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Large Amounts of Vascular Endothelial Growth Factor at the Site of Hemostatic Plug Formation In Vivo

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Abstract—Vascular endothelial growth factor (VEGF) is important for the proliferation, differentiation, and survival of microvascular endothelial cells. It is a potent angiogenic factor and a specific endothelial cell mitogen that increases fenestration and extravasation of plasma macromolecules. Recently, large quantities of VEGF were detected in human megakaryocytes. Incubation of human platelets with thrombin in vitro resulted in the release of large amounts of VEGF. To investigate whether VEGF is released from platelets during coagulation activation in vivo, we measured in human subjects VEGF at the site of plug formation, ie, in blood emerging from a standardized injury made to determine bleeding time (shed blood). VEGF was also determined in the same volunteers after treatment with the specific thrombin inhibitor recombinant hirudin (r-hirudin). In a double-blind, randomized, crossover study, 17 healthy male volunteers (aged 20 to 35 years) were investigated. VEGF concentrations were measured in venous blood and in shed blood by the use of an immunoassay 10 minutes after intravenous administration of r-hirudin (0.35 mg/kg of body weight) or physiological saline. Prothrombin fragment f1.2 (f1.2) and β-thromboglobulin (β-TG) were determined as indicators of coagulation and platelet activation, respectively. Concentrations of VEGF, f1.2, and β-TG in shed blood 4 minutes after injury were significantly higher than in venous blood (VEGF, 55.8±9.2 versus <20 pg/mL, P<0.001; f1.2, 71.3±10.4 versus 0.78±0.03 nmol/L, P<0.001; β-TG, 2290±170 versus 53.2±14.0 ng/mL, P<0.001). Administration of r-hirudin caused a >50% inhibition of the β-TG and f1.2 levels in shed blood. In a similar manner, much lower amounts of VEGF were detectable at the site of plug formation after r-hirudin treatment (69.0±9.5 versus 37.8±2.6 pg/mL per minute; P=0.0015). Our data indicate that substantial quantities of VEGF are released from platelets during the interaction with the injured vessel wall in vivo. This finding may be relevant with respect to wound healing and tissue repair, tumor vascularization, or arterial thrombus formation. (Arterioscler Thromb Vasc Biol. 1999;19:1757-1760.)

Key Words: vascular endothelial growth factor ■ coagulation activation ■ prothrombin fragment 1.2 ■ β-thromboglobulin ■ recombinant hirudin

Vascular endothelial growth factor (VEGF) is a cytokine that regulates proliferation, differentiation, and survival of microvascular endothelial cells.1–3 VEGF is a potent angiogenic factor and a specific endothelial cell mitogen that increases fenestration and extravasation of plasma macromolecules.5,5 Furthermore, VEGF supports trans endothelial migration of monocytes and is chemotactic for mast cells and monocytes.6,7 So far, 4 isoforms of VEGF, with 121, 165, 189, and 206 amino acids, have been identified.8 VEGF121 and VEGF165 are secreted by various malignant and nonmalignant cells, including peripheral mononuclear blood cells.9,10 In malignant tumors, overexpression of VEGF supports development of tumor vascularization and may be responsible for tumor growth.11,12 Monoclonal anti-VEGF antibodies inhibit the vascularization and growth of human tumors in nude mice.13

Recently, large quantities of different VEGF isoforms were detected in human megakaryocytes.14,15 Further, incubation of human platelets with thrombin resulted in a release of large amounts of VEGF121 and VEGF165.14 From these in vitro experiments, the authors surmised that release of VEGF from activated platelets at the site of vascular injury could play an important role in wound healing and neovascularization.

To investigate whether VEGF is released from platelets during the early stages of coagulation activation in vivo, we applied a recently described technique that allows investigating the mechanisms of plug formation close to the in vivo situation in human subjects.16–21 This method consists of a standardized injury of the microvasculature (template bleeding time incision) and subsequent measurement of platelet and coagulation activation markers (β-thromboglobulin [β-TG] and prothrombin fragment f1.2 [f1.2]), as indicators of platelet and coagulation activation, respectively, in blood emerging from the injury site (shed blood). By using this technique, we first quantified the release of VEGF at the site of platelet–vessel wall interaction in vivo. Second, we inves-
tigated if reduction of platelet function by blocking thrombin activity (via administration of the direct thrombin inhibitor recombinant hirudin [r-hirudin]) causes a decrease of VEGF release into shed blood.

Methods

Subjects
Studies were conducted in 17 healthy male volunteers (median age, 27 years; range, 20 to 35 years) who were nonsmokers and drug-free for at least 3 weeks. The study was approved by the Ethics Committee of Vienna University School of Medicine. Written informed consent was obtained from all volunteers before inclusion into the study.

