Aggregating Human Platelets Stimulate Expression of Vascular Endothelial Growth Factor in Cultured Vascular Smooth Muscle Cells Through a Synergistic Effect of Transforming Growth Factor-$\beta_1$ and Platelet-Derived Growth Factor$_{AB}$

Nicola Kronemann, PhD; Anne Bouloumié, PhD; Steffen Bassus, MD; Carl M. Kirchmaier, MD; Rudi Busse, MD, PhD; Valérie B. Schini-Kerth, PhD

**Background**—Vascular endothelial growth factor (VEGF), an endothelial mitogen and chemoattractant, has been implicated in the recovery of the endothelium after balloon injury. The increased expression of VEGF in vascular smooth muscle cells (SMC) at sites of injury suggests that this cell type may be a major cellular source of VEGF. This study examined whether aggregating platelets stimulate VEGF expression in cultured SMC.

**Methods and Results**—VEGF expression in SMC was assessed by Northern blot analysis and by reverse transcription followed by polymerase chain reaction and the release of VEGF by Western blot analysis and immunoassay. Platelet-derived products (PDP) released by aggregating human platelets time-dependently and concentration-dependently enhanced VEGF mRNA levels, mainly that coding for the soluble splice variant VEGF$_{165/164}$, and stimulated the release of VEGF protein. These effects were potentiated by transient acidification of PDP, which release bioactive transforming growth factor (TGF)-$\beta_1$, and mimicked by platelet-derived growth factor (PDGF)$_{AB}$ and TGF-$\beta_1$ in a synergistic manner. Both a TGF-$\beta$–neutralizing antibody and a PDGF-neutralizing antibody significantly attenuated the effect of acidified PDP on VEGF production.

**Conclusions**—Aggregating human platelets induce VEGF mRNA expression in cultured SMC and the subsequent release of VEGF protein. This effect can be attributed to a supra-additive action of PDGF$_{AB}$ and TGF-$\beta_1$ and may represent a novel mechanism by which platelets contribute to the recovery of the endothelial lining at sites of balloon-injured arteries. *(Circulation. 1999;100:855-860.)*

**Key Words:** platelet-derived factors ■ angiogenesis ■ muscle, smooth ■ arteriosclerosis

Vascular endothelium is now well recognized to play a fundamental role in the local regulation of vascular tone and homeostasis, in part, by the release of short-lived potent vasoactive autacoids such as nitric oxide and prostacyclin.1 During therapeutic angioplasty, the endothelium of the targeted artery is disrupted and the underlying smooth muscle layer is severely damaged by the balloon catheter.2 As a consequence of the exposure of the subendothelial matrix to circulating blood, an instantaneous local activation of the coagulation cascade and of platelets as well as the formation of a mural thrombus occur. This response to injury is associated with the local release of potent vasoactive factors such as thrombin and platelet-derived products [ie, platelet-derived growth factor AB (PDGF$_{AB}$), epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I), transforming growth factor (TGF)-$\beta_1$, serotonin, and thromboxane A$_2$]. Moreover, the arterial wall itself generates vasoactive factors such as PDGF, basic fibroblast growth factor (FGF), IGF-I, endothelin, and angiotensin II in response to balloon injury. Blood-derived and vascular wall–derived factors contribute to limiting the success of the angioplasty by causing local vasospasm and by stimulating the migration, proliferation, and synthesis of extracellular matrix molecules by vascular smooth muscle cells (SMC) that ultimately lead to the development of intimal thickening.

