On the Mechanism of Thrombin-induced Angiogenesis

POTENTIATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR ACTIVITY ON ENDOTHELIAL CELLS BY UP-REGULATION OF ITS RECEPTORS*

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Many of the cellular actions of thrombin may contribute to the angiogenesis-promoting effect of thrombin reported previously. In this study, we investigated the interaction between thrombin and vascular endothelial growth factor (VEGF), the specific endothelial cell mitogen and key angiogenic factor. Exposure of human umbilical vein endothelial cells to thrombin sensitizes these cells to the mitogenic activity of VEGF. This thrombin-mediated effect is specific, dose-dependent and requires the activated thrombin receptor. Quantitative reverse transcription-polymerase chain reaction analysis reveals a time- and dose-dependent up-regulation of mRNA for VEGF receptors (KDR and flt-1). Optimal thrombin concentration for maximal expression of mRNA for KDR is 1.5 IU/ml (170% over controls) and appears 8–12 h after thrombin stimulation. Nuclear run-on experiments demonstrate that the up-regulation of KDR mRNA by thrombin occurred at the transcriptional level. In addition, functional protein of KDR receptor is increased to about 200% over control after 12 h of thrombin treatment. The up-regulation of KDR and flt-1 mRNA is also mimicked by the thrombin receptor activating peptide. These findings could explain at least in part the potent angiogenic action of thrombin.

The original observation made by Trousseau in 1872 (1), that there is frequent blood coagulation in cancer patients, has been verified by many investigators. Clinical, laboratory, histopathological, and pharmacological evidence support the notion that a systemic activation of blood coagulation is often present in cancer patients (2). This may be explained by the fact that many tumor cells elicit procoagulant activity either directly or through interaction with platelets, leukocyte and endothelial cells (3). Zacharsky et al. (4) have shown recently the presence of thrombin in a variety of tumor types. The presence of thrombin in these tumors explains the hypercoagulability in cancer but does not answer the question whether thrombin contributes directly to the tumor promotion and metastasis. It has been shown in a recent large clinical study that primary thromboembolism increases by 3-fold the risk of overt cancer diagnosis within the next 6–12 months after thrombosis (5). These clinical observations are in line with animal experiments, where thrombin treatment of B16 melanoma cells increases dramatically the number of lung metastasis in rats (6). More recently, it has been shown that the metastatic ability of human breast cancer cells is related to the number of thrombin receptors on these cells (7).

These tumor-promoting effects of thrombin may be related to our previous finding that thrombin is a potent stimulator of angiogenesis. This was shown in the chick chorioallantoic membrane system (8) and the mouse Matrigel system (9). In view of the pivotal role of angiogenesis in tumor progression and metastasis (10), this new action of thrombin on angiogenesis may provide an explanation for the aforementioned observations in animal models of cancer and in the clinic. In addition to cancer in many other conditions, where angiogenesis is activated, there is bleeding, and therefore blood coagulation and thrombin generation (e.g. wound healing, diabetic retinopathy, within the atherosclerotic plaque, endometrium, etc.). Thrombin has many actions on various cell types (11), which may support the angiogenic process in all these conditions. However, the specific molecular mechanism(s) by which thrombin activates the angiogenic cascade have not been elucidated.

Vascular endothelial growth factor (VEGF)‡ and its two tyrosine kinase receptors (the kinase insert domain-containing receptor, KDR; and Fms-like tyrosine kinase, flt-1), play important roles in mediating physiological and pathological angiogenesis (12). Although VEGF is expressed in various cell types, KDR and flt-1 expression is primarily restricted to vascular endothelial cells (13–15). Up-regulation of VEGF and its receptors has been observed in tumors and in various conditions such as hypoxia and wound healing (16–20), whereas relatively low levels are expressed in the blood vessels of normal adult tissues (21). The loss of even a single VEGF gene results in embryonic lethality, showing the central role of this factor in vascular system development (22, 23). In the majority of human tumors, the overexpression of VEGF has been correlated with high vascularity, lymph node and liver metastasis, and a poorer prognosis than VEGF-negative tumors (24). Antibodies to VEGF or expression of a dominant-negative VEGF receptor inhibit tumor growth in vivo without affecting tumor cell proliferation in vitro, showing that the inhibitory effect on tumor growth is mediated by blockage of the angiogenic activity of VEGF (25–28). These findings implicate VEGF as the most important angiogenesis factor so far identified.

‡ The abbreviations used are: VEGF, vascular endothelial growth factor; TRAP, thrombin receptor activating peptide; PKC, protein kinase C; MAP, mitogen-activated protein; HUVEC, human umbilical vein endothelial cell; BSA, bovine serum albumin; FBS, fetal bovine serum; RT, reverse transcription; PCCR, polymerase chain reaction; bp, base pair(s); PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide; PPACK, phenylalanyl-propyl-arginine chloromethyl ketone.

