Identification of functional VEGF receptors on human platelets

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Abstract Platelets secrete platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) upon stimulation. We have demonstrated that platelets have functionally active PDGF α-receptors, a transmembrane tyrosine kinase involved in negative feedback regulation. Here we demonstrate the presence of the related VEGF receptors fms-like tyrosine kinase-1 and kinase-insert domain region on human platelets. VEGF itself did not cause platelet aggregation. However, addition of exogenous VEGF to SFRLLN or thrombin-stimulated platelets potentiated platelet aggregation. Moreover, thrombin-induced phosphoinositide 3-kinase and mitogen-activated protein kinase activity were enhanced in the presence of VEGF. **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Vascular endothelial growth factor; Fms-like tyrosine kinase; Kinase-insert domain region; Mitogen-activated protein kinase; Phosphoinositide 3-kinase

1. Introduction

Platelet adhesion to endothelial surfaces in response to vascular injury or during pathophysiological conditions results in secretion of substances from platelet dense granules (e.g. ADP, ATP and serotonin), lysosomes (acid hydrolases) and α granules (proteins only, including coagulation factors, fibrinogen, platelet-derived growth factor (PDGF), transforming growth factor-β (reviewed in [1]). More recently it has been shown that platelets also secrete vascular endothelial growth factor (VEGF) during platelet activation [2,3]. VEGF is a key regulator of angiogenesis, the formation of new capillaries from pre-existing vessels, and for the progression of several disease states including tumor growth and metastasis [4,5].

VEGF-A is a strong inducer of vascular permeability and a potent mitogen for endothelial cells [5]. It binds to and exerts its biological effect through two tyrosine kinase receptors, VEGF receptor-1 (VEGFR1) (Flt-1, fms-like tyrosine kinase) and VEGFR2 (KDR, kinase-insert domain region) [5,6]. The VEGFRs are structurally related to type III transmembrane tyrosine kinase receptors belonging to the PDGF receptor family [5].

We previously demonstrated that human platelets have functionally active PDGF α-receptors, but not β-receptors [7], and that an autocrine inhibition pathway is transduced through such tyrosine kinase receptors during platelet activation [7,8]. In this study we give evidence for the expression of the related Flt-1 and KDR receptors in platelets. Moreover, we show here that VEGF has a potentiating effect on platelet activation.

2. Materials and methods

2.1. Materials

Recombinant human VEGF was purchased from Pharmingen (San Diego, CA, USA). Monoclonal anti-VEGFR1 (Flt-1 receptor) and general laboratory reagents were supplied by Sigma (St. Louis, MO, USA). The VEGFR2 (KDR receptor) specific antiserum was kindly provided by Prof. Carl-Henrik Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden). Thrombin was purchased from Parke-Davis (Morris Plains, NJ). Prof. Nils Olav Solum (Rikshospitalet, Oslo, Norway) generously provided the synthetic thrombin receptor agonist peptide Ser-Phe-Arg-Leu-Leu-Asn (SFLLRN). Mitogen-activated protein kinase (MAPK) detection kit was from Upstate Biotechnology (Lake Placid, NY, USA). Protein A–Sepharose and Sepharose CL-2B were from Pharmacia Biotech, Sweden. [^{32}P]-Orthophosphate ([^{32}P]P_i) and [γ-^{32}P]ATP were from Amersham (Amersham, UK).

2.2. Methods

2.2.1. Platelet isolation, labelling and incubation. Platelet-rich plasma was prepared from fresh human venous blood anticoagulated with acid citrate dextrose obtained at the blood bank, Haukeland Hospital, Bergen, Norway. Achievement of highly marked [^{32}P]phosphoinositides and isolation of platelet by gel-filtration was done as previously described [9]. Aggregation of gel-filtered platelets was measured in a chronolog dual channel aggregation module interfaced to a PC (Chrono-log Whole Blood-Lumi ionized calcium aggregometer, Havertown, PA, USA).

2.2.2. Extraction and analysis of 3-phosphorylated glycerophosphoinositides. Extraction of 3-phosphorylated glycerophosphoinositides was done as described previously [10]. The radioactive fractions of phosphatidylinostol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) were deacylated and subjected to high performance liquid chromatography eluted with a 0–2 M gradient of $(NH4)_3PO_4$ (pH 3.8) according to Auger et al. [11]. The radioactive peak was detected directly in the eluate by Cerenkov counting. The output from the monitor was recorded online with a standard PC.