Recently, complications of balloon catheterization have been prevented by strategies aimed at expediting the recovery of the endothelial lining at sites of arterial injury. Indeed, local application of vascular endothelial growth factor (VEGF), an endothelial cell–specific mitogen that is also able to promote endothelial cell migration,3,4 either as recombinant protein or by VEGF gene transfer to balloon injured...
arteries, accelerated reendothelialization. This latter effect was associated with reduced intimal thickening, increased thromboreistance, and normalization of vasomotor reactivity. More recently it was recognized that balloon injury to arteries caused a marked upregulation of VEGF expression predominantly in the neointima and media. Because continuous administration of a neutralizing VEGF antibody impaired reendothelialization, endogenous SMC-derived VEGF appears to play a critical role in the recovery of the endothelium at sites of balloon injury. Although the stimulus responsible for the enhanced smooth muscle VEGF expression at sites of vascular injury remains to be determined, this upregulation shortly after the injury suggests a role for events occurring during the initial hemostatic and thrombotic responses. To understand the role of platelets as potential inducers of VEGF expression, the effect of aggregating platelets on VEGF expression in cultured rat and human SMC was examined. The current findings indicate that aggregating platelets stimulate smooth muscle VEGF expression predominantly through a concerted effect of PDGF-AB and TGF-β1.

**Methods**

**Materials**

Recombinant human PDGF-AB, recombinant human TGF-β1, TGF-β–neutralizing antibody, and normal rabbit IgG and goat IgG were provided by R&D Systems; PDGF-neutralizing antibody was from Upstate Biotechnology Inc; recombinant human IGF-I and EGF from Prepro Tech EC Ltd; minimum essential medium (MEM) containing 0.1% fatty acid–free BSA for 1 day before treatment. Human cells were kindly provided by Dr T. Scott-Burden (Texas Heart Institute, Houston), Rat and human SMC were cultured serially in MEM containing 2 mmol/L l-glutamine, 5 mmol/L TES, 5 mmol/L HEPES (both at pH 7.3), 100 U/mL of penicillin and 50 μg/mL of streptomycin, and 10% FCS. All experiments were performed on SMC passages 5 to 24. When SMC reached confluence, the culture medium was replaced by serum-free medium containing 0.1% fatty acid–free BSA for 1 day before treatment.

**Platelet Preparation**

One volume of platelet-rich plasma (2.6×10^10 platelets) obtained from healthy human subjects was collected into 25% (vol/vol) trisodium citrate, 124 mmol/L glucose, pH 4.5) and centrifuged at 1 mmol/L CaCl2; platelet buffer].

**Preparation and Treatment of Platelet-Derived Products**

Platelet-derived products (PDP) were prepared and treated as previously described. Briefly, suspensions of washed human platelets (3.5×10^9 platelets/mL) were stimulated with α-thrombin (1 U/mL) for 2 minutes before addition of hirudin (10 thrombin-inactivating U/mL). Platelet buffer was processed in a manner similar to platelet suspensions. After removal of platelet aggregates by centrifugation, PDP and platelet buffer were collected and stored in aliquots at −70°C until use. The protein content of PDP amounted to 170 to 390 μg protein/mL (16 different preparations). In some instances, PDP and platelet buffer were transiently acidified (a condition known to release bioactive TGF-β1 by addition of HCl (10N) to pH 2.0 to 2.5 for 30 minutes at 22°C, followed by neutralization to pH 7.4 with NaOH (2N). In some experiments, transiently acidified PDP and acidified platelet buffer were incubated with either PDGF-neutralizing or TGF-β-neutralizing antibodies or their respective nonimmune control IgGs (0.2 mg/mL) for 60 minutes at 22°C.

**Cell Culture**

SMC were isolated by elastase and collagenase digestion of thoracic aortas from male Wistar rats and from a piece of human aorta. Human cells were kindly provided by Dr T. Scott-Burden (Texas Heart Institute, Houston). Rat and human SMC were cultured serially in MEM containing 2 mmol/L l-glutamine, 5 mmol/L TES, 5 mmol/L HEPES (both at pH 7.3), 100 U/mL of penicillin and 50 μg/mL of streptomycin, and 10% FCS. All experiments were performed on SMC passages 5 to 24. When SMC reached confluence, the culture medium was replaced by serum-free medium containing 0.1% fatty acid–free BSA for 1 day before treatment.