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In this study, we explored the possibility that thrombin is involved in the well defined and specific VEGF-mediated angiogenesis. We demonstrate that thrombin greatly potentiates VEGF-induced endothelial cell mitogenesis and that this potentiation is accompanied by up-regulation of mRNA of KDR and flt-1. We also show for KDR that this up-regulation is taking place at the transcriptional level and is accompanied by an increase in immunoprecipitable functional KDR protein. The transduction mechanisms for these thrombin-receptor-mediated events seem to proceed via protein kinase C (PKC) and mitogen-activated protein (MAP) kinases.

EXPERIMENTAL PROCEDURES

Endothelial Cell Culture—HUVECs were obtained by established methods (29) from freshly delivered umbilical cords from caesarean births. Cells were cultured as described previously (9) and were used for experiments from passages 4–6.

³HThymidine Incorporation Assay—HUVECs were seeded sparsely (10,000 cells/well) into 24-well plates and cultured for 2 days. Cells were then made quiescent by incubation in M199/4% FBS for 24 h. After two washes with serum-free M199, cells were preincubated with M199 supplemented with 1% bovine serum albumin (BSA, fraction V, Sigma) alone or with thrombin (1.5 U/ml) or with VEGF (kindly provided by Dr. H. Weich, Braunsewieg, Germany) for 8 h. All cells were pulsed with 1 μCi/ml [³H]thymidine (Amersham Pharmacia Biotech, Buckinghamshire, UK) for 2 days and then starved by incubation (with MTT, Sigma) method (30). HUVECs were grown in 24-well gelatin-coated plates (5000 cells/well) for 2 days and then starved by incubation in M199/4% FBS for 24 h. The cells were then preincubated with M199/1% BSA alone or with thrombin for 8 h. Subsequently, the cells were incubated with either M199/4% FBS alone or with VEGF (kindly provided by Dr. H. Weich, Braunsewieg, Germany) for 18 h. All cells were pulsed with 1 μCi/ml [³H]thymidine (Amersham Pharmacia Biotech, Buckinghamshire, UK) for 6 h. DNA synthesis was stopped by removing the radioactive media, washing the cells with phosphate-buffered saline, and fixing them with ice-cold methanol and 5% trichloroacetic acid. Finally, the acid-insoluble fractions were lysed by 0.3 N NaOH (0.2 ml/well) and the radioactivity was determined in liquid scintillation counter. Each experiment included six wells for each condition tested. All results are expressed as mean ± S.E. percentage over that of control, which is taken as 0%, from one representative experiment. Results were compared by unpaired t test.

Cell Proliferation Assay—Cell proliferation assays were performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) method (30). HUVECs were grown in 24-well gelatin-coated plates (5000 cells/well) for 2 days and then starved by incubation in M199/4% FBS for 24 h. The cells were then preincubated with M199/1% BSA alone or with thrombin for 8 h. Subsequently, the cells were incubated with either M199/4% FBS alone or with VEGF for 2 days. MTT stock solution (5 mg/ml) was added to each well equal to 0.1 mg/ml final concentration and incubated at 37 °C for 4 h. Then, the wells were washed and stained with the reduced dye, and the amount of formazan produced was determined colorimetrically at 570 nm.

RNA Isolation from HUVECs—After reaching confluence and 3 days after the last medium change, HUVECs were grown on 60-mm diameter tissue culture petri dishes (Nunc) and were harvested at varying time points, ranging from 1 to 8 h. Total RNA was extracted (31) from cells, and the levels of KDR and GAPDH were determined by quantitative RT-PCR as described above. To correct for differences in RNA used in RT-PCR reactions, the signal intensity for each PCR product of KDR and flt-1 was divided by that of GAPDH, for which reverse transcription and cDNA amplification was performed in the same PT-PCR reaction tube with KDR or flt-1. To exclude potential genomic DNA contamination in the RNA preparations, samples were either first treated with DNase and then used in RT-PCR, or RNA was directly used in PCR amplification. In the former case, the obtained PCR products were observed, while in the latter case, nonspecific PCR products were detected (data not shown).

Nuclear Run-on Assay—Nuclei of endothelial cells were isolated, and run-on transcription experiments were performed as described by Kanaan et al. (34) with modifications. Three days after the last medium change, starved confluent HUVECs were incubated with M199/1% BSA alone or with thrombin (1.5 U/I/ml) for 8 h. Cells were then harvested at varying time points, ranging from 1 to 8 h. Total nuclear RNA was isolated by the guanidinium thiocyanate-phenol-chloroform method (31), and the determination of KDR and GAPDH transcription rates was performed using quantitative RT-PCR, as described above in detail.

Measurement of RNA Stability—Confluent and starved endothelial cells were incubated with M199/1% BSA alone or with thrombin (1.5 U/I/ml) for 8 h, before addition of actinomycin D (10 μg/ml). Cells were harvested at 0, 1, 2, 3, and 4 h, and total RNA was extracted (31) from cells, and the levels of KDR and GAPDH transcripts were determined by quantitative RT-PCR as described above.

