In vitro release of vascular endothelial growth factor during platelet aggregation

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Maloney, James P., Christopher C. Silliman, Daniel R. Ambruso, J un Wang, Rubin M. Tudor, and Norbert F. Voelkel. In vitro release of vascular endothelial growth factor during platelet aggregation. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H1054–H1061, 1998.—Platelet aggregation is a cardinal feature of both vascular repair and vascular disease. During aggregation platelets release a variety of vasoactive substances; some of these promote angiogenesis, endothelial permeability, and endothelial growth, actions shared by vascular endothelial growth factor (VEGF). This study was undertaken to investigate the hypothesis that VEGF is released by aggregating platelets. We found that VEGF was secreted during the in vitro aggregation of platelet-rich plasma induced by thrombin, collagen, epinephrine, and ADP (range 23–518 pg/ml). Furthermore, serum VEGF levels were elevated compared with plasma (230 ± 63 vs. 38 ± 8 pg/ml), indicative of VEGF release during whole blood coagulation. Lysates of apheresed, leukocyte-poor platelet units contained significant amounts of VEGF (2.4 ± 0.8 pg/mg protein). VEGF message and protein were also present in a megakaryocytic cell line (Dami cell). These results suggest constitutive roles for platelet VEGF in the repair of intimal vessel injury and in the altered permeability and intimal proliferation seen at sites of platelet aggregation and thrombosis.

PLATELET AGGREGATION occurs characteristically at endothelial surfaces in response to vascular injury and leads to the secretion of an array of platelet proteins (6). Despite the known role of platelets in the repair of damaged vascular intima, there is little known regarding the endothelial mitogens within platelets. It is likely that the repair of the thin endothelial cell lining of the intima requires the appearance of endothelial mitogens; and the platelet would be a logical source of such mitogen release because platelet granules are repositories for a variety of vasoactive substances, including known mitogens for smooth muscle and fibroblasts. For example, platelets contain the vascular smooth muscle mitogen platelet-derived growth factor (PDGF) (19), the angiogenic factor platelet-derived endothelial cell growth factor (22), endothelial permeability-enhancing activity (14), high- and low-molecular-weight endothelial mitogenic activity (5, 18), and more recently described, vascular endothelial growth factor (VEGF) (23). Platelet aggregation as a process can generate a high concentration of a secreted protein at the site of aggregation (6). However, the presence of a mediator within platelets does not necessarily imply that it is secreted during aggregation; for instance, platelets store but do not secrete platelet-derived endothelial cell growth factor (22).

VEGF is a 34- to 42-kDa dimeric peptide growth factor with remarkable specificity for endothelial cells (16). VEGF was first described as a potent endothelial permeability factor (31) and later was also identified as an endothelial mitogen with effects in the nanomolar range (9), identifying it as one of the most potent endothelial permeability and mitogenic factors known (7). Of note, VEGF receptors are localized almost exclusively on endothelial cells (28). Firm roles for VEGF have been established in tumor angiogenesis (17, 32) and neovascularization (21). On the basis of these properties, VEGF may have a role in the normal host repair of damaged blood vessels; indeed, some investigators have already reported beneficial effects using VEGF as a therapeutic angiogenic agent (34). Although VEGF is expressed and secreted by a variety of cell types and tissues (3, 4, 24), secretion of VEGF from circulating blood cells such as T lymphocytes, plasma cells, and platelets may be important mechanisms of VEGF action as well (10, 15, 23). VEGF protein has been detected in thrombin-treated platelet suspensions, and the presence of VEGF mRNA and protein within human megakaryocytes provides strong evidence that VEGF synthesis during thrombopoiesis is the origin of platelet VEGF (12, 23). As platelet aggregation is a crucial event in vascular repair and vascular disease, it is of importance to know whether platelet aggregation leads to release of this potent mitogen and permeability factor.