**Reverse Transcription–Polymerase Chain Reaction and Northern Blot Analyses**

Total RNA was isolated by guanidinium isothiocyanate and phenol extraction. For the reverse transcription (RT), 2 μg total RNA was incubated with 200 U reverse transcriptase (Gibco), dNTP (175 μmol/L), oligo (dT) (200 ng), dithiothreitol (1 mmol/L), and reaction buffer in a final volume of 20 μL at 37°C for 60 minutes. In some reaction mixtures, reverse transcriptase or total RNA was omitted to determine the amplification of contaminating genomic DNA or cDNA. After a final denaturation at 94°C for 7 minutes, 6 μL of cDNA was subjected to polymerase chain reaction (PCR) consisting of denaturation at 94°C for 1 minute followed by 90 seconds of annealing at 65°C and 2 minutes of elongation at 72°C for 30 cycles. The last cycle was ended by 7 minutes of elongation at 72°C. The oligonucleotide primers used for amplification of VEGF cDNAs were derived from the sequence of the cloned human cDNA (sense primer: 5’GGAGAGATGAGCTTCCTACAG3’; antisense primer: 5’TCACCCCGCTTGCTTGTCAACA3’) and have previously been shown to amplify all reported VEGF splice variants.11 The PCR contained 0.4 μmol/L of each primer, dNTP (200 μmol/L), MgCl2 (1 mmol/L) reaction buffer, and 2.5 U Taq DNA-polymerase (Promega) in a final volume of 50 μL. The amplified cDNAs were size-fractionated by PAGE (12%), visualized under UV after ethidium bromide staining, and transferred to nylon membranes (Porablot NY amp, Macherey-Nagel). VEGF PCR products were identified by Southern blot analysis with a rat 32P-labeled VEGF cDNA probe. Northern blots were performed with 20 to 25 μg total RNA, which was electrophoresed on a 1.2% formaldehyde–denatured agarose gel, visualized with ethidium bromide, transferred to nylon membranes, and hybridized with either a rat 32P-labeled VEGF cDNA probe or a 32P-labeled 18S ribosomal RNA fragment. Autoradiography was performed with Fuji RX film with intensifying screens (DuPont de Nemours) at −70°C. The autoradiographs were analyzed by scanning densitometry. VEGF mRNA levels were normalized to their respective 18S ribosomal RNA levels and expressed in arbitrary units as a fold increase of the signal obtained with untreated cells.

**Western Blot Analysis**

Proteins present in the conditioned medium of SMC were precipitated by the addition of trichloroacetic acid (7%) for 30 minutes at 4°C. Samples were centrifuged (15 000 g for 30 minutes at 4°C) and pellets washed with ethanol (70%) followed by centrifugation (15 000 g for 15 minutes at 4°C). Pellets were resuspended in 2% (wt/vol) SDS, 5% (vol/vol) β-mercaptoethanol, 10% (vol/vol) glycerin, and 0.001% (wt/vol) bromophenol blue and 63 mmol/L Tris-HCl (pH 6.8), heated at 95°C for 5 minutes, and subjected to 12% SDS-PAGE. The separated proteins were electrophoretically transferred to nitrocellulose membranes. Nitrocellulose blots were incubated overnight at 4°C with a polyclonal rabbit antibody directed against human VEGF, which recognizes VEGF isoforms of rat and...
human origin (dilution 1:1000; Santa Cruz Biotechnology), and then with a secondary polyclonal goat anti-rabbit antibody conjugated to horseradish peroxidase (1:10 000, Calbiochem). VEGF immunoreactivity was visualized by exposing an x-ray film to blots incubated with the ECL reagent.

### Determination of VEGF

A commercially available immunoassay (human VEGF immunoassay, R&D Systems) was used for the determination of VEGF content in PDP and in rat SMC-conditioned medium. The amount of VEGF present in PDP and in acidified PDP was subtracted from that measured in conditioned medium of PDP-treated and acidified PDP-treated SMC, respectively. Because the cross-reactivity of rat VEGF with the antibody directed against human VEGF is unknown, the amount of VEGF in conditioned medium of PDP-treated SMC was arbitrarily defined as 100%.

