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Human microvascular endothelial cells express receptors for platelet-derived growth factor

(angiogenesis/neovascularization/tyrosine kinases)

JULIE G. BEITZ, IN-SOON KIM*, PAUL CALABRESI, AND A. RAYMOND FRACKELTON, JR.†

Department of Medicine, Roger Williams General Hospital and Brown University, Providence, RI 02908

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ABSTRACT **Endothelial cells have been widely thought to be unresponsive to platelet-derived growth factor (PDGF, a major growth factor released from stimulated platelets at the sites of vascular insults) and devoid of PDGF receptors. Nevertheless, in examining the growth-factor responses of microvascular endothelial cells isolated from human omental adipose tissue, we were surprised to detect PDGF-induced tyrosine phosphorylation of a 180-kDa glycoprotein, subsequently identified as the cellular receptor for PDGF by specific immunoprecipitation. Scatchard analysis of ^{125}I-labeled PDGF binding to human microvascular endothelial cells revealed 30,000 PDGF receptors per cell with a K_d of 0.14 nM. PDGF stimulated tyrosine phosphorylation of PDGF receptors and other cellular proteins in a dose- and time-dependent manner, with half-maximal receptor phosphorylation occurring at 0.3 nM recombinant human PDGF (B chain) and a ≤1-min exposure to PDGF. Normal cellular consequences of receptor activation were also observed, including tyrosine phosphorylation of a 42-kDa protein and serine phosphorylation of ribosomal protein S6. Furthermore, PDGF was mitogenic for these cells. Microvascular endothelial cells play a central role in neovascularization required for wound healing and solid tumor growth. Thus, the discovery of functional PDGF receptors on human microvascular endothelial cells suggests a direct role for PDGF in this process.**

Neovascularization by the microvascular endothelium plays a critical role in several physiologic and pathologic conditions, including wound healing, ovulation, diabetic retinopathy, and tumor angiogenesis (for review, see ref. 1). Observations of new capillary growth *in vitro* and *in vivo* demonstrate that the microvascular endothelium participates in a cascade of events beginning with the dissolution of endothelial-cell basement membrane, endothelial-cell migration toward the angiogenic stimulus, cell proliferation, sprout development, and eventual capillary loop formation (2–5). Neovascularization appears to be controlled by an interplay of several polypeptide growth factors, primarily fibroblast growth factor and transforming growth factors α and β (1). However, endothelial cells have been widely believed to be unresponsive to platelet-derived growth factor (PDGF), a major growth factor released at the sites of vascular insults (6), and to lack PDGF receptors (7–11). Although PDGF receptors are absent from macrovascular endothelial cells, microvascular endothelial cells grown at low serum concentrations had not been examined (7–11). Therefore, we sought to examine direct actions of PDGF, as well as other growth factors, on human capillary endothelial cells.

We chose to examine cultures of human microvascular endothelial cells (HMVECs) isolated from adipose tissue and grown in medium that allows subcultivation in the absence of matrix-coated flasks, tumor-conditioned medium, or high serum concentrations (12, 13).

Herein, we report the surprising discovery of functional PDGF receptors on microvascular cells of human origin.

MATERIALS AND METHODS

Cell Culture. HMVECs were obtained from Clonetics (San Diego), were maintained at 37°C and 5% CO_2/95% air in MCDB-131 medium supplemented with 2% (vol/vol) fetal calf serum, 1.7 nM epidermal growth factor (EGF), and 2.5 μM hydrocortisone, and were used between passages 1 and 5. The HMVECs were isolated from omental adipose tissue obtained from adults undergoing abdominal surgery. After tissue was dissected qualitatively free of large blood vessels and mesothelial tissue, endothelial cell clusters were released from the tissue by collagenase digestion and were subsequently enriched by filtration and differential sedimentation to remove contaminating adipocytes and stromal cells, as described in detail by Kern *et al.* (12). The endothelial origin of the our HMVECs was demonstrated by positive staining for factor VIII antigen, *Ulex europeaus I* lectin binding, and expression of angiotensin-converting enzyme (14). Furthermore, these cells exhibited a cobblestone morphology and stained strongly with antibodies to type IV (but not type I) collagen, thus distinguishing them from fibroblasts, epithelial cells, and smooth muscle cells. Most importantly, the HMVECs formed capillary-like structures when seeded on Matrigel (Collaborative Research)-coated tissue culture plates. This characteristic alone uniquely identifies endothelial cells and thus distinguishes microvascular endothelial cells from mesothelial cells (15).

