 ± 17	94 ± 13
55	280 ± 13	216 ± 6	207 ± 15	130 ± 14

Figure 2. Checkerboard analysis of SMC migration. The data represent cell numbers per 0.37 mm² field (mean ± SD). The values on the diagonal indicate chemokinetic migration and the values below the diagonal indicate chemotaxis.

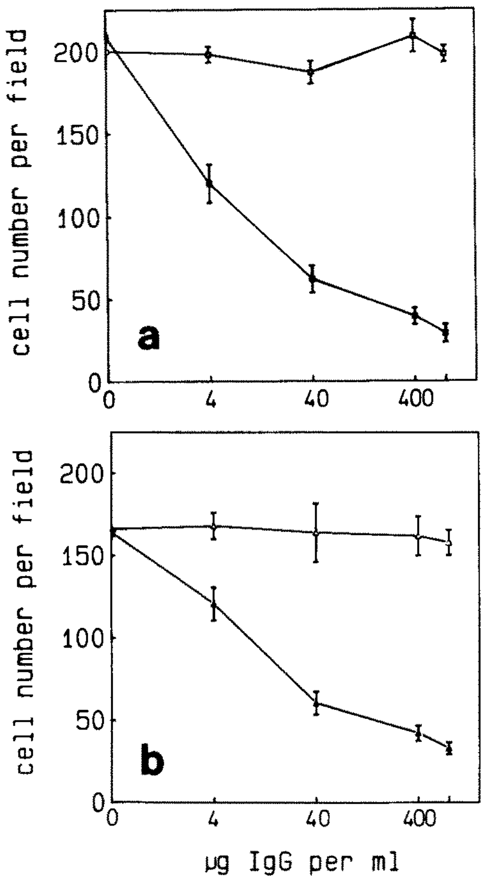

Figure 3. Inhibition of migration by anti-PDGF antibodies. (*a*) Inhibition of migration of SMCs (■) was tested at a constant dose of 0.03 mg/ml protein of the BAEC-conditioned medium while increasing the concentration of anti-PDGF IgG. (*b*) Inhibition of AF migration (▲) was tested at a constant dose of 0.06 mg/ml protein while increasing the concentration of anti-PDGF IgG. Normal rabbit IgG at the same concentration was added as a control (□, △).

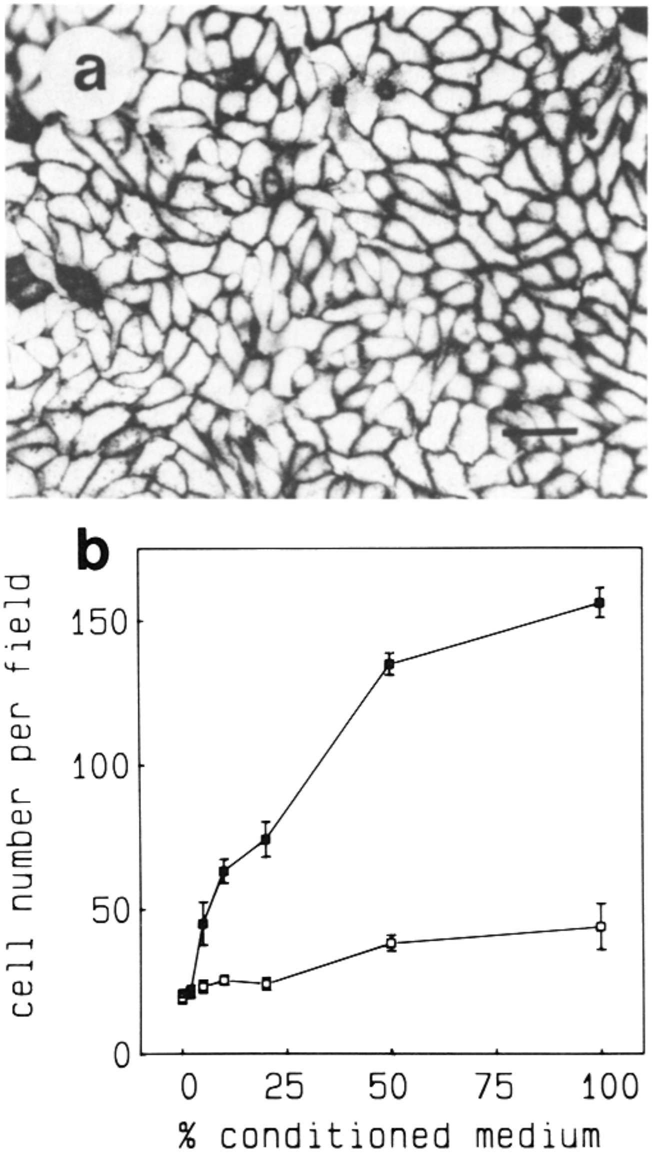

Figure 4. (*a*) Silver staining of a confluent monolayer of BAECs cultured on nitrocellulose membranes in Millicell chambers. (*b*) Chemotactic activity for SMCs of the BAEC-conditioned medium from the upper (□) and lower (■) compartments of Millicell chambers. Medium was collected every 3 h, pooled, dialyzed against distilled water, and concentrated 75-fold by lyophilization. This concentrated preparation was designated 100% BAEC-conditioned medium. Dilutions thereof were used to test SMC chemotaxis in a dose-dependent manner. The protein concentration of 100% BAEC-conditioned medium from the lower compartment was 0.25 mg/ml; from the upper compartment, 0.15 mg/ml. Bar, 50 μm.