### Statistical Analysis

Results are shown as mean±SEM. Statistical analyses were performed with a Student’s paired t test (2-tailed) or an ANOVA followed by Fisher’s protected least significant difference test to compare 2 treatments. A value of $P<0.05$ was considered statistically significant.

### Results

#### Expression of VEGF mRNA in SMC

VEGF mRNA levels in serum-deprived quiescent rat and human aortic SMC were low and sometimes even barely detectable (Figures 1 and 2). Steady-state levels of the transcript were markedly increased after exposure of rat and human SMC to products released by aggregating washed human platelets, whereas exposure of cells to the same volume of buffer had only minor effects (Figures 1 and 4). The stimulatory effect of PDP was transient. After a delay of 30 minutes, VEGF mRNA levels increased to reach maximum values after ~2 hours and thereafter returned to baseline within the next 13 hours (Figure 1A). The stimulatory effect of PDP was concentration dependent, with a consistent increase obtained at concentrations ≥5 μg protein/mL (Figure 1B).

To identify putative mediators of the stimulatory PDP effect, the effect of several exogenous platelet-derived factors was investigated at concentrations previously shown to evoke maximal activation of SMC. Exposure of rat and human SMC to either TGF-β1 (the only isoform present in human platelets) or PDGFAB (the predominant isoform in human platelets) significantly increased VEGF mRNA levels, whereas IGF-I and EGF had only minor effects (Figure 2). No stimulatory effect was found with serotonin (1 μmol/L; 1.4±0.3, n=5), platelet factor-4 (250 ng/mL; 0.7±0.2, n=4), or the thromboxane A2 mimetic U46619 (3 μmol/L, 1.4±0.5, n=3). In addition, simultaneous treatment of rat SMC with TGF-β1 and PDGFAB caused a supra-additive increase in VEGF mRNA levels (Figure 3).

Thrombin-activated human platelets secrete TGF-β1 predominantly in a biologically latent form in which the precursor peptide (the latency-associated peptide) remains covalently associated with the mature 25-kDa TGF-β1 dimer. Biologically active TGF-β1 is released from the latency-associated peptide in vitro by transient acidification or alkalization. To determine whether platelet-derived TGF-β1 regulates VEGF expression, PDP were transiently acidified

---

**Figure 1.** Time-dependent (A) and concentration-dependent (B) effects of PDP on VEGF mRNA expression in cultured rat aortic SMC. Depicted are representative Northern blots showing VEGF mRNA (top), 18S ribosomal RNA (center), and cumulative data (bottom). Results are shown as mean±SEM of 5 to 6 different experiments. *Significant effect of PDP.

**Figure 2.** Effect of several exogenous platelet products on VEGF mRNA expression in cultured rat and human SMC (4-hour treatment period). A, Representative Northern blots showing VEGF mRNA and 18S ribosomal RNA in human SMC; B, cumulative data obtained from rat and human SMC. Results are shown as mean±SEM of 5 to 7 different experiments. *Significant stimulatory effect.
(pH 2 to 2.5 for 30 minutes) before their addition to SMC. Transient acidification significantly enhanced the stimulatory effect of PDP on VEGF mRNA expression in rat and human SMC, whereas exposure to the same volume of transiently acidified buffer only slightly increased VEGF mRNA levels (Figure 4).

Next, selective neutralizing antibodies against the most potent inducers of VEGF expression, TGF-β1 and PDGFAB, were used to identify the role of these 2 factors in the stimulatory effect of acidified PDP. Treatment of acidified PDP with either a TGF-β–neutralizing antibody or a PDGF–neutralizing antibody significantly reduced their stimulatory effect by 43% and 65% in rat SMC, respectively (Figure 5), whereas control IgGs had only minor effects (data not shown).