Immunoprecipitation and Western Blot—Three days after the last medium change, starved confluent endothelial monolayers were incubated with M199/1% BSA alone or with thrombin (1.5 U/I/ml) for 12 h. Cells were then lysed at 4 °C by scraping in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 0.14 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.02% NaN₃, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.02% sodium dodecyl sulfate, 0.8 μg/ml leupeptin, 0.5 μg/ml pepstatin, and 10 μg/ml leupeptin. Protein samples (1 mg) were precleared with 100 μl of protein A-Sepharose (Sigma) conjugated with normal rabbit IgG (Sigma) for 3 h at 4 °C. After a brief centrifugation, the supernatants were mixed with 10 μg of protein A-Sepharose conjugated with affinity-purified anti-KDR rabbit polyclonal antibody (generous gift from Dr. H. Weich, National Research Center for Biotechnology, Braunschweig, Germany) and rotated overnight at 4 °C. The antigen-antibody-protein A-Sepharose conjugates were removed by centrifugation and washed five times with wash buffer (10 mM Tris-HCl, pH 7.4, 0.14 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.02% NaN₃, 1 mM phenylmethylsulfonyl fluoride, 1 mM β-mercaptoethanol, 1 mM EDTA, 1 mM β-mercaptoethanol, 10 μg/ml leupeptin). Protein were then denatured by boiling in Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol) for 5 min and electrophoresed on 7.5% SDS-polyacrylamide gels (SDS-PAGE) under reducing conditions by method of Laemmli (35). After

23970
electrophoresis, proteins on gels were transferred to nitrocellulose membranes (Bio-Rad, München, Germany). The obtained membranes were blocked with 5% skim milk in PBS, overnight at 4°C and then incubated with anti-KDR rabbit polyclonal antibody at 1 μg/ml in blocking buffer for 2 h at room temperature. After washing with PBS, the membranes were incubated with anti-rabbit IgG conjugated with horseradish peroxidase (Sigma) in PBS containing 0.05% Tween 20 for 1 h at room temperature. The membranes were then washed with Tween-PBS, and the blots were developed using enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech) according to the manufacturer’s protocol.

In order to quantitate functional KDR receptors present in HUVECs treated with thrombin, phosphorylated forms of KDR were identified after exposure to VEGF. Starved endothelial cells were incubated for 12 h with M199/1% BSA alone or with thrombin and stimulated for the last 15 min with VEGF. Lysis of cells, immunoprecipitation with anti-KDR rabbit polyclonal antibody, SDS-PAGE protein separation, and transfer to nitrocellulose membrane were performed as described in details above. The blocked membranes were then immunoblotted with 0.1 μg/ml RC20H anti-phosphotyrosine monoclonal antibody conjugated with horseradish peroxidase. (kindly provided by Dr. E. Dejana, Mario Negri, Milan, Italy) for 3 h at room temperature. After extensive washing with Tween-PBS immunoreactive bands were visualized using the ECL reagent.

RESULTS

Thrombin Potentiates VEGF-induced Mitogenesis in HUVECs—Mitogenic activity of endothelial cells can be stimulated by a variety of growth factors, including VEGF and thrombin (11, 12). In the experiments described in this report, thrombin (1.5 IU/ml) caused a significant increase in DNA synthesis by HUVECs ranging from 40% to 90% over that of controls. Similarly, VEGF (5 ng/ml) caused an even greater stimulation of DNA synthesis by HUVECs ranging from 160% to 280% above the controls. When HUVECs were preincubated with thrombin for 8–12 h and subsequently exposed to VEGF, the increase in DNA synthesis by these cells was greater than the additive effect expected from thrombin and VEGF alone (Fig. 1A). The thrombin-treated cells responded to VEGF-induced DNA synthesis in this synergistic way only at least 8 h after exposure to thrombin. At earlier times: 0.5, 1.5, and 4 h, this potentiating effect of thrombin on VEGF-induced DNA synthesis was not evident (Fig. 1A). The effect is specific to thrombin since hirudin completely cancels out this effect (Table I). Additionally, PPACK-thrombin (chemically inactivated thrombin at the active site) is without effect, thus establishing the requirement for a proteolytic activation of thrombin receptors on endothelial cells (Table I). This effect of thrombin was dose-dependent and reached a plateau at about 1.5 IU/ml thrombin (Fig. 2A).

Many of the effects of thrombin are mimicked by TRAP, the decapeptide representing the NH2-terminal sequence of the activated thrombin receptor (36). TRAP bypasses the requirement for proteolysis of the thrombin receptor for activation and acts as tethered agonist peptide of the activated receptor (37). As shown in Figs. 1B and 2B, TRAP has the same effects as thrombin on the VEGF-induced DNA synthesis. As with thrombin, incubation of cells for at least 8 h subsequent to TRAP treatment is essential for the increase of VEGF-induced DNA synthesis to become evident (Fig. 1B). The synergistic effect of thrombin on VEGF-induced DNA synthesis was also investigated at the level of endothelial cell proliferation. Cells were preincubated with thrombin (1.5 IU/ml) for 8 h and subsequently with VEGF (5 ng/ml) for 2 days. The cell growth was determined colorimetrically. Thrombin or VEGF alone caused about 20% and 100% increase in cell proliferation rate, respectively (Fig. 3). When cells were pretreated with thrombin and subsequently exposed to VEGF, the increase in cell proliferation was about 220% over controls (Fig. 3). This is in line with the results obtained by monitoring DNA synthesis.