During initial clinical studies measuring blood VEGF levels in normal volunteers, we observed consistently higher serum VEGF concentrations compared with plasma concentrations. We hypothesized that this difference was due to VEGF release from platelets during activation of clotting and more specifically that VEGF is secreted during platelet aggregation. Experiments were designed to test the hypotheses that platelets secrete VEGF during platelet aggregation and to further verify that platelets and their precursors contain VEGF protein. The results of this work provide evidence that VEGF is secreted from human platelets during platelet aggregation and that this is a general response of platelet aggregation that is not specific to a particular aggregation agonist and does not occur due to agonist stimulation if aggregation is blocked. VEGF protein content within highly purified, leukocyte-depleted human platelet lysates and within a megakaryocytic cell line is also demonstrated, supportive of significant platelet VEGF content with an origin from de novo VEGF synthesis during thrombopoiesis.
METHODS

Reagents and supplies. General laboratory reagents were from Sigma Chemical (St. Louis, MO). Blood collection tubes were from Becton-Dickinson (Franklin Lakes, NJ). Ficoll Paque reagent grade was purchased from Pharmacia Biotech (Uppsala, Sweden). Abciximab for human use (Reopro) was obtained from Eli Lilly (Indianapolis, IN), and diluent (pH 7.2, 0.01 M sodium phosphate, 0.15 M NaCl, 0.001% Polysorbate-80 in H2O) was used as the corresponding control.

Human plasma and serum collection. Ten healthy volunteers (5 male, 5 female) were recruited under a protocol approved by the University of Colorado Multiple Institutional Review Board. All subjects avoided aspirin or nonsteroidal anti-inflammatory drug ingestion for 2 wk before blood collection. Preparation of plasma and serum followed published procedures (25). Ten milliliters of blood were drawn by venipuncture for plasma and serum from each volunteer. Blood was allowed to stand for 30 min at 22°C to ensure full clotting of serum. The samples were centrifuged at 750 g for 10 min at 4°C, and the supernatants were then aliquoted and frozen at −70°C pending analysis.

Platelet-rich plasma isolation. Fresh platelet-rich plasma (PRP) was obtained from four volunteers following a published procedure (25). A platelet count was completed on EDTA-treated whole blood from each donor using an automated cell counter.

Platelet isolation for lysis experiments. Apheresis platelet concentrates were obtained from four healthy adult volunteers (each donated 1 unit) after informed consent was obtained as above. COBE spectra cell separation system equipped with the LRS system for producing platelet concentrates containing very low levels of residual leukocytes was used (1). Platelets were stored at 20–24°C with constant agitation under standard conditions. Samples of 15–60 ml were withdrawn on the day of collection through couplers using sterile technique. These platelet concentrates contained 1.29 ± 0.17 × 10^10 platelets/ml and 201 ± 320 total leukocytes/ml (mean ± SD) in volumes averaging 267 ± 19 ml (n = 4, mean ± SD).

Human leukocyte isolation. Leukocytes were separated from heparinized (1 U/ml) whole blood from five healthy adults after informed consent as above (33). Neutrophils and mononuclear cells were isolated by standard techniques (2). The final mononuclear and granulocyte cell fractions were then counted and aliquoted for further studies.

Cell lysis. Freshly isolated cells were washed in PBS (in endotoxin-free H2O, pH 7.4), then pelleted at 180 g for 10 min at 22°C and lysed as described (29). Platelet pellets containing 10^11 cells were disrupted by incubation in 5 ml of lysis buffer containing (in mmol/l) 10 HEPES, 137 NaCl, 2.9 KCl, 12 NaHCO3, 25 deoxycholic acid (1%), and 7 SDS (0.2%), as well as 2% Nonidet P-40 with protease inhibitors (0.1 mmol/l phenylmethylsulfonyl fluoride, 20 µg/ml leupeptin, and 20 µg/ml aprotinin) at 4°C for 30 min. Resuspended pellets represented a 50-fold concentration of the platelet suspensions with an attendant increase in contaminating leukocyte concentrations to 10^4 leukocytes/ml. Leukocytes isolated from whole blood were similarly lysed at 2 × 10^7 neutrophils/ml and 2 × 10^6 mononuclear cells/ml. These leukocyte concentrations were chosen as they were higher than those contaminating platelet concentrates. Lysates were then centrifuged at 14,000 g in an Eppendorf microcentrifuge at 4°C for 10 min, and the supernatants were then aliquoted and frozen at −70°C for later analysis. Protein concentration was measured by a modified Lowry assay (Sigma; St. Louis, MO).