RT-PCR analysis was performed to characterize the VEGF isoforms expressed in rat and human SMC. Low levels of a single 219 bp VEGF cDNA corresponding to the amplification of the VEGF165 transcript were found in quiescent human SMC (Figure 6A). In contrast, in RNA samples from PDP-treated or acidified PDP-treated SMC, levels of the 219 bp VEGF transcript were markedly increased, and 2 additional faint cDNAs of 87 bp and 291 bp, corresponding to VEGF121 and VEGF189, respectively, were amplified (Figure 6A). Similar findings were also obtained with the use of rat SMC (Figure 6B).

**Release of VEGF Protein From SMC**

Western blot analysis indicated the presence of an immunoreactive band of ∼21 kDa in conditioned medium of PDP-treated rat SMC (Figure 7A). The abundance of the VEGF signal was increased by transient acidification of PDP, whereas no such signal was found in conditioned medium of SMC exposed to either buffer or acidified buffer (Figure 7A) and in fresh medium containing PDP (10 μg protein/mL; data not shown). The TGF-β1 and PDGFAB treatments of SMC were also associated with a VEGF signal, and a synergistic effect was found in response to the combined treatment with the 2 factors (Figure 7A).

An immunoassay indicated the presence of human VEGF (23.6±0.7 pg/mL, n=7) in fresh medium containing PDP (10 μg protein/mL), and this level was not significantly changed by transient acidification (22.4±1.3 pg/mL). Detectable levels of VEGF were also found in conditioned medium of PDP-treated rat SMC from which the amount of VEGF present in PDP has been subtracted, and this level was increased by ∼2.9-fold after transient acidification of PDP (Figure 7B). Moreover, significant levels of VEGF were detected in conditioned medium of TGF-β1-treated or PDGFAB-treated SMC, and a more than additive effect was found in response to TGF-β1 and PDGFAB (Figure 7B).

**Discussion**

This study demonstrates that aggregating human platelets markedly stimulate the expression of VEGF in rat and human SMC, as indicated by the time-dependent and concentration-dependent increases in the steady-state levels of VEGF mRNA and the secretion of VEGF protein.

To date, molecular cloning of cDNAs for human VEGF has revealed the existence of 4 major closely related members.
of the VEGF family, each formed by alternative splicing from a single 8-exon gene. VEGF mRNAs encode proteins of 121, 165, 189, and 206 amino acids. Nonhuman VEGFs are expected to be shorter by 1 amino acid. They comprise 34- to 42-kDa homodimeric, disulfide-bonded glycoproteins able to evoke biological responses in endothelial cells, but they differ predominantly in their cellular distribution. The shorter VEGF isoforms (VEGF121/120 and VEGF165/164) are readily secreted by producer cells, whereas the 2 larger ones (VEGF189/188 and VEGF206) are retained on the cell surface and in the extracellular matrix by heparin-like molecules.

Consistent with previous observations, quiescent SMC expressed a single VEGF mRNA transcript corresponding to VEGF165/164. In contrast, 3 distinct VEGF mRNA transcripts were found in SMC exposed to aggregating human platelets, demonstrating a marked upregulation of VEGF165/164 mRNA and the appearance of VEGF mRNA signals corresponding to VEGF189/188 and VEGF121/120. These findings indicate that aggregating platelets increase the expression of secreted VEGF isoforms, mostly VEGF165/164, but also of a cell-associated VEGF isoform, VEGF189/188.