The cell growth was determined colormetrically. Thrombin or TRAP treatment is essential for the increase of VEGF-induced DNA synthesis to become evident (Fig. 1B). Mitogenic activity of endothelial cells can be stimulated by a variety of growth factors, including VEGF and thrombin (11, 12). In the experiments described in this report, thrombin (1.5 IU/ml) caused a significant increase in DNA synthesis by HUVECs ranging from 40% to 90% over that of controls. Similarly, VEGF (5 ng/ml) caused an even greater stimulation of DNA synthesis by HUVECs ranging from 160% to 280% above the controls. When HUVECs were preincubated with thrombin for 8–12 h and subsequently exposed to VEGF, the increase in DNA synthesis by these cells was greater than the additive effect expected from thrombin and VEGF alone (Fig. 1A). The thrombin-treated cells responded to VEGF-induced DNA synthesis in this synergistic way only at least 8 h after exposure to thrombin. At earlier times: 0.5, 1.5, and 4 h, this potentiating effect of thrombin on VEGF-induced DNA synthesis was not evident (Fig. 1A). The effect is specific to thrombin since hirudin completely cancels out this effect (Table I). Additionally, PPACK-thrombin (chemically inactivated thrombin at the active site) is without effect, thus establishing the requirement for a proteolytic activation of thrombin receptors on endothelial cells (Table I). This effect of thrombin was dose-dependent and reached a plateau at about 1.5 IU/ml thrombin (Fig. 2A).

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Taken together, these data show that thrombin enhances effectively the mitogenic potency of VEGF on endothelial cells.

Thrombin Up-regulates VEGF Receptor mRNA in HUVECs—We next determined whether the potentiating effect of thrombin on VEGF-induced endothelial cells mitogenesis could be related, at least in part, to regulation by thrombin of VEGF receptors. We employed a sensitive quantitative RT-PCR technique to examine KDR and flt-1 gene expression in HUVECs.

Table I

| Treatment                    | Percentage over control (S.E.) | Treatment                      | Percentage over control (S.E.) |
|------------------------------|---------------------------------|---------------------------------|---------------------------------|
| None/control                 | 0 (0)                           | None/control                   | 0 (0)                           |
| Thr (1.5 IU/ml)              | 90 (4)                          | Thr (1.5 IU/ml)                | 59 (2)                          |
| PPACK-Thr (25 μg/ml)         | 29 (14)                         | Hirudin (1.5 IU/ml)            | 33 (6)                          |
| VEGF (5 ng/ml)               | 259 (21)                        | VEGF (5 ng/ml)                 | 215 (15)                        |
| Thr/VEGF                     | 659 (33)                        | Thr + hirudin                  | 11 (9)                          |
| PPACK-Thr/VEGF               | 303 (13)                        | Thr + VEGF                     | 489 (23)                        |
| Thr + hirudin/VEGF           | 268 (12)                        | Thr + hirudin/VEGF             | 268 (12)                        |

The synergistic effect of thrombin on VEGF-induced DNA synthesis was also investigated at the level of endothelial cell proliferation. Cells were preincubated with thrombin (1.5 IU/ml) for 8 h and subsequently with VEGF (5 ng/ml) for 2 days. The cell growth was determined colorimetrically. Thrombin or VEGF alone caused about 20% and 100% increase in cell proliferation rate, respectively (Fig. 3). When cells were pretreated with thrombin and subsequently exposed to VEGF, the increase in cell proliferation was about 220% over controls (Fig. 3). This is in line with the results obtained by monitoring DNA synthesis.
Primers for KDR, flt-1, and GAPDH were chosen so that they would correspond to the regions where sequence homologies among the three primers are relatively low and would generate products of differing lengths. This allowed us to perform RT-PCR with the housekeeping gene GAPDH into the same reaction tubes with KDR or flt-1. Data presented in this report show that each set of primers worked well in specifying their corresponding mRNA. Single bands at about 312, 596, and 1080 bp were obtained for KDR, GAPDH, and flt-1 mRNA, respectively. Titration curves of RT-PCR products have been employed for determining the quantitative range in which the reactions proceeded exponentially (data not shown). Signal intensities of the products obtained were plotted as functions of RNA template amount and cycle number. Thus, we established the optimal conditions for RT-PCR, which were performed with 250 ng of total RNA for 25 cycles for KDR/GAPDH and 500 ng of total RNA and 30 cycles for flt-1/GAPDH. As shown in Fig. 4, treatment of HUVECs with thrombin (1.5 IU/ml) resulted in increase in the message for KDR as compared with untreated cells (about 170% over of that controls). The up-regulation of KDR mRNA was evident 8–12 h after thrombin stimulation. At earlier times (2 and 4 h) or after 16 h of thrombin treatment, the mRNA levels of KDR in the thrombin-treated cells were comparable to control endothelial cells (Fig. 4). A similar increase in mRNA for flt-1 was also evident 8 h after thrombin stimulation (Fig. 8).