Human foreskin fibroblasts (HFFs), obtained from C. Eil (Roger Williams General Hospital), were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, nonessential amino acids, glutamine, and antibiotics and used between passages 19 and 23.

For stimulation with growth factors, cells were seeded in 33-mm dishes at 25% confluency; between 4 and 6 days later, the spent medium was removed, and cells were rinsed twice with phosphate-free minimal essential medium (GIBCO), then incubated in this medium with [^{32}P]orthophosphate (ICN; 1 mCi/ml in each dish; 1 Ci = 37 GBq) for 3 hr at 37°C, exposed to growth factors [recombinant human PDGF B chain (Amgen Biologicals) or PDGF, purified from outdated human platelets (16), could be used interchangeably, and recombinant EGF (Amgen Biologicals) or EGF, purified from male mouse submaxillary glands (17), could be used interchangeably] at 37°C for the times indicated, and ex-

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Abbreviations: PDGF, platelet-derived growth factor; EGF, epidermal growth factor; HMVEC, human microvascular endothelial cell; HFF, human foreskin fibroblast; WGA, wheat germ agglutinin.

*Present address: Department of Pharmacology, Catholic University Medical College, Seoul, Korea.
†To whom reprint requests should be addressed.

tracted at 0°C with 1% Triton X-100 in a buffer containing proteinase, kinase, and phosphatase inhibitors as we have described (16, 18–20).

Immunoaffinity Purification of Phosphotyrosyl Proteins. Tyrosine-phosphorylated proteins were isolated from the extracts by microbatch affinity chromatography on the highly specific 1G2 monoclonal antibody to phosphotyrosine as we have described (16, 18–20). Tyrosine-phosphorylated proteins were specifically eluted from the immunosorbant with 1 mM phenyl phosphate in 40 μl of extraction buffer. (Specificity was verified by the ability of phenyl phosphate, a close analog of the phosphotyrosyl residue, to inhibit protein isolation, and by the stability of ^{32}P on these proteins to 1 M NaOH at 56°C for 1 hr.) In some cases, tyrosine-phosphorylated glycoproteins were purified further by wheat germ agglutinin (WGA) affinity chromatography. For this purpose, phosphotyrosine-containing proteins were diluted to 0.5 ml with extraction buffer and then mixed for 1 hr with 25 μl of WGA-Sepharose 4B (Pharmacia). After unadsorbed proteins were removed by two washes with 1 ml of extraction buffer, proteins bound to lectin were competitively eluted with 40 μl of 0.3 M *N*-acetylglucosamine in extraction buffer (16). PDGF receptors were immunoprecipitated from the WGA eluate using a 1:50 dilution of a rabbit antisera (antiserum 88) specific for PDGF receptors and coprecipitated using protein A-Sepharose (21). This antisera was generated by immunizing a rabbit with a synthetic peptide corresponding to amino acids 738–760 of the β-PDGF receptor (only 50% homologous to the α-PDGF receptor subunit) cloned from BALB/c 3T3 cells (21–27), is specific for the β-PDGF receptor subunit, reacts with both mouse and human PDGF receptors by Western blot analysis, and immunoprecipitates mouse PDGF receptors (21–24). Specificity was determined by the lack of immunoprecipitation with preimmune sera and by the ability of the peptide containing amino acids 738–760 to specifically inhibit PDGF receptor precipitation by rabbit antiserum 88 (21–24). The purified proteins from 1.6×10^4 HMVECs and from 1×10^5 HFFs were resolved by SDS/polyacrylamide gel electrophoresis on 7.5% gels, visualized by autoradiography using Kodak XAR-5 preexposed film with Lightning Plus intensifying screens at −70°C for 1–4 days, and quantitated by video densitometry. PDGF stimulation of HMVECs has been performed >20 times.