Platelet aggregation. Platelet aggregation was performed in a standard manner (25). Human PRP was freshly prepared and then mixed in a 1-to-1 dilution with filtered Tyrode buffer with 2 mmol/l CaCl2. After standing at 22°C for 30 min, 0.5 ml of PRP was then added to each of two tubes in a dual-chamber lumiaggregometer (Payton Scientific model 300-S) set at 37°C with constant stirring at 900 rpm. After 7 min of preequilibration the experiment was begun by the paired addition of the aggregating agent or vehicle (PBS). The aggregation time for each experiment was 7 min. The final concentrations of platelet aggregation agents in solution were selected to achieve maximal aggregation (8, 11, 20, 25): 0.5 U/ml of human thrombin (Sigma T-7009), 10 µmol/l of ADP, 10 µmol/l of (−)-epinephrine, and 125 µg/ml of bovine skin collagen. Agonist-induced aggregation was verified by characteristic tracings. As control, the lack of aggregation for paired vehicle addition was likewise verified. Samples were centrifuged at 10,000 g at 4°C for 10 min, and the supernatants were frozen at −70°C for later analysis. In three individuals, a series of four incremental ADP concentrations was used to investigate dose-response effects on VEGF secretion. There were three to five experiments using all four agonists for each donor. The age and sex of the donors were as follows: donor 1, 55-yr-old male; donor 2, 36-yr-old male; donor 3, 36-yr-old male; and donor 4, 32-yr-old female.

In separate experiments, platelet aggregation was performed using a graded series of concentrations of ADP and thrombin. Seven concentration points were used for each agonist to generate a dose-response curve for VEGF release from PRP under these conditions. The final concentrations of the agonists were as follows: 1) ADP: 20, 10, 5, 2.5, 1.25, 0.63, and 0 µmol/l; 2) thrombin: 5, 2.5, 1.25, 0.63, 0.31, 0.16, and 0.05 U/ml. Platelet aggregation on the lumiaggregometer tracing was graded as none, primary wave only (reversible and transient), or full aggregation (secondary wave present). Presence of platelet aggregation was defined as a secondary aggregation wave verified by the lumiaggregometer tracings. Three donors were tested three times for each agonist series on separate days, and the data were pooled for each donor.

In other experiments, a monoconal antibody to the human platelet glycoprotein (GP) IIb/IIIa receptor (abciximab, Reopro, 300-350 µg/ml final concentration) (31) was added to PRP 30 min before the addition of one of three agonists (final concentrations 10 µmol/l ADP, 10 µmol/l epinephrine, and 0.5 U/ml thrombin) with the aim of preventing platelet aggregation. The presence of a secondary wave was again used to define aggregation in these experiments. For thrombin, up to 50 µg/ml abciximab were used in an attempt to prevent platelet aggregation. The PRP of each of two subjects was tested on three separate days for each agonist and paired control, and the results were pooled for each subject.

ELISA for VEGF. VEGF concentrations were measured in duplicate for each sample with a commercial ELISA kit (R&D Systems; Minneapolis, MN). This assay is sensitive to 9 pg/ml (0.2 µM) VEGF and does not cross-react with PDGF or other homologous cytokines. Optical density at 450 nm was measured on a Titerk Multiskan MC plate reader (Flow Laboratories; Helsinki, Finland) and VEGF concentration was determined by linear regression from a standard curve using the kit VEGF standard and GraphPad software (San Diego, CA) for analysis.

Dami cell culture. Dami cells, a cell line with megakaryocytic features (13), were grown at 37°C with 5% CO2 and 21% O2 in a Forma Scientific model 3326 incubator (Marietta, OH) to subconfluence on polystyrene flasks or sterile multichamber microscope slides after first coating surfaces with 1% bovine skin gelatin. Growth media were 10% bovine calf
serum, 25 mmol/l HEPES, 10 mmol/l NaHCO₃, and 1% penicillin-streptomycin in Iscove's modified Dulbecco's medium. Media were changed every 2 days. Conditioned media, defined as media bathing subconfluent cultures for 2 days, were analyzed by centrifugation of day 2 media aliquots at 10,000 g for 10 min at 4°C. Supernatants were then aliquoted and frozen at −70°C until assay for VEGF content. Unconditioned media consisted of growth media that had never been added to Dami monolayers.