Thrombin stimulation increased KDR mRNA of HUVECs in a dose-dependent fashion. As shown in Fig. 5A, thrombin at 1.5 IU/ml concentration increased expression of KDR mRNA to maximal levels. At 5 IU/ml, the stimulatory effect of thrombin declined to lower levels. This bell-shaped effect of thrombin is observed in many of the effects of thrombin including angiogenesis (8, 38). These effects of thrombin on KDR and flt-1 mRNA are receptor-mediated events. TRAP, the synthetic decapeptide, mimics the effects of thrombin (Figs. 5B and 8).

To exclude the possibility that up-regulation of the two VEGF receptors was due to a generalized increase in mRNA production induced by thrombin, we performed RT-PCR with the same RNA preparations using primers for fibroblast growth factor receptor 1 (FGFR1). Under the same conditions, thrombin did not cause any change in mRNA levels of FGFR1 in the thrombin-treated cells as compared with controls cells (data not shown).

Thrombin Increases the Rate of Transcription but Not the Stability of KDR mRNA—In order to determine whether thrombin affected the steady-state level of KDR mRNA by decreasing its rate of degradation, we measured KDR mRNA in the presence of actinomycin D, to inhibit transcription from control and thrombin-treated endothelial cells. As shown in Fig. 6A, thrombin did not affect the rate of decay of KDR mRNA. The estimated half-life of KDR mRNA was approximately 2.8 h and identical for controls and thrombin-treated cells (Fig. 6A).
We also performed nuclear run-on experiments to determine the rate of KDR gene transcription in the presence and absence of thrombin as compared with the rate of the transcription of the constitutively expressed GAPDH gene. As shown in Fig. 6B, in a representative experiment (of three) the rate of transcription for KDR and GAPDH in HUVECs nuclei is linear for at least 1 h. In addition, thrombin (1.5 IU/ml) increases the rate of KDR gene transcription, which reach approximately 80% over that of control at 1 h after the beginning of in vitro transcription (Fig. 6B). These results imply that thrombin-induced increase of KDR mRNA is due to increases in the rate of transcription of KDR gene and not to changes in the stability of its mRNA.

**Thrombin Promotes the Expression of VEGF Receptors through PKC and MAP Kinase-dependent Pathways—**To define the signaling pathways responsible for the up-regulation of KDR and flt-1 expression by thrombin, we treated HUVECs with agents that modulate the above key cellular transduction mechanisms involved in thrombin cellular actions (39). We used PMA as PKC activator, calphostin C as selective PKC inhibitor (40), PD98059 as selective MAP kinase inhibitor (41, 42), and forskolin as selective activator of adenylyl cyclase (43). As shown in the Figs. 7 and 8, PMA (50 ng/ml) potently up-regulates the mRNA levels of KDR and flt-1, whereas calphostin C (0.5 μg/ml) does not effect the basal expression of KDR. When HUVECs were preincubated with calphostin C for 30 min and then coincubated with thrombin (1.5 IU/ml) for 8 h, calphostin C completely abolished the thrombin-promoting effect of KDR and flt-1 expression. Similarly, treatment with PD98059 (20 μM) abolished the thrombin-induced increase of KDR and flt-1 mRNA. In contrast, the activation of adenylyl cyclase by forskolin (5 μg/ml) and the resulting elevation of cellular cAMP did not effect the levels of KDR and flt-1 mRNA induced by thrombin. Taken together, these results suggest that thrombin up-regulates KDR and flt-1 mRNA, possibly via activation of PKC and MAP kinase signaling pathways.

**Thrombin Increases New Functional KDR Protein Synthesis in HUVECs—**To determine whether the increase in KDR mRNA was accompanied by an increase in protein synthesis, total endothelial cell lysates were immunoprecipitated using an affinity-purified rabbit anti-KDR polyclonal antibody, which recognizes a peptide in the extracellular domain of KDR receptor. Immunoprecipitates were electrophoresed, transferred onto nitrocellulose membranes, and immunoblotted with the same anti-KDR antibody. A major band of about 210 kDa was detected. A faint band also appears at about 190 kDa, which possibly corresponds to a differently glycosylated form of KDR (Fig. 9A). No signal was detectable if the antibody used for Western blotting was pre-adsorbed with the corresponding peptide (from the extracellular domain of KDR), thus demonstrating the specificity of both bands (data not shown).