RESULTS

PDGF Receptors on HMVECs. The binding of PDGF to its cellular receptor, a 180-kDa transmembranous glycoprotein, initiates a cascade of events beginning with rapid activation of the protein-tyrosine kinase activity of the PDGF receptor, autophosphorylation of the receptor on tyrosine residues, near stoichiometric phosphorylation on tyrosines and serines of a 42-kDa cytoplasmic protein (recently identified as a microtubule-associated-protein kinase), serine phosphorylation of ribosomal protein S6, and ultimately cell proliferation (2–5, 16, 28–34). Therefore, to assess the growth factor responses of HMVECs and, as controls, normal human fibroblasts (HFFs), cells were incubated with [^{32}P]orthophosphate, exposed briefly to either PDGF or EGF, and then extracted with a solution containing 1% Triton X-100 and inhibitors of proteinases, kinases, and phosphatases. Phosphotyrosine-containing proteins were then purified from these extracts by immunoaffinity chromatography using the 1G2 monoclonal antibody, which is highly specific for phosphotyrosine-containing proteins (16, 18–20). Surprisingly, exposure of HMVECs to PDGF resulted in the appearance of a prominent phosphoprotein of 180 kDa that comigrated with the 180-kDa PDGF receptor from PDGF-stimulated HFFs (Fig. 1A).

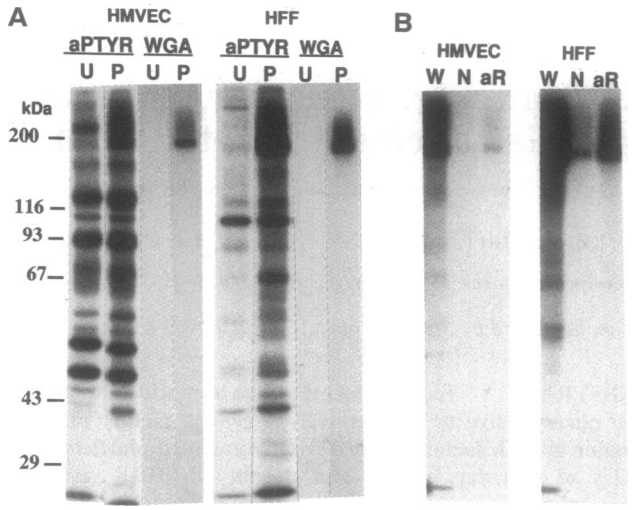

FIG. 1. PDGF-induced tyrosine phosphorylation in HMVECs. Phosphotyrosyl proteins from HMVECs (1.6×10^4 cells per lane) and HFFs (1×10^5 cells per lane) are shown in representative autoradiograms of the ^{32}P-labeled phosphotyrosyl proteins, resolved by SDS/polyacrylamide gel electrophoresis. (*A*) Lanes: aPTYR, proteins purified from cell extracts by anti-phosphotyrosine affinity chromatography; WGA, proteins first purified by anti-phosphotyrosine immunosorbant and then secondarily purified by WGA affinity chromatography; U, unstimulated cells; P, cells exposed to 1 nM PDGF for 5 min at 37°C. (*B*) WGA-purified phosphotyrosyl proteins (lanes W) from PDGF-stimulated cells were subsequently immunoprecipitated with either rabbit antisera specific for PDGF receptors (lanes aR) or normal rabbit serum (lanes N).