In situ hybridization for VEGF protein in Dami cells. Dami cell monolayers were grown to subconfluence under the above conditions on eight-well chamber slides (Nunc, Naperville, IL) coated with 1% bovine skin gelatin. The cells were washed twice with Hanks' balanced salt solution, fixed in 4% paraformaldehyde for 10 min, and frozen at −70°C. In situ hybridization was performed as previously described (35). VEGF transcripts in specimens were detected using the subclone rat VEGF-2 (316 bp) as a probe, which was generously provided by Dr. G. Breier already cloned into a Bluescript KS+ vector (Stratagene; La Jolla, CA). Digoxigenin labeling of antisense and sense cRNA probes for VEGF was performed using a Genius IV kit (Boehringer-Mannheim, Indianapolis, IN). This rat probe was previously shown to bind with the human VEGF mRNA in Northern Blot and in situ hybridization studies. Hybridized probe was detected by sheep antidigoxigenin polyclonal Fab fragments conjugated to alkaline phosphatase, with detection by 4-nitroblue tetrazolium. The human glioblastoma cell line 9L (known to produce VEGF) (32) was the positive control; human umbilical vein endothelial cells were the negative control.

Data analysis. Statistical differences among blood, platelet aggregation, and cell culture specimens for VEGF concentration were measured by Kruskal-Wallis analysis, paired ANOVA with a post hoc Student-Newman-Keuls test, and Kruskal-Wallis analysis, respectively (30).

RESULTS

Comparison of VEGF levels between matched human plasma and serum. Serum levels of VEGF from healthy volunteers were significantly higher at 5.3 ± 1.5 pmol/l (230 ± 63 pg VEGF/ml) than those of paired plasma at 0.9 ± 0.2 pmol/l (38.2 ± 7.7 pg VEGF/ml) (mean ± SD, n = 10; P < 0.05; using a molecular mass of 42 kDa for VEGF). This compares with mean serum VEGF levels of 5.2 pmol/l (224 pg/ml) in volunteers as reported by the manufacturer in the ELISA kit.

VEGF content in human platelet preparations and analysis for VEGF content within contaminating leukocytes. Apheresis concentrates were used to obtain the lowest practical leukocyte contamination of a platelet product. Pellets of apheresed platelet units (n = 4) were subjected to isotonic lysis, and substantial amounts of VEGF were found in the lysate supernatants at 2.4 ± 0.8 pg VEGF/mg protein (mean ± SD, n = 4) as shown in Table 1. As these platelet preparations had a low but measurable number of contaminating leukocytes, experiments were performed to evaluate the contribution of contaminating leukocyte VEGF to total VEGF content in the platelet preparations. Suspensions of granulocytes (n = 3) and mononuclear cells (n = 4) from human donors at leukocyte concentrations greater (2 × 10⁵ cells/ml and 2 × 10⁶ cells/ml, respectively) than those seen in the lysates of platelet pellets were analyzed for VEGF content. There was no detectable VEGF in any of these leukocyte preparations, as shown in Table 1.