We also evaluated the functionality of KDR receptors present in HUVECs under control conditions and their modulation after thrombin stimulation. The phosphorylated KDR receptor
was identified as a single band of approximately 210 kDa in immunoprecipitates of VEGF-stimulated endothelial cells but not in unstimulated HUVECs (Fig. 9B). Immunoprecipitates from endothelial cells that have been treated with thrombin (1.5 IU/ml) for 12 h showed an increase in functional KDR receptor levels as compared with controls that were not exposed to thrombin (Fig. 9B).

**DISCUSSION**

In this report we have studied a novel action of thrombin on endothelial cells, which may be the major contributor to the angiogenesis-promoting effect of thrombin. Exposure of HUVECs to thrombin causes an amplification of their response to VEGF, the key angiogenic factor. VEGF is secreted and up-regulated when angiogenesis is activated and is specific for endothelial cells inducing migration, proliferation, and tube formation (12). The appearance of the phenotype of thrombin-treated endothelial cells, which has increased sensitivity to VEGF, requires at least 8 h of incubation of cells after exposure to thrombin. The activated thrombin receptor is involved, since TRAP, which acts as agonist to the activated thrombin receptor, has similar effects. The delayed appearance of synergistic effect between thrombin and VEGF and the involvement of the activated thrombin receptor imply that early transduction mechanisms subsequent to thrombin receptor activation trigger downstream events. We have established that a result of these events is the up-regulation of VEGF receptors (KDR and flt-1). To monitor the effect of thrombin on KDR and flt-1 gene expression, we used RT-PCR technology. This approach allows accurate and reproducible quantification of gene expression. Our data show that mRNA for both KDR and flt-1 is increased in human endothelial cells treated with thrombin or TRAP. The time required after thrombin treatment for maximum KDR and flt-1 mRNA synthesis is 8–12 h. The effect is also dose-dependent for thrombin with optimal concentration of about 1.5 IU/ml, which is consistent with the concentration required for most of the cellular actions of thrombin (11). We have also shown that KDR mRNA up-regulation is accompanied by an increase in immunoprecipitable KDR protein. It was further shown that this KDR protein is functional; hence, it can be...
phosphorylated after exposure of the cells to VEGF. This finding further establishes the identity of KDR receptor protein.

We have investigated the possibility that thrombin may effect the stability of mRNA for KDR. However, when actinomycin D was added to prevent new RNA synthesis, the half-life of the already formed mRNA remains the same in presence or absence of thrombin. In addition, in nuclear run-on experiments, we have established that thrombin acts at the transcriptional level. The nuclear transcription factors involved in thrombin-induced KDR and flt-1 gene expression regulation are under investigation. Recently, Scarpati and DiCorletto (46) reported that in endothelial cells the thrombin-stimulated transcription of platelet-derived growth factor-B (PDGF-B) is associated with the presence of thrombin responsive elements within PDGF-B promoter region. These elements, which consist of a repeat of CCACCC sequence, interact with the thrombin-inducible nuclear factor (46). If this region is in fact the site responsive to thrombin-induced transcriptional activation, then one can anticipate the presence of CCACCC motif within the promoter region of other thrombin-responsive genes. Indeed, CCACCC motif was also found to be present in the promoter elements of several genes, which are known to be activated by thrombin, such as PDGF-A, bFGF, thrombomodulin, von Willebrand factor, etc., and to be absent in various other genes that are not known to be modulated by thrombin (11, 46). Recently, Patterson et al. (47) reported the sequence of the promoter region of KDR receptor gene. It is of interest that this thrombin-responsive sequence CCACCC also exists in the region −4 to −10 of the transcription start site in the promoter region of KDR receptor gene.

Thrombin receptor is a member of a seven-transmembrane domain receptor family coupled to G-proteins (39). This receptor is proteolytically activated by thrombin generating a new NH2 terminus, which acts as a tethered ligand and promotes the interaction between the receptor and the G-proteins on the intracellular side of the membrane. An exchange of GDP for GTP bound to the α-subunit of the G-protein leads probably to dissociation of the βγ-heterodimers. After that the GTP-bound G-protein α-subunits or βγ-complexes initiate the intracellular signaling responses (48). Because many types of α- and β-subunits exist, this provides a diversity in the intracellular signals that can be generated by G-proteins. The repertoire of G-proteins coupled to the thrombin receptor, and the cellular effectors activated, determine the nature of the cellular response generated. As studied extensively by others (11, 39), in most cell types thrombin stimulates phospholipase C and A2, PKC, MAP kinases, tyrosine kinases, and modulates the activity of adenylyl cyclase. Our experiments suggest that the activation of PKC and MAP kinases are involved in the thrombin-mediated events leading to up-regulation of KDR and flt-1 mRNA. This is based on the findings that PMA, which activates PKC and thrombin increase mRNA levels of both VEGF receptors. Conversely, calphostin C, a specific PKC inhibitor, blocks the thrombin-promoting effect. Similarly, the specific inhibitor of MAP kinases PD98059, abolishes the KDR and flt-1 mRNA synthesis induced by thrombin. These findings are in agreement with the results obtained by Shen et al. (49). They have shown that the up-regulation of KDR by VEGF is mediated by activation of phospholipase C-γ, PKC, and MAP kinases.