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cal and basolateral compartments. Aortic endothelial cells form a monolayer when cultured on nitrocellulose (Fig. 4 *a*). This culture system is, therefore, comparable to the in vivo situation where the apical surface forms a nonthrombogenic surface, whereas a basement membrane-like extracellular matrix is laid down basally. A further indication of an in vivo-like situation is the silver staining of cell junctions of confluent monolayers (14). Fig. 4 *a* shows the "silver lining" of a confluent monolayer of aortic endothelial cells cultured on nitrocellulose. Serum-free conditioned medium from the upper and lower compartments of such cultures was collected and tested for chemotactic activity.

In our initial experiments using medium conditioned for 48 h, we found nearly equal amounts of chemotactic activity in the two compartments. To give an indication of the rate of exchange of macromolecules between the two compartments, we added different amounts of radiolabeled ovalbumin to the lower or the upper compartments and measured the permeability of the endothelial monolayer over time. We found that after ~20 h the radioactive tracer had equilibrated between the two compartments. We therefore collected conditioned medium from both compartments every 3 h during which time only 5% of the radioactive tracer was found on the other side of the monolayer (data not shown). Using conditioned medium collected in this manner, we found the major amount of chemotactic activity in the basal compartment (Fig. 4 *b*). We have obtained this result with BAECs derived from different primary cultures used at different passages (p4, p7, and p8). The fraction of total chemoattractant found in the lower compartment was 66-80% in all experiments.

In a comparative study, we assessed the chemotactic activity of unconcentrated medium conditioned for 3 h by BAECs cultured on plastic vs. nitrocellulose. The total level of activity released by cells grown on plastic was lower (38 ± 5 migrated cells per field) than that secreted by cells grown in Millicell chambers (37 ± 6 cells per field for the upper compartment and 64 ± 7 cells per field for the lower compartment); the number of cells that migrated in the absence of chemoattractant was 14 ± 3.

Discussion

Our results demonstrate that endothelial cells in vitro secrete a potent chemotactic factor for vascular SMCs and fibroblasts. The factor is immunologically related to PDGF and exhibits the same target cell specificity as PDGF. Purified human PDGF is undistinguishable from the PDGF-like factor secreted by BAECs in vitro as far as chemotactic activity and inhibition by anti-PDGF antibodies is concerned. This is consistent with the data for the mitogenic activity of an endothelial cell-derived PDGF-like factor which competes with PDGF in a radio receptor assay (9). Endothelial cells do not secrete autocrine motility factors, such as those described recently for tumor cells (27). Although other factors are present in endothelial cell-conditioned medium (8, 29), our results suggest that endothelial cell growth factors, or fibroblast growth factors, which are known to stimulate endothelial cell migration and chemotaxis (1, 31, 39), are not secreted by endothelial cells in vitro. We could not detect, in the conditoned medium, any fibroblast growth factor activity.

The weak response of pericytes suggests that these cells may need other chemotactic factors, such as platelet factor 4 (3), which are not produced by endothelial cells in vitro. According to our model of vascular wall development, mesenchymal cells would be expected to be candidate motile cells capable of differentiation into pericytes or SMCs. We have observed that the endothelial cell-derived PDGF-like factor is strongly chemotactic for mouse embryo fibroblasts (our unpublished observations).

We conclude that the PDGF-like factor is the principal chemotactic factor, because we observed more than 75% inhibition of chemotaxis using specific neutralizing antibodies.

A prerequisite for our hypothesis—that an endothelial cell-derived chemotactic factor could be responsible for the development of the vascular wall—is the secretion of this factor albuminally. Taking advantage of a nitrocellulose culture system frequently used for epithelial cell cultures (13), we were able to show that endothelial cells, cultured as a confluent in vivo-like monolayer on nitrocellulose, secreted chemotactic factors almost exclusively into the basal compartment. Aortic endothelial cells do not form an impermeable barrier as do tight epithelia. The exchange of labeled ovalbumin between the two compartments probably takes place either through junctions or holes in the monolayer generated by dead cells under serum-free culture conditions. Taking into account the exchange of macromolecules even during a 3-h culture period, one could calculate that the little chemotactic activity found in the upper compartment could be due to molecules derived from the basal compartment or to dead cells. Previous biochemical and histochemical work has indicated that endothelial cells, in vivo as well as in vitro, are polar cells (28, 38). Our results show that BAECs also secrete a chemotactic factor in a polar fashion.

The role of PDGF-like factors in vivo is unknown. Apart from their role as products of oncogenes in neoplastic transformation (19), several potential roles have been proposed in wound repair, atherosclerosis, and other diseases taking into account both its mitogenic and chemotactic properties (34, 35). Our results of a PDGF-like chemotactic factor basally secreted by endothelial cells implies its further involvement in vascular wall development as well as atherosclerosis.

Provided that the PDGF-like chemotactic factor is homologous to PDGF, it will be important to determine whether the B-chain gene of PDGF, which is homologous to the sis-oncogene, or the A-chain gene, which is likely to give rise to a secreted PDGF molecule (20), or both, are required for the chemotactic factor. In vivo and in vitro studies on the expression of these genes, like those performed using the sis-gene (2, 7, 23) would provide valuable information about the regulation of mitogenic and chemotactic factors during development and disease.

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