2.2.3. In vitro immune complex MAPK kinase assay. MAPK immunoprecipitation kinase assay was carried out according to the manufacturer's instructions (Upstate Biotechnology). Briefly, platelets were solubilized in lysis buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.5 mM Na_3VO_4, 0.1% 2-mercaptoethanol, 1% Triton X-100, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 10 mM sodium β-glycerol phosphate, 0.1 mM phenylmethylsulfonyl fluoride

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Abbreviations: Flt-1, fms-like tyrosine kinase; KDR, kinase-insert domain region; MAPK, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; PI3K, phosphoinositide 3-kinase; PtdIns(3,4,5)P_3, phosphatidylinostol 3,4,5-trisphosphate; PMSF, phenylmethylsulfonyl fluoride; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor

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(PMSF), 1 μg/ml of aprotinin, pepstatin and leupeptin, and 1 μM microcystin) and MAPK was immunoprecipitated with anti-rat MAPK-R2 (cross-reactive with human p42/erk1 and p42/erk2). Protein A–Sepharose CL-4B (Pharmacia Biotech) was used to collect the immune complexes. The protein A–Sepharose/MAPK immune complex kinase assay was performed in 40 μl assay dilution buffer (20 mM morpholinopropanesulfonic acid, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol) containing MAPK inhibitor cocktail, MAPK substrate cocktail (myelin basic protein), and 10 μCi [γ-^{32}P]ATP, for 20 min in a 30°C shaking incubator. The protein A–Sepharose/MAPK immune complex was pelleted and 30 μl of the supernatant was spotted onto the center of P81 phosphocellulose paper squares. The assay squares were washed three times in 0.75% phosphoric acid and once in acetone. Finally, the radioactivity of the assay squares was determined by liquid scintillation counting.

2.2.4. In vitro immune complex VEGFR kinase assay. Platelets were solubilized in lysis buffer (0.5 M NaCl, 0.02 M Tris–HCl, pH 7.4, 0.5% Triton X-100, 1% Trasylol, 1 mM PMSF, 1 mM dithiothreitol) for 20 min at 4°C. The lysates were centrifuged at 13 000×*g* for 15 min and the supernatants were then subjected to immunoprecipitation with anti-VEGFR1 (Flt-1 receptor) or anti-VEGFR2 (KDR receptor) specific antiserum for 2 h. Protein A–Sepharose CL-4B was used to collect the immune complexes. Kinase assay were performed in 35 μl of 20 mM HEPES, pH 7.5, 10 mM $MnCl_2$, 1 mM dithiothreitol, containing 10 μCi [γ-^{32}P]ATP for 30 min at room temperature. After eluting the immune complexes by heating the beads at 95°C for 4 min in 35 μl sample buffer (4% SDS, 2% mercaptoethanol, 0.2 M Tris–HCl, pH 8.8, 0.5 M sucrose, 5 mM EDTA, 0.01% bromophenol blue), the samples were subjected to electrophoresis on a 5–15% gradient reducing SDS–polyacrylamide gel electrophoresis (PAGE). The ^{32}P-labelled proteins were detected by autoradiography.

3. Results

3.1. Human platelets express Flt-1 and KDR receptors

We have previously reported that human platelets have functionally active PDGF α-receptors, but not β-receptors [7]. The related c-Kit receptor is also found in human platelets [12]. To determine whether platelets also contain VEGFRs, which are members of the same subfamily of type III transmembrane tyrosine kinase as the PDGF and c-Kit receptors, human platelets were preincubated with recombinant VEGF (250 ng/ml) or vehicle for 1 min at 37°C before incubation with thrombin (0.1 U/ml) or vehicle for a further 5 min. Platelets were lysed and immunoprecipitated with antisera specific for the Flt-1 or KDR receptors. The precipitates were washed and incubated with [γ-^{32}P]ATP before SDS–PAGE. As shown in Fig. 1A, stimulation of platelets by thrombin in the presence of VEGF produced a heavily phosphorylated protein of approx. 180 kDa (Fig. 1A, lane 4), corresponding to the Flt-1 receptor, which was immunoprecipitated with monoclonal anti-Flt-1. VEGF alone (Fig. 1A, lane 2) showed small or no effect on the autophosphorylation of the 180 kDa protein. Thus, active VEGF-binding Flt-1 receptors only seem to be exposed on the platelet surface after stimulation of the platelets with thrombin. The KDR antiserum identified a doublet of approx. 190 and 170 kDa (Fig. 1B). The 190 kDa and 170 kDa proteins correspond to glycosylated and non-glycosylated form of the KDR as recently described [13]. The relative high amount of radioactivity in the absence of added VEGF suggests that the putative KDR receptor is constitutively tyrosine phosphorylated. Ligand-independent receptor autophosphorylation has previously been reported for both Flt-1 and KDR [13,14]. It is also possible that this phosphorylation partly is caused by dimerization of the receptors by the KDR antiserum as previously observed for the PDGF receptors [15]; however, platelets coincubated with VEGF and thrombin showed markedly increased tyrosine phosphorylation of the putative KDR receptor (Fig. 1B, lanes 3 and 5).