To determine if the 180-kDa protein might be the heavily glycosylated PDGF receptor, we tested for its well-known ability to bind to the lectin WGA (16). When phosphotyrosyl proteins from PDGF-stimulated HMVECs were incubated with WGA-Sepharose, the 180-kDa protein bound to WGA and was specifically eluted by *N*-acetylglucosamine; elution was identical to that of the 180-kDa PDGF receptor from HFFs (Fig. 1*A*). Notice in addition that the WGA eluates from both HMVECs and HFFs contain comparable high molecular weight diffuse bands, characteristic of the PDGF receptor [partial proteolysis using *Staphylococcus aureus* V8 protease of the ≈300-kDa diffuse band from PDGF-stimulated BALB/c 3T3 cells yielded peptides on SDS/polyacrylamide gel electrophoresis that were identical to those of the BALB/c 180-kDa PDGF receptor (A.R.F., unpublished data)]. As a final test of the identity of the 180-kDa protein, the WGA eluates were subjected to immunoprecipitation with a rabbit antibody specific for the PDGF receptor (21–24). Although prepared against a murine PDGF receptor, the antisera was able, albeit not very efficiently, to immunoprecipitate the PDGF receptor from human fibroblasts (Fig. 1*B*, lane HFF aR) and the 180-kDa candidate PDGF receptor from the HMVECs (Fig. 1*B*, lane HMVEC aR). The reason for the relatively low efficiency with which this sera precipitated PDGF receptors from both the human fibroblasts and endothelial cells is unknown but may reflect the peptide origin of this antisera and, therefore, its preference for recognizing the β-PDGF receptor in its denatured form on Western blots rather than in its native form in immunoprecipitations. This may also reflect some preference for mouse compared with human PDGF receptors. Because human fibroblasts typically have 5–10 times more β-PDGF receptors than α-PDGF receptors (35), it is unlikely that the inefficient immunoprecipitation results from the presence of a preponderance of α-PDGF receptors on our human fibroblasts or endothelial cells.

PDGF receptors on these cells were characterized further by ligand binding using a radioreceptor assay (10). Scatchard analysis of ^{125}I-labeled PDGF binding to HMVECs revealed 30,000 receptors per cell, with a single population of high-affinity receptors having a K_d of 0.14 nM (Fig. 2).

Maximal phosphorylation of the HMVEC PDGF receptor was extremely rapid, occurring within 1 min of PDGF addition and then diminishing gradually over the course of 1 hr (Fig. 3). Similar kinetics of receptor phosphorylation were observed in HFFs (Fig. 3) and in 3T3 cells (16). Thus, the time course of the PDGF response in HMVECs is consistent with PDGF receptor autophosphorylation.

Dose–response measurements for PDGF-induced phosphorylation of the PDGF receptors revealed an ED_{50} of about 0.3 nM using either recombinant human PDGF (B chain) or PDGF isolated from human platelets (data not shown), comparable to the K_d of 0.14 nM determined by Scatchard analysis (Fig. 2) and consistent with values reported earlier in 3T3 cells (16). Maximal intensity of phosphorylation of this protein in HMVECs ranged from 30% to 60% of the HFF response to PDGF on a per cell basis. This finding could reflect differences in receptor affinity, subtype, serine phosphorylation, metabolism, or receptor number. The last possibility seems most likely in that Scatchard analysis revealed about 30,000 PDGF receptors per HMVEC and 50,000 receptors per human fibroblast (Fig. 2 and data not shown).