Release of VEGF during platelet aggregation. The lumiaggregometer tracing pairs in Fig. 1 are representative of the platelet aggregation induced by the four chosen agonists used at concentrations known to cause maximal aggregation. Notably, thrombin at 0.5 U/ml uniformly caused platelet agglutination (reflected by a flatter curve plateau) after initial platelet aggregation. The other three agents (10 µmol/l ADP, 0.125 mg/ml collagen, and 10 µmol/l epinephrine) all aggregated platelets without apparent agglutination. In the four subjects studied (each on 3 separate occasions) the VEGF release triggered by these four agonists displayed interindividual variation [range 0.5–11.9 pmol/l (23–518 pg VEGF/ml)] (Fig. 2), which was statistically significant between donor 1 and donor 4 for all of the agonists except collagen (P < 0.05). The interday variation for each individual was usually small, as denoted by each respective SE in Fig. 2. Vehicle (PBS) treatment was associated with comparatively minor VEGF release [range 0–2 pmol/l (0–87 pg VEGF/ml)]. This likely occurred because of platelet disruption and activation from in vitro conditions (stir-bar agitation and activation by tube surfaces), as there was never graphical evidence of platelet aggregation with vehicle addition. In all subjects, VEGF release was greater for agonist than for paired control, and in the great majority of cases these differences were statistically significant (Fig. 2). The highest VEGF release in response to each of the four agonists occurred in the donor with the highest circulating platelet count (donor 4, 281 × 10⁹ platelets/l; vs. donors 1–3, 179, 180, and 203 × 10⁹ platelets/l, respectively). Platelet counts were not performed on the actual PRP suspensions.

The use of a graded series of concentrations of the agonists ADP and thrombin revealed that significant release of VEGF from platelets occurred only when the agonist concentration was high enough to cause platelet aggregation (as defined by the presence of a secondary aggregation wave). Agonist concentrations below the threshold dose associated with aggregation did not lead to significant VEGF release. Furthermore, agonist concentrations above the first dose associated with a complete aggregation response did not result in significantly greater VEGF release (Fig. 3, B and C).

| Table 1. VEGF content within human blood cell types subjected to isotonic lysis |
|-----------------|---|---------|-----------------|
| Cell Type | n | Source | VEGF, pg/mg protein | Cell Density, cells/ml |
| Platelets | 4 | Platelet concentrates (apheresis) | 2.35 ± 0.8 | 1.3 ± 0.2 × 10⁹ |
| Mononuclear leukocytes | 4 | Whole blood | ND | 2 × 10⁶ |
| Granulocytes | 3 | Whole blood | ND | 2 × 10⁶ |

Values are expressed as means ± SD; n = no. of individual blood collections for each category of cellular element. VEGF, vascular endothelial growth factor; ND, nondetectable.
Experiments designed to prevent platelet aggregation using the monoclonal antibody abciximab targeted against the GP IIb/IIIa platelet receptor revealed that VEGF release did not occur in the presence of abciximab even when high concentrations of the agonists ADP and epinephrine were used. In contrast, paired experiments with vehicle revealed typical platelet aggregation with ADP and epinephrine with associated VEGF release. Of note is that a concentration of abciximab even five times higher than that which abrogated platelet aggregation in the presence of ADP and epinephrine did not...
prevent platelet aggregation in the presence of thrombin (not shown). Platelet aggregation was limited in some cases, but never prevented, and associated VEGF release was always seen. Higher concentrations of abciximab were not used because of limitations imposed by the stock concentration of the drug and because 50 µg/ml was already a high concentration for in vitro use of a monoclonal antibody. However, the thrombin/abciximab experiments remained consistent with the other data, indicating that platelet aggregation was necessary for VEGF release.

VEGF activity in Dami cells. Dami cell production of VEGF was measured by analysis of VEGF secretion into media overlying subconfluent Dami cell cultures (conditioned media, n = 3) and was further documented by in situ hybridization studies of VEGF protein mRNA content within paraformaldehyde-fixed Dami cell monolayers (n = 2). Dami-conditioned media contained significant VEGF at 160 ± 115 pmol/l (6.96 ± 5 ng/ml) vs. 4 ± 6 pmol/l (0.18 ± 0.26 ng/ml) of unconditioned media (mean ± SD; n = 3 for each; P < 0.01). The trace VEGF detected in unconditioned media may have been secondary to bovine VEGF from serum. In situ hybridization for VEGF with anti-sense probe demonstrated a clear staining signal for VEGF mRNA in Dami cells, whereas no staining was seen with the control (sense) probe (Fig. 5).

**DISCUSSION**

The clear association of VEGF release with platelet aggregation is the most important finding of this work. These studies also clarify that platelet VEGF content is not an artifact of contaminating leukocytes and that a megakaryocytic cell line contains VEGF, providing further evidence that platelets contain VEGF on the basis of de novo synthesis.