3.2. VEGF enhances thrombin- and SFLLRN-induced platelet activation

In order to investigate whether VEGF responsive tyrosine kinase receptors could modulate platelet activation, we performed platelet aggregation studies. Fig. 2A shows that the (0.05 U/ml) thrombin-induced platelet aggregation was enhanced by addition of VEGF (250 ng/ml). When platelets were incubated with a SFLLRN (5 μM) concentration too

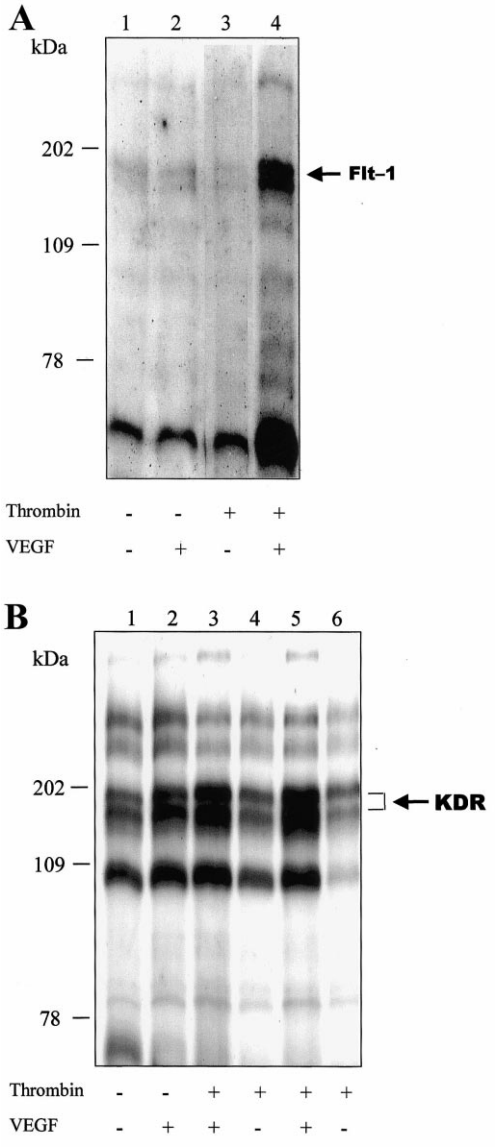

Fig. 1. Human platelets express functionally active KDR and Flt-1 receptors. Human platelets were preincubated with 250 ng/ml VEGF or vehicle for 1 min before activation with thrombin for 5 min at 37°C. Platelet lysates were immunoprecipitated with antiserum specific for the Flt-1 (A) or the KDR (B) receptors and protein A–Sepharose; the receptor immune complexes were then subjected to an in vitro protein kinase assay by incubation with [γ-^{32}P]ATP. ^{32}P-labelled proteins were separated by SDS–PAGE and detected by autoradiography. Flt-1 (180 kDa) and KDR (200 and 170 kDa) receptor bands are indicated by arrows.