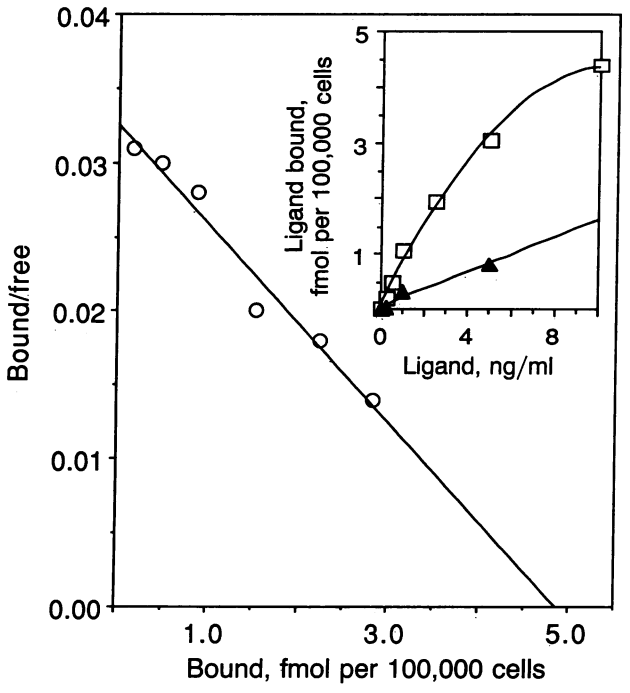

FIG. 2. Scatchard analysis of ^{125}I-labeled PDGF binding to HMVECs. HMVECs were seeded into 6-well tissue culture plates at 1×10^6 cells per well and grown until confluent in MCDB 131 medium containing 2% fetal calf serum. Recombinant human PDGF (Amersham; 1087 Ci/mmol) was labeled by the Bolton–Hunter reagent and subsequently purified by gel-filtration HPLC; labeled PDGF retained >90% of its activity. ^{125}I-labeled PDGF binding was carried out using a radioreceptor assay (10). Briefly, cell monolayers were washed with ice-cold medium containing 0.1% bovine serum albumin, and then incubated with various concentrations of ^{125}I-labeled PDGF in this medium for 4 hr at 4°C. The cell monolayers were then washed four times with ice-cold isotonic phosphate-buffered saline to remove unbound ^{125}I-labeled PDGF and remaining cell-associated radioactivity was extracted with 1% Triton X-100 and 0.1% bovine serum albumin and quantitated by γ counting. Nonspecific binding was determined by inhibiting specific binding using a 1000-fold excess of nonradiolabeled recombinant PDGF B chain. (*Inset*) Total (□) and nonspecific (▲) binding.

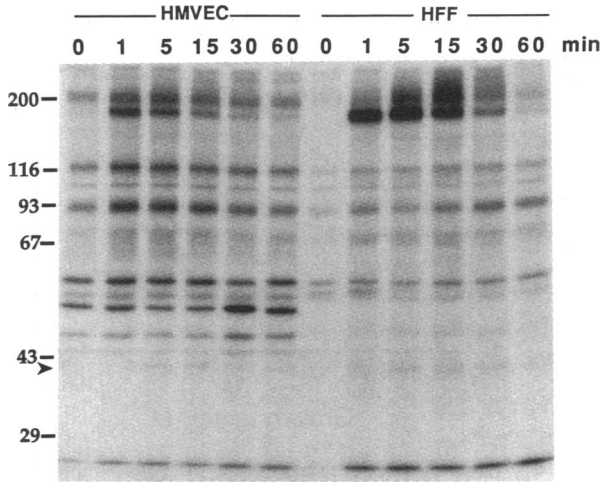

FIG. 3. Time course of PDGF-stimulated phosphorylations in HMVECs and HFFS. ^{32}P-labeled HMVECs and HFFs were exposed to 1 nM PDGF for the indicated times and then extracted, and phosphotyrosine-containing proteins were purified as described in Fig. 1. Molecular masses in kDa are shown to the left. Arrowhead indicates the PDGF-stimulated 42-kDa protein.

PDGF Stimulates Intracellular Cascades of Biochemical Events in HMVECs. Exposure of both HMVECs and HFFs to PDGF (and to EGF, data not shown) resulted in prominent tyrosine phosphorylation of a 42-kDa protein (Fig. 1), the latter an expected finding characteristic of diverse mitogenic agents (36). In contrast to phosphorylation of the PDGF receptor, which reached maximal intensity within 1 min, phosphorylation of the 42-kDa protein in PDGF-stimulated HMVECs (see Fig. 1*A* and Fig. 3) did not reach maximal intensity until 5 min and then diminished rapidly, resembling the time course of tyrosine phosphorylation of the 42-kDa protein in PDGF-stimulated fibroblasts. This delay in attaining maximal intensity is consistent with the 42-kDa protein becoming tyrosine phosphorylated by the activated PDGF receptor, or as a result of a cascade of intracellular events placed in motion by the activated PDGF receptor kinase. Phosphorylation of the 42-kDa protein is known to result from a cascade of events that includes activation of a Ca^{2+}/phospholipid-dependent serine/threonine kinase (protein kinase C) as well as activation of an as yet unidentified cellular tyrosine kinase (32).