Among the many factors reported to promote angiogenesis (50), VEGF has a pattern of spatial and temporal expression that establishes its pivotal role both in physiological and pathological angiogenesis (12). Through interactions with its endothelial cell receptors, VEGF promotes the growth and maintenance of endothelial cells and the development of new blood vessels. VEGF has also been shown to increase the cell surface expression of several other receptors, such as urokinase-type plasminogen activator receptor (51), endothelial receptor tyrosine kinase tie-1 (52), and tissue factor (53), all of which are implicated in angiogenesis. In view of these effects of VEGF, it is reasonable to assume that the thrombin-mediating up-regulation of VEGF receptors may play a key role in angiogenic cascade.

The discovery of many endogenous modulators of angiogenesis led many to believe that activation of angiogenesis may be the result of an imbalance of angiogenic and anti-angiogenic factors (54). However, angiogenesis is an important physiological process to be controlled only by algebraic additions of the effects of redundant promoters and inhibitors. Strict controls must exist, and immediate activation of angiogenesis at a short notice must be possible. This can only be accomplished by intricate interactions of the modulators of angiogenesis. Specific interactions that modulate key molecules such as VEGF and its receptors are likely to be involved. However, little is known about such interaction of VEGF and its receptors with other angiogenic factors. Recently, bFGF has been shown to increase KDR receptor expression (55). The mechanisms responsible for the elevated expression of KDR by bFGF may be an indirect effect due to elevated endogenous VEGF. Indeed, it has been shown that bFGF increases endogenous VEGF expression (56), which in turn may increase the levels of its receptors (49).

Our findings reported in this paper provide another paradigm of such an interrelation and interaction of thrombin as angiogenic factor with VEGF. We propose that a primary event in many angiogenic processes is the generation of thrombin, which through direct and/or indirect mechanisms activates the expression of VEGF receptors. In addition, thrombin has been reported to increase the release of VEGF from platelets (57) and the expression and release of bFGF from endothelial cells. Herbert et al. (58) have shown that the mitogenic effect of thrombin in human endothelial cells is largely due to bFGF. Activation of gelatinase A by thrombin may facilitate the initial local dissolution of basement membrane and cell migration at the early steps of angiogenesis (59). The decrease of endothelial cells attachment to extracellular matrix by thrombin that we have reported previously (60) may stimulate migration and cell survival. All these effects of thrombin on endothelial cells, as well as in other cell types involved in angiogenesis, may have synergistic effects in the activation of angiogenesis under physiological and pathological conditions. The relative importance of the aforementioned cellular effects of thrombin in the promotion of angiogenesis is likely to depend on the particular site and pathology involved. Thrombin thus may orchestrate these events temporally and spatially in order to activate, amplify, and maintain the angiogenic cascade.

Many of these processes as well as angiogenesis can be promoted by TRAP, the agonist peptide for the activated thrombin receptor (36). This opens the possibility of using thrombin-peptide mimetics to promote angiogenesis. Such non-thrombogenic analogs of the activated thrombin receptor may have potential therapeutic applications in wound healing, ischemic conditions, and other clinical situations where promotion of angiogenesis is desirable. Conversely, inhibitors of thrombin or peptide antagonists to the activated receptor, which are not interfering with blood coagulation, may be useful agents for anti-angiogenic therapy in cancer and other angiogenic diseases.

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REFERENCES

1. Trousseau, A. (1872) Lectures in Clinical Medicine, Delivered in Hotel-Dieu, Paris, pp. 281-285, New Sydenham Society, London.

2. Rickles, P. R., and Edwards, R. L. (1968) Blood 44, 14-31.

3. Sloan, B. F., Rozhin, J., Johnson, K., Taylor, H., Crissman, J. D., and Honn, K. V. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 2438-2447.

4. Zacharsky, L. R., Memoli, V. A., Morain, W. D., Schlaeppi, J. M., and Zacharsky, L. (1996) Cancer Res. 56, 4032-4038.

5. Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K. S., Pawling, J., Moos, L., Collen, D., Risau, W., and Nagy, A. (1996) Nature 380, 435-439.

6. Maeda, K., Chung, Y. S., Ogawa, Y., Takatsuka, S., Kang, S. M., Ogawa, M., Sawada, T., and Sowa, M. (1996) Cancer Res. 56, 858-863.

7. Chomzynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156-159.

8. Folkman, J. (1985) Adv. Cancer Res. 12, 179-203.

9. Maier, A. J., M. Voulalas, P., Roeder, D., and Maciag, T. (1990) Science 249, 1570-1574.

10. Klagsbrum, M., and D'Amore, P. A. (1991) Annu. Rev. Physiol. 53, 339-355.

11. Seghetti, G., Patel, S., Ren, C.-J., Guandalin, A., Pintucci, G., Robbins, E. S., Megnatti, P., and Pepper, M. S. (1995) J. Biol. Chem. 270, 20801-20807.