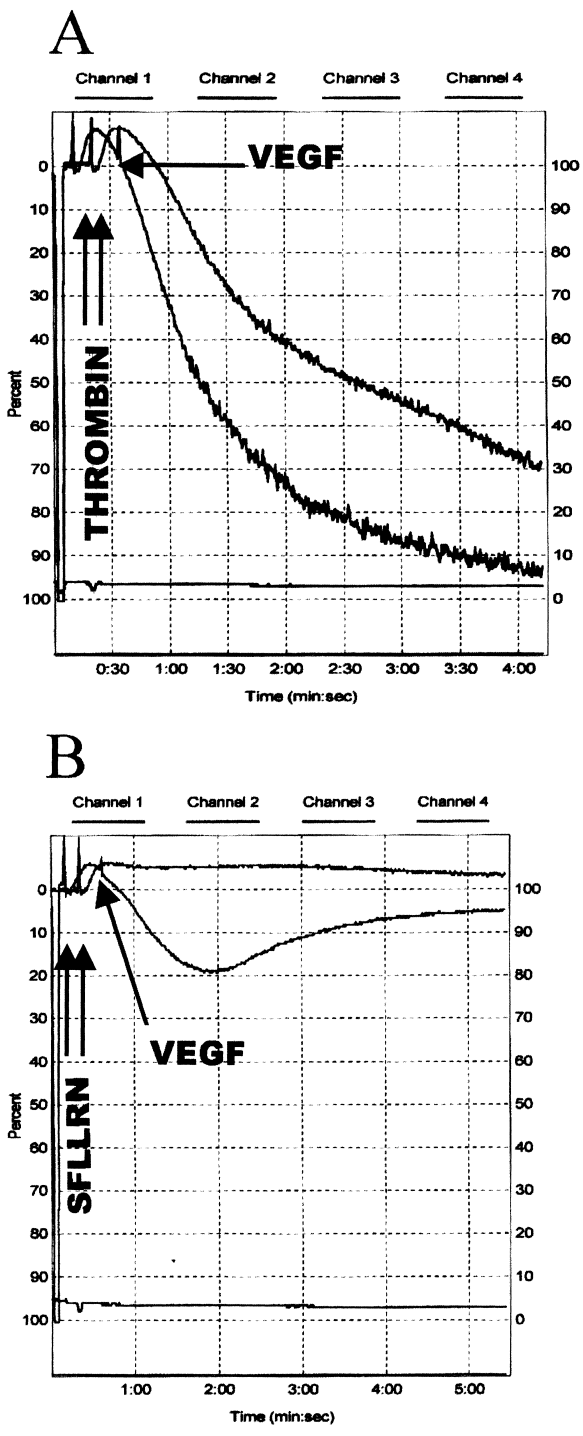

Fig. 2. Stimulatory effect of VEGF on thrombin- and SFLLRN-induced platelet aggregation. Gel-filtered platelets were stirred at 1000 rpm measured in a Chronolog dual channel aggregation module interfaced to a PC. Arrows show additions. A: Thrombin (0.04 U/ml)+vehicle versus thrombin (0.04 U/ml)+VEGF (250 ng/ml); B: SFLLRN (40 μM)+vehicle versus SFRLLN (40 μM)+VEGF (250 ng/ml).

low to induce platelet aggregation, addition of VEGF (250 ng/ml) induced platelet reversible aggregation (Fig. 2B). VEGF by itself failed to induce platelet aggregation (data not shown). Thus, VEGF enhanced the thrombin-induced platelet aggregation.

Table 1
VEGF enhance thrombin-induced MAPK activity

	MAPK activity (cpm)
Vehicle	2 832 ± 316
VEGF	5 362 ± 2 010
Thrombin	12 997 ± 3 276
Thrombin+VEGF	22 630 ± 3 722

Gel-filtered platelets were preincubated with 250 ng/ml VEGF or vehicle for 5 min before activation with thrombin (0.1 U/ml) for 5 min at 37°C. Platelet lysates were immunoprecipitated with anti-MAPK-R2. The immunoprecipitated MAPK was used to phosphorylate myelin basic protein in vitro as described in Section 2. The data are representative of two separate experiments, bars show mean ± S.E.M.

3.3. *Effect of VEGF on thrombin-induced phosphoinositide 3-kinase (PI3K) and MAPK activity*

PI3K and MAPK signalling pathways have been reported to be crucial elements in VEGF-activated endothelial cell survival and proliferation [4,16–18]. We therefore decided to study if VEGF had any effects on these signalling molecules in platelets. For measurement of the PI3K product PtdIns(3,4,5)P_3, [^{32}P]Pi-labelled platelets were preincubated with VEGF (250 ng/ml) or vehicle for 1 min before stimulation with thrombin (0.04 U/ml). VEGF alone had no effect on synthesis of PtdIns(3,4,5)P_3, however, the thrombin-induced synthesis of PtdIns(3,4,5)P_3 was markedly increased in the presence of VEGF (Fig. 3).

To investigate if VEGF had any effect on platelet MAPK, we immunoprecipitated MAPK1/erk1 and MAPK2/erk2 from VEGF and thrombin-stimulated platelets and subsequently measured the activity of the MAPKs in the immune complex. As shown in Fig. 3, VEGF (250 ng/ml) clearly enhanced (0.1 U/ml) thrombin-induced MAPK activity. Platelet incubated