The binding of diverse mitogenic agents to cells causes serine phosphorylation of ribosomal protein S6, believed to be involved in up-regulating protein synthesis (32–34, 37). Further demonstrating that sequelae of the PDGF signal distal to PDGF receptor autophosphorylation were evolving in these cells, PDGF (and EGF) markedly stimulated serine phosphorylation of ribosomal protein S6 (Fig. 4). Interestingly, this effect occurred at PDGF (and EGF) concentrations well below concentrations required for half-maximal receptor occupancy and half-maximal receptor tyrosine phosphorylation. These cells may have "spare receptors" for S6 phosphorylation, analogous to some insulin-stimulated responses. For example, insulin stimulates glycogen synthase activity and glucose transport with ED_{50} values at as little as 1% insulin receptor occupancy (for review, see ref. 38).

PDGF Mitogenicity for HMVECs. Because of the potential importance of these findings (Figs. 1–4) in forcing reassessment of the role of PDGF as an endothelial-cell mitogen, we examined the ability of PDGF to cause HMVECs to proliferate in tissue culture. HMVECs were seeded into wells of tissue-culture plates and cultured in the presence or absence of 1 nM PDGF for 3 days, after which cells were recovered by trypsin treatment and their number was determined.

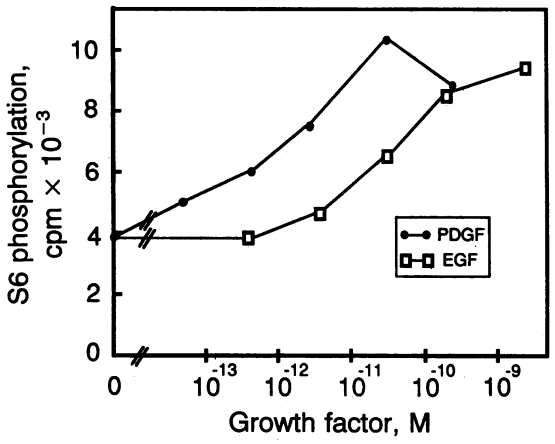

FIG. 4. Dose–response relationship of PDGF- and EGF-stimulated S6 phosphorylation. HMVECs were incubated for 3 hr with $^{32}P_i$ and exposed to the indicated concentrations of PDGF or EGF for 30 min. Ribosomes were extracted with detergent and isolated by ultracentrifugation, and the phosphorylated ribosomal protein S6 was resolved by electrophoresis on 10% polyacrylamide gels containing SDS and located by autoradiography. The S6 proteins were excised and their radioactivity was quantitated by Cerenkov counting.

These cultures were carried out in the presence of low concentrations of serum to help minimize effects of PDGF present normally in serum. PDGF caused a marked and highly significant ($P < 0.001$) increase in HMVEC cell numbers (Fig. 5).

DISCUSSION

Because of the long-held belief that endothelial cells lack PDGF receptors (7–11), we were surprised by our initial finding of PDGF receptor phosphorylation in human endothelial cells. Nevertheless, the data clearly demonstrate that these endothelial cells isolated from human adipose microvasculature possess respectable numbers (≈30,000 per cell) of high-affinity ($K_d \approx 0.14$ nM, see Fig. 2) PDGF receptors and, furthermore, that these cells respond to PDGF both biochemically (with rapid tyrosine phosphorylation of pp42 and serine phosphorylation of ribosomal protein S6, see Figs. 1, 3, and 4) and mitogenically (Fig. 5). However, in contrast to the microvascular cells used here, the many previous studies reporting endothelial cells to lack PDGF responsiveness and PDGF receptors dealt primarily with macrovascular endothelial cells (7–11, 39). We similarly fail to detect PDGF responses or receptors in endothelial cells derived from large vessels such as bovine aorta or human umbilical vein (unpublished data).