12. Ferreira, N., Carver, C. U., Steinhoff, M., and Bunnett, N. W. (1998) J. Biol. Chem. 273, C1429-C1452.

13. Kobayashi, E., Nakano, H., Morimoto, N. M., and Tamaoki, Y. (1989) Biochem. Biophys. Res. Commun. 159, 548-553.

14. Dery, O., Corcoran, K. T., and Maragoudakis, M. E. (1998) Angiogenesis Models, pp. 257-272.

15. Nierodzik, M. L., Kajumo, F., and Karpatikin, S. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 514.

16. Kuthanou, C., Kakkar, V. V., and Benzakour, O. (1998) in Maragoudakis, M. E., ed Vol. 298, 7533-7537.

17. Svarcova, J., Jirasek, J., and Jelencik, J. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 9109-9113.

18. Tuder, R. M., Fleck, B. E., and Voelkel, N. F. (1995) J. Clin. Invest. 95, 1350-1357.

19. Maier, A. J., M. Voulalas, P., Roeder, D., and Maciag, T. (1990) Science 249, 1570-1574.

20. Jaffe, E. A., Nachman, R.-L., Becker, C. G., and Minick, C. R. (1973) J. Clin. Invest. 52, 2745-2756.

21. Jaffe, E. A., Nachman, R.-L., Becker, C. G., and Minick, C. R. (1973) J. Clin. Invest. 52, 2745-2756.

22. Carmichael, J., Degriff, W. C., Gazdar, A. F., Minna, J. D., and Mitchell, J. B. (1987) Cancer Res. 47, 943-946.

23. Jaffe, E. A., Nachman, R.-L., Becker, C. G., and Minick, C. R. (1973) J. Clin. Invest. 52, 2745-2756.

24. Vlodavsky, I., and Bar-Shavit, R. (1998) Cell 90, 909-914.

25. Leemans, P. J., and Bohlen, P. (1992) Cancer Res. 52, 9159-9166.

26. Millauer, B., Shawner, L. K., Plate, K. H., Risau, W., and Ullrich, A. (1994) J. Biol. Chem. 269, 15547-15554.

27. Mohile, R., Green, D., Moore, M. A., Nachman, R. L., and Rajfi, S. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 663-668.

28. Herbert, J. M., Dupuy, E., Laplace, M. C., Ziu, J. M., Bar-Shavit, R., and Toebel, G. (1994) Biochem. J. 303, 227-231.

29. Zucker, S., Conner, C. E., Lorenz, A. F., Drews, M. H., and Bahou, W. F. (1998) Int. J. Cancer 75, 780-786.

30. Folkman, J. (1985) Nat. Med. 1, 27-31.

31. Herbert, J. M., Dupuy, E., Laplace, M. C., Ziu, J. M., Bar-Shavit, R., and Toebel, G. (1994) Biochem. J. 303, 227-231.

32. Zacharsky, L. R., Memoli, V. A., Morain, W. D., Schlaeppi, J. M., and Zacharsky, L. (1996) Cancer Res. 56, 858-863.

33. Maeda, K., Chung, Y. S., Ogawa, Y., Takatsuka, S., Kang, S. M., Ogawa, M., Sawada, T., and Sowa, M. (1996) Cancer Res. 56, 858-863.

34. Kim, K. J., Winer, J., Armanini, M., Gillet, N., Phillips, H. S., and Ferrara, N. (1993) Nature 362, 841-844.

35. MILLAUER, B., SHAWNER, L. K., PLATE, K. H., RISAU, W., AND ULLRICH, A. (1994) J. Biol. Chem. 269, 15547-15554.

36. Bergstrom, P., Hillan, K. J., Svarcova, J., and Ferrara, N. (1999) Cancer Res. 59, 1790-1797.

37. Svarcova, J., Jirasek, J., and Jelencik, J. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 9109-9113.

38. SEGHERTI, G., PATEL, S., REN, C.-J., GUANDALIN, A., PINTUCCI, G., ROBBINS, E. S., SHAPIRO, R. L., GALLOWAY, A. C., RIFKIN, D. B., AND MIGNATTI, P. (1995) J. Biol. Chem. 270, 23111-23118.

39. Seghetti, G., Patel, S., Ren, C.-J., Guandalin, A., Pintucci, G., Robbins, E. S., Shapiro, R. L., Galloway, A. C., Rifkin, D. B., and Mignatti, P. (1995) J. Cell Biol. 131, 1659-1673.

40. Segheriti, G., Patel, S., Ren, C.-J., Guandalin, A., Pintucci, G., Robbins, E. S., Shapiro, R. L., Galloway, A. C., Rifkin, D. B., and Mignatti, P. (1995) J. Cell Biol. 131, 1659-1673.