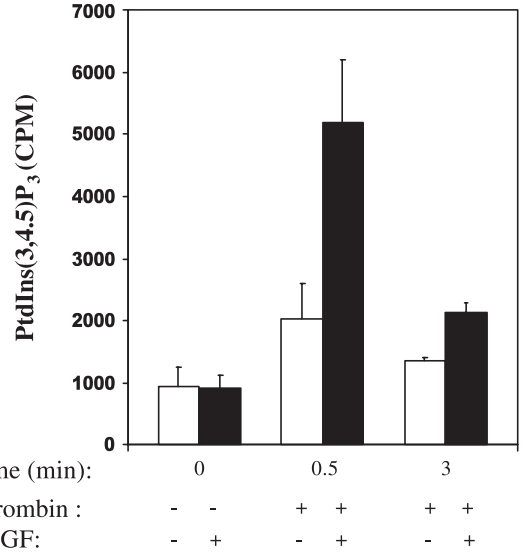

Fig. 3. Thrombin-induced synthesis of PtdIns(3,4,5)P_3 is augmented by VEGF. Gel-filtered platelets were stirred at 1000 rpm in a two-channel aggregometer at 37°C with thrombin (0.04 U/ml) or VEGF (250 ng/ml) plus thrombin together. VEGF was added 10 s after stimulation with thrombin. Radiolabelled deacylated phospholipids were analyzed by high performance liquid chromatography as described in Section 2. The data are representative of two separate experiments, bars show mean ± S.E.M.

with VEGF alone showed a small increase in MAPK activity (Table 1).

4. Discussion

In this study we show by immune complex kinase assay that platelets also have functional Flt-1 and KDR receptors. Enhanced tyrosine phosphorylation of the putative Flt-1 and KDR receptors were only observed on platelets that where coactivated with VEGF and thrombin. Thus, the platelet VEGFRs seem to be cryptic receptors which become exposed on the platelet membrane during platelet activation. Consistent with this hypothesis, we found no effect on platelet aggregation when platelets were incubated with VEGF alone. However, addition of VEGF to SFLLRN- and thrombin-stimulated platelets clearly potentiated platelet aggregation. In concordance with our findings, the presence of Flt-1 mRNA has just been identified in platelets [19].

Regarding the above discussion, the granulocyte colony-stimulating factor and stem cell factor (c-Kit) only potentiate platelet activation induced by physiological agonists, and have no effect by themselves [12,20]. An active, ligand binding c-Kit receptor was only observed after stimulation of platelets with agonist. Ligation of the tyrosine kinase receptors c-Kit [12], KDR and Flt-1 (present study) are all found to have a stimulatory effect on human platelets. In contrast, the PDGF α-receptor transduces inhibitory signals for platelet activation [7,8,21]. However, exposure of specific PDGF binding sites on the platelet membrane has been shown to appear only after platelets activation [21]. Thus, the requisite for platelet prestimulation for exposure of a conformational active ligand binding receptor seems to be a common feature for the transmembrane tyrosine kinase receptors on platelets.

PI3K and MAPK are key players in VEGF-induced endothelial cell survival and proliferation [4,16–18]. We show here that VEGF increases the thrombin-induced synthesis of the PI3K product PtdIns(3,4,5)P_3. Moreover, VEGF markedly enhanced the MAPK activity in thrombin-stimulated platelets. Thus, platelets VEGFRs seem to activate the same signalling pathways as the endothelial Flt-1 and KDR receptors.

It has recently been reported that cancer patients with soft tissue sarcomas show high VEGF expression and the presence of activated platelets within the tumor vasculature [22]. Verheul et al. [23] recently reported that VEGF-stimulated endothelial cells promoted adhesion of non-activated platelets. Platelet adhesion was found to be dependent on VEGF-induced endothelial tissue factor (TF) expression and subsequent generation of thrombin from prothrombin by the activated coagulation cascade. In addition, it has been shown that thrombin-activated platelet secretes proangiogenic VEGF [2,3]. Our results showing a stimulatory effect of exogenously added VEGF on thrombin-induced platelet activation, suggest that, in addition to its major role as an angiogenic factor, endogenously secreted platelet VEGF may function as a positive feedback regulator during platelet activation.

VEGF has many vascular effects. It stimulates production of the PGI_2 and NO in the endothelium [5], agents that counteract platelet activation. Conversely, VEGF activates TF which initiates the intrinsic coagulation cascade, culminating in production of thrombin, a potent platelet agonist. The secretion of VEGF from platelets also points to a role of platelets in angiogenesis, which has both physiological (healing) and pathological roles (tumor growth).

In conclusion, the present work shows that VEGF, in addition to the known effects listed above, also has a potentiating effect on platelet activation. Obviously, VEGF is involved in the fine-tuning of platelet activation both through its autocrine platelet behavior and through VEGF–endothelial cell interactions.

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