Study Design
The study was conducted in a double-blind, randomized, crossover design. After an overnight fast and a 30-minute resting period, each subject received an intravenous injection of either r-hirudin (0.35 mg/kg of body weight; Refludan, Hoechst Marion Roussel) or placebo (physiological saline). Venous blood was drawn 10 minutes after the injection. Blood collection from bleeding time incisions was performed after 15 minutes. The interval between treatments was at least 4 days.

Blood Sampling
Template bleeding time incisions were performed as described by Mielke et al. In brief, a sphygmomanometer cuff was placed at the upper arm and inflated to 40 mm Hg. Four incisions, 5 mm long and 1 mm deep, were placed on the lateral aspect of the forearm by the use of a disposable standard device (Simplate II, Organon Teknika Corp). The blood was collected in plastic tubes containing 75 U of aprotinin. After mixing, the tubes were centrifuged at 12 000 g for 10 minutes. The supernatant was removed and stored at −80°C. The procedure was performed by the same investigator each time.

Venous blood was collected by a puncture of an antecubital vein and drawn into 1/10 volumes of 3.8% sodium citrate. After mixing, the tubes were centrifuged at 3500g for 10 minutes. The supernatant was removed and stored at −80°C. Three milliliters of venous blood was collected into glass tubes containing EDTA for determination of platelet counts. Determinations of activated partial thromboplastin time (APTT) and platelet count were performed on the day of blood sampling.

Assays
Determinations of APTT (Pathrombin, Behringwerke AG) and platelet count were performed by routine laboratory procedures. F1.2, β-TG, and VEGF were measured by using the following commercially available assay kits: Enzygnost F1+2 (Behringwerke AG), Asserachrom β-TG (Diagnostica Stago), and Quantikine human VEGF (R&D Systems Europe).

Data Analysis
To quantify generation of f1.2, β-TG, and VEGF in shed blood, their respective concentrations were measured in two 2-minute aliquots over 4 minutes. The area under the concentration versus time curve (AUC) was calculated by the trapezoidal rule and regarded as the measure of mediator production in the microcirculation. Effects of r-hirudin on f1.2 (AUC), β-TG (AUC), and VEGF (AUC) in shed blood and on f1.2, β-TG, VEGF, and APTT in venous blood were compared with placebo treatment. For comparison between the data sets the Wilcoxon matched pairs test was used. P<0.05 was considered significant. When the VEGF concentration was below the limit of sensitivity of the assay system (20 pg/mL), 20 pg/mL was assumed, to perform statistical evaluation of the data. Thus, the minimum area under the VEGF concentration curve was 30 pg/mL per minute. Data are presented as mean ± SEM values.

Results
No adverse events were encountered in any of the volunteers.

APTT, β-TG, f1.2, and VEGF in Venous Blood Before and After r-Hirudin
Compared with placebo, intravenous administration of r-hirudin resulted in a significant prolongation of the APTT from 35.0±0.8 to 125.3±4.3 seconds (P<0.001). No significant difference between placebo and r-hirudin administration was observed for f1.2 and β-TG plasma levels (0.78±0.03 versus 0.79±0.04 nmol/L and 53.2±14.0 versus 38.8±4.7 ng/mL, respectively).

VEGF was not detectable in venous blood after placebo or after r-hirudin administration (limit of sensitivity of the assay, 20 pg/mL).

VEGF, β-TG, and f1.2 in Shed Blood Before and After r-Hirudin
Compared with venous blood, significantly higher concentrations of f1.2 were found in shed blood at 2 and 4 minutes after the injury (0.78±0.03 nmol/L [venous blood] versus 16.0±2.8 nmol/L [2nd minute] and 71.3±10.4 nmol/L [4th minute]; P<0.001 for all comparisons. In a similar manner, much higher levels of β-TG were detected at 2 and 4 minutes in shed blood compared with venous blood (53.2±14.0 ng/mL [venous blood] versus 1428±134 ng/mL [2nd minute] and 2290±170 ng/mL [4th minute]; P<0.001 for all comparisons).

Concentrations of VEGF in shed blood at 2 and 4 minutes after injury of the microvasculature were higher than in venous blood (34.3±4.4 pg/mL [2nd minute, P=0.005] and 55.8±9.2 pg/mL [4th minute] versus <20 pg/mL in all subjects [venous blood]; P=0.001 for both comparisons). The difference between VEGF levels at 2 and 4 minutes was statistically significant (P=0.002).