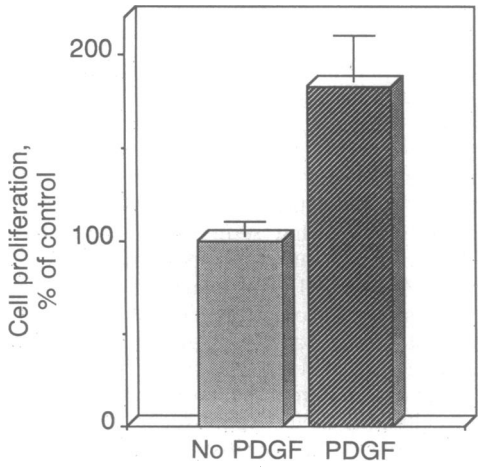

FIG. 5. PDGF-induced proliferation of HMVECs. HMVECs were seeded into wells of 48-well tissue culture plates at 7×10^3 cells per well (≈20% confluence) in 0.5 ml of medium containing low concentrations of fetal calf serum (the lowest effective serum concentration was 0.7%) and, where indicated, recombinant human PDGF B chain (Amgen Biologicals) at 30 ng/ml. After 72 hr, cells were treated with trypsin and counted. Each column depicts the number of cells expressed as a percentage of the number of cells in the absence of exogenous PDGF ($n = 10$; bars indicate standard errors). For PDGF-treated HMVECs, $P < 0.001$ by Student's t test.

The macro- and microvascular endothelium exhibit many differences in structure and function, nutritional requirements, migratory behavior, responses to modulators of protein kinase C, and abilities to form three-dimensional capillary-like networks in tissue culture (12, 13, 40–42). The finding of PDGF receptors on cultured HMVECs further underscores the dissimilarity between the micro- and macrovasculature. Endothelial cells of the microvasculature (but not the macrovasculature) are uniquely involved in the events of neovascularization, including basement membrane degradation, cell proliferation and migration, and finally capillary tubule formation. Formation of new capillary networks is critical for such diverse processes as wound healing, tumor angiogenesis, diabetic retinopathy, and ovulation (for review, see ref. 1).

Because PDGF is a major growth factor released by platelets at the site of vascular insults (6) and because PDGF is produced by many tumors (10, 42–44), the ability of microvascular endothelial cells to respond to PDGF suggests a direct role for PDGF in neovascular events. Type A and B PDGF chains are also synthesized and released by activated endothelial cells isolated from large vessels and from the microvasculature (45–47), affording an opportunity for paracrine activation of the HMVECs described here. An interesting and potentially important question, then, is whether the HMVECs themselves synthesize either A or B chain PDGF, resulting in autocrine activation. This question becomes most relevant when considering PDGF receptor subunits displayed by these cells. We were able to stimulate tyrosine phosphorylation in HMVECs with either recombinant PDGF (homodimeric B chains) or platelet-derived PDGF [a mixture of isoforms, predominantly PDGF AB-chain heterodimers, but perhaps containing as much as 30% homodimeric PDGF AA and 30% PDGF BB (48–50)]. PDGF AB and AA bind to cells expressing either only α or both α and β PDGF receptor subunits, whereas PDGF BB binds to cells expressing either only β or both α and β PDGF receptor subunits (35). Thus, from data we present, HMVECs may express either PDGF receptor subunits β or both subunits α and β.

Several additional reports provide evidence consistent with our findings of PDGF receptors on at least some microvascular endothelial cells. Shortly after our initial observation (Fig. 1) of PDGF receptor phosphorylation in HMVECs, Hermansson *et al.* (51) reported PDGF receptor mRNA *in situ* in hyperproliferating microvascular endothelial cells growing in the vicinity of malignant glioma cells secreting PDGF. More recently, Bar *et al.* (52) demonstrated PDGF binding to mouse brain and bovine fat microvascular cells. In these cells, PDGF stimulated uptake of glucose and neutral amino acids, as well as thymidine incorporation into DNA. Furthermore, Smits *et al.* (53) reported β-type PDGF receptor subunits on rat brain microvascular cells and Streeten *et al.* (54) have observed PDGF (albeit, not recombinant)-stimulated DNA synthesis in cloned endothelial cells from fetal bovine bone. Our results, then, extend these findings to human endothelial cells and more rigorously

define and characterize the presence of PDGF receptors and their responsiveness to PDGF.

We fully expect microvascular endothelial cells of differing tissue origins to display heterogeneity in their responses to various growth factors. For example, we have been unable to detect PDGF responses or receptors in several independent cultures of bovine adrenal capillary endothelial cells (unpublished data). Obviously, it will be important to examine microvascular endothelial cells derived from a wide variety of tissues for their responsiveness to PDGF *in vitro* and, ultimately, to assess the role of PDGF in neovascularization *in vivo*.

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