Aggregating platelet–induced upregulation of VEGF expression in SMC appears to be mediated by factors released during the thrombin-stimulated activation and degranulation of platelets. Moreover, the kinetics of VEGF expression suggest that the stimulatory effect of platelet-derived products is due to a direct effect on SMC. The study of several authentic platelet-derived factors on VEGF expression indicated that PDGFAB and TGF-β1 are potent inducers of VEGF expression in SMC, whereas IGF-I, EGF, serotonin, platelet factor-4, and thromboxane A2, which was studied with its stable mimic U46619, were inactive (all growth factors were tested at concentrations previously shown to evoke maximal activation of cultured SMC). The hypothesis that PDGFAB and TGF-β1 are the physiologically relevant mediators of VEGF expression in SMC by aggregating platelets is supported by the following findings: (1) Transient acidification, which releases biologically active TGF-β1 from its latency-associated peptide, markedly increased PDP-induced VEGF mRNA expression and the secretion of VEGF protein. (2) Selective TGF-β–neutralizing and PDGF–neutralizing antibodies significantly prevented the stimulatory effect of acidified PDP on VEGF mRNA expression. (3) TGF-β1 and PDGFAB levels in acidified PDP amounted to 6 ng and 3 ng/10 μg protein, respectively. Such growth factor levels are within the range of those eliciting biological responses in cultured SMC. (4) Although the upregulation of VEGF expression in SMC by PDGFAB and TGF-β1 alone has been previously described, the current findings indicate that the combination of the 2 growth factors was markedly more effective. Altogether, this study indicates that aggregating platelets are potential endogenous stimulators of vascular smooth muscle VEGF expression after balloon catheter injury of arteries, mainly through a concerted effect of PDGFAB and TGF-β1.

The platelet response at vascular sites of balloon injury has been shown to rapidly subside 24 hours after the in vivo injury, suggesting that although aggregating platelets may provide a stimulus for the early enhanced smooth muscle VEGF expression, they are unlikely to explain its upregulation throughout the development of intimal thickening.

**Figure 6.** Effect of PDP and acidified (Ac) PDP on VEGF expression in human (A) and rat (B) SMC as assessed by RT-PCR analysis (4-hour treatment period). Representative findings obtained from 2 different experiments.

**Figure 7.** VEGF protein levels as assessed by Western blot analysis (A) and an immunoassay (B) in conditioned medium of rat SMC exposed to either PDP, acidified (Ac) PDP, or exogenous platelet products for 24 hours. Similar observations were made in 2 additional experiments. *P<0.05 vs PDP; #supra-additive effect of TGF-β1 and PDGFAB.
expression may be controlled by endogenous factors possibly released from injured cells within the arterial wall. Indeed, an increased production of TGF-β, and PDGF-A has been found in balloon-injured rat carotid arteries during the development of intimal thickening. In addition, basic FGF, angiotensin II, and endothelin, all involved in the proliferative response to balloon injury, and the proinflammatory mediator interleukin-1β are other potential endogenous stimuli as these factors increased VEGF expression in cultured SMCs. Although SMCs are the major source of these factors at sites of vascular injury, they are also produced and secreted by inflammatory cells present within the neointima and by surrounding endothelial cells.

In conclusion, this study demonstrates that aggregating platelets stimulate VEGF expression in cultured SMC mainly through a synergistic effect of TGF-β, and PDGFAB. Thus it is conceivable that the local activation of platelets at sites of endothelial denudation after balloon catheterization might provide an important endogenous stimulus for VEGF production in the arterial smooth muscle during the early phase of the vascular response to injury. Thereafter, the long-term upregulation of smooth muscle VEGF expression during the development of intimal thickening may be due to factors generated within the arterial wall itself. Smooth muscle–derived VEGF might act as a paracrine hormone to stimulate migration and proliferation of surrounding endothelial cells and hence contribute to the recovery of the protective endothelial lining at sites of balloon-injured arteries.

Acknowledgments

This study was supported by grants from the Institut de Recherches Internationales Servier (Paris) and from the Commission of the European Communities (BMH4-CT96-0979).

References

Nicola Kronemann, Anne Bouloumié, Steffen Bassus, Carl M. Kirchmaier, Rudi Busse and Valérie B. Schini-Kerth

Circulation. 1999;100:855-860
doi: 10.1161/01.CIR.100.8.855

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/100/8/855

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/