Compared with placebo, r-hirudin treatment resulted in significantly lower f1.2 and β-TG shed blood levels (f1.2 [placebo], 51.7±7.9 nmol/L per minute, f1.2 [hirudin]: 21.2±3.0 nmol/L per minute, P<0.001; β-TG [placebo]: 1290±97 ng/mL per minute, β-TG [hirudin]: 392±38 ng/mL per minute, P<0.001). This effect was seen in all volunteers (Figure, A and B). After administration of r-hirudin, significantly lower amounts of VEGF in shed blood were detected compared with placebo (37.8±2.6 versus 69.0±9.5 pg/mL per minute; P=0.0015). In 3 of the 17 subjects, VEGF levels in shed blood were below the detection limit of the assay both after placebo and after hirudin treatment. These volunteers were not considered for this particular evaluation. In 13 of the 14 remaining individuals, VEGF shed blood levels were lower after hirudin application than after placebo (Figure, C).

Discussion
There is evidence from recent studies that VEGF is stored in human megakaryocytes and is released from platelets on stimulation with thrombin. These studies were performed under in vitro conditions in the absence of important determinants of in vivo clotting such as the vessel wall, blood flow, and clotting proteases. Thus, it is uncertain if their in vitro observations are of relevance for the in vivo circumstances. The technique applied in our study covers many of these mechanisms relevant for hemostatic plug formation in vivo. It
is performed directly on human subjects and takes into account the interaction between blood cells, endothelium, and various plasma proteins under physiological flow conditions. Thus, we believe that the experimental approach applied in this study is appropriate for investigating the mechanisms important for plug formation under conditions close to the in vivo circumstances.

The principal finding of our study was that much higher concentrations of VEGF are detectable in blood emerging from a human microvascular injury site than in venous blood. Moreover, VEGF levels 4 minutes after vascular injury were significantly higher than at 2 minutes. Thus, a gradual increase in the concentration of VEGF at the local injury site increases as hemostatic system activation progresses with time (which is reflected by substantially higher f1.2 and β-TG levels in the 4th minute compared with those detected in the 2nd minute).

Under the experimental conditions applied in this study, the hemostatic system is investigated in an activated state as indicated by the very high levels of the platelet- and coagulation-specific activation markers β-TG and f1.2 in shed blood. The large amounts of thrombin are generated most probably through the extrinsic pathway, resulting in both feedback activation of the coagulation cascade as well as in massive platelet activation. Because thrombin is a very potent platelet activator, we surmised that inhibition of thrombin activity might result in a reduction of platelet activation and, consequently, in a decrease of VEGF in shed blood. Indeed, treatment of our volunteers with r-hirudin, a selective and strong thrombin inhibitor, resulted in a significant reduction not only in the levels of platelet- and coagulation-specific indicators, but also in the amount of VEGF at the microvascular injury site. Our data thus strongly suggest that large quantities of VEGF are released from blood platelets during hemostatic plug formation at the site of an injury of the microcirculation.

Our observations may have several important clinical and therapeutic implications. VEGF elicits several mitogenic and nonmitogenic responses of the vascular system, such as endothelial cell growth, chemotaxis, expression of tissue factor, plasminogen activators, and collagenases, and enhancement of vessel wall permeability. Our finding of large amounts of VEGF at the site of a microvascular injury emphasizes the potential importance of VEGF in wound healing and tissue repair.

Malignant cells exhibit enhanced expression of VEGF mRNAs.9,12 VEGF expression seems to be greatest in regions of tumors adjacent to vascular areas of necrosis.23,24 This is consistent with the possibility that tumor angiogenesis might be driven, at least in part, by hypoxic induction of VEGF regardless of the underlying genetic mutations. Furthermore, anti-VEGF monoclonal antibodies inhibit the vascularization and growth of human tumors in animals.13,25 There is evidence from large clinical trials that treatment with antithrombotic drugs, regardless of their antithrombotic properties, has a beneficial effect on the incidence and course of malignant diseases.26–30 So far, no ready explanation has surfaced for these observations. One may speculate from our data that the “antitumor effect” of some antithrombotics is linked, at least in part, to the lower amounts of VEGF at the tumor site. This could result from a reduced release of VEGF by blood platelets as the consequence of inhibition of platelet and coagulation activation by the antithrombotic therapy.

In patients with acute coronary syndromes, the arterial injury, either spontaneously or induced by coronary interventions such as percutaneous transluminal coronary angioplasty, is associated with massive platelet activation and thrombin generation often followed by the formation of an occlusive thrombus. Under these circumstances, large amounts of VEGF are most likely released by platelets, predominantly at the site of thrombus formation. The role of VEGF in acute coronary syndromes remains to be clarified.

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