The finding that platelet VEGF release occurs as a principal consequence of platelet aggregation adds new information to recent work demonstrating VEGF release in platelet suspensions after thrombin addition (23). In that study VEGF release was measured after 30 min of thrombin exposure in a serum-free medium, yet platelet aggregation was not investigated or characterized, and other agonists were not utilized. As thrombin is a potent agonist capable of causing platelet
secretion directly, independently of aggregation (20), it is unclear whether the results reflected VEGF release due to platelet aggregation, platelet agglutination, or direct platelet secretion (without platelet aggregation). We describe the uniform release of VEGF by aggregated (and agglutinated) platelets after exposure to a number of agonists in a well-defined system of in vitro platelet aggregation, with optical confirmation of platelet aggregation in our experiments.

In our work VEGF release occurred with thrombin-induced aggregation/agglutination as well as with aggregation due to the agonists collagen, epinephrine, and ADP. The great majority of the platelet aggregation experiments were associated with significant VEGF release compared with vehicle (Fig. 2). All the tested aggregation agonists promoted VEGF release when used at concentrations known to produce platelet aggregation, and each of the volunteer platelets released VEGF during aggregation. In addition, experiments with a graded series of concentrations of ADP and thrombin revealed a threshold effect of these agonists in relation to VEGF release (Fig. 3). The threshold dose of each agonist that was associated with VEGF release was the dose necessary to trigger platelet aggregation (secondary aggregation wave, as described in Fig. 2). Agonist concentrations below this threshold concentration did not result in significant VEGF release beyond that of vehicle alone. Moreover, agonist concentrations above this threshold resulted in no significant additional VEGF release (Fig. 3, B and C). No single agonist was clearly more efficacious in generating VEGF release than another, yet there were apparent differences between individual subjects in the magnitude of VEGF release during aggregation. For instance, the amount of VEGF release was significantly different (P < 0.05) between donors 1 and 4 for all agonists except collagen (Fig. 2). The subject with the highest VEGF release to all agonists was interestingly the subject with the highest circulating platelet count (donor 4, Fig. 2). Presently it remains unclear whether quantitative (platelet number) or qualitative (intrinsic platelet properties) factors predominate in creating the interindividual differences in platelet VEGF secretion during aggregation.

Apheresed platelet units were utilized for platelet lysis studies as they allow the lowest level of white cell contamination, which is an important issue as leukocytes produce VEGF (10). That the VEGF detected in these platelet lysates reflects platelet release (but not leukocyte release) is apparent on the basis of the findings that VEGF is present in lysates of highly purified platelets, in which leukocyte contamination is minimal, and by the finding that VEGF is not measurable in lysates of isolated leukocytes even at leukocyte concentrations two orders of magnitude greater than those that contaminated these platelet preparations. Similarly, the agonists used to aggregate platelets in plasma are not useful leukocyte secretagogues and therefore are unlikely to have effects on any residual leukocytes in platelet-rich plasma. Additionally, the findings that Dami cells express and secrete VEGF are in agreement with work demonstrating that Dami cells, human CD41a+ cells, and ex vivo-generated megakaryocytes express VEGF (23).

Platelets secrete proteins during aggregation that are important for coagulation, inflammation, and vessel repair. It is particularly noteworthy that VEGF is secreted by aggregating platelets given the potent activities of VEGF as both an endothelial cell growth factor and as a permeability mediator. Together with the fact that platelet aggregation occurs predominantly at sites of endothelial injury (6), these data suggest that platelet VEGF is an important constitutive mediator of intimal repair. Platelet VEGF may also contribute to the microvascular leak and angiogenesis that is associated with wound repair, where there is characteristically platelet aggregation and thrombosis (26). Similarly, it is conceivable that platelet-borne VEGF may
contribute to the heightened endothelial permeability seen in processes such as sepsis with disseminated intravascular coagulation (27). Platelet VEGF may also be involved in the progression of endothelial proliferative disease often seen at sites of chronic microthrombosis, such as in the lungs of patients with primary pulmonary hypertension (36).

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