Thrombin induces increased expression and secretion of angiopoietin-2 from human umbilical vein endothelial cells

Yao-Qi Huang, Jian-Jun Li, Liang Hu, Merlin Lee, and Simon Karpatkin

Angiogenesis is required for tumor growth and metastasis. It has recently been suggested that thrombin is a potent promoter of angiogenesis. We therefore examined the possibility that thrombin could be inducing the expression of angiopoietin-2 (Ang-2), necessary for remodeling. Human umbilical vein endothelial cells were incubated with or without thrombin (1 U/mL) for 1 to 24 hours and then examined for messenger RNA (mRNA) by Northern analysis. Enhanced mRNA expression (about 4-fold over baseline) was noted at 4 hours. Enhanced expression of Ang-2 mRNA was secondary to enhanced transcription (about 4-fold), with no effect on stabilization. Enhanced Ang-2 mRNA transcription was inhibited by H7 and PD98059, indicating the requirement of serine/threonine kinases as well as the mitogen-activated protein kinase pathway. Up-regulation of mRNA was associated with enhanced Ang-2 protein synthesis and secretion as assayed by immunoblot. Thrombin-induced secreted Ang-2 inhibited the binding of recombinant 185–Ang-1 to its Tie-2–Fc receptor, demonstrating functionality. Hirudin reversed this effect, demonstrating thrombin specificity. Thus, thrombin-induced tumorigenesis and metastasis is associated with enhanced Ang-2 protein synthesis and secretion via enhanced transcription of Ang-2. This could help explain how thrombin promotes angiogenesis. (Blood. 2002;99:1646-1650) © 2002 by The American Society of Hematology

Materials and methods

Cell culture and materials

Human diploid primary culture fibroblasts (FS4) were kindly provided by Dr J. Vilcek (New York University Medical Center, New York, NY) and cultured in Dulbecco modified Eagle medium with 10% fetal calf serum and 1% penicillin and streptomycin. Human umbilical vein endothelial (HUVE) cells were derived from fresh umbilical cord. The cord was washed 3 times with phosphate-buffered saline, treated with 0.5% trypsin (Sigma, St Louis, MO) for 3 to 5 minutes, and the HUVE cells isolated by washing and centrifugation in phosphate-buffered saline. HUVE cells were cultured in EBM-2 media (Clonetics) containing 10% fetal calf serum, 1% penicillin/streptomycin, and endothelial growth supplements (Sigma). Cells were starved for 4 hours in Dulbecco modified Eagle medium and then treated with different concentrations of thrombin for various time intervals. Thrombin, wortmannin, H7, and PD98095 were obtained from Sigma.

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Northern Blot analysis
Total RNA was extracted, fractionated by electrophoresis on a 1% agarose gel in 6.7% formaldehyde, and transferred onto a Genescreen Plus nylon membrane (NEN Life Science Products, Boston, MA). The membrane was hybridized at 42°C overnight with 32P-dCTP–labeled probes specific for Ang-2 (1.5 kb reverse transcriptase–polymerase chain reaction product, which contains the complete coding region). The blots were sequentially washed with varying dilutions of SSC, the last being 0.1× SSC at 65°C for 30 minutes. Autoradiography was carried out at ~70°C with an intensifying screen. The autoradiographic signals were quantified by densitometric analysis (Personal Densitometer, Molecular Dynamics/Amersham).

Immunoblotting of secreted Ang-2
Cells were treated with 1 U/mL thrombin for 8 hours. Culture media were collected and concentrated with Centricom (Sigma), and cells were lysed and nuclei isolated by centrifugation at 12,000 g. HUVE cells were stimulated with or without thrombin (1 U/mL) for 1 hour. With ECL reagents (Amersham Life Science). Antibodies were applied and chemiluminescence generated by incubation with blocking solution (2% powdered milk, 0.2% Tween 20 in phosphate-buffered saline), and reacted with a specific goat anti–Ang-2 polyclonal antibody (Santa Cruz, CA). After washing, peroxidase-conjugated secondary antibody was applied and chemiluminescence generated by incubation with ECL reagents (Amersham Life Science).

Nuclear run-on analysis of transcription
HUVE cells were stimulated with or without thrombin (1 U/mL) for 1 hour. They were then lysed and nuclei isolated by centrifugation at 12,000g for 15 minutes at 4°C. The nuclear suspension was incubated with 0.5 mM each of CTP, ATP, and GTP and with 250 μCi (9.25 MBq) of 32P-UTP. The samples were extracted with phenol/chloroform, precipitated, and resuspended at equal counts per milliliter in hybridization buffer. Denatured plasmid DNA harboring the Ang-2 fragment as well as GAPDH were blotted onto nitrocellulose filters and then hybridized with the radiolabeled samples. The filters were washed as described above for Northern analysis and the membranes subjected to autoradiography.

Actinomycin D chase studies
Confluent cells were incubated in the presence of thrombin (1 U/mL) for 1 hour at 37°C. The cells were then treated with media containing actinomycin D (5 μg/mL) and incubated for an additional 1 to 7 hours. Total RNA was extracted and analyzed for Ang-2 mRNA levels by Northern blot analysis.

Ang-2 inhibition of binding of Ang-1 to Tie-2–Fc
35S-labeled recombinant Ang-1 was prepared by in vitro translation of the complementary DNA using the coupled TNT transcription/translation ribosomal system (Promega, Madison, WI). The recombinant Tie-2–Fc fusion protein was prepared from 293 cells and purified with protein A beads as previously described. Cultured HUVE cells were treated with thrombin (1 U/mL) or thrombin plus hirudin (1:1) for 8 hours at 37°C. The media were removed and concentrated 10-fold with Centricom (AMICON, Beverly, MA). A total of 1 mL concentrated media was mixed with 5 μL recombinant 35S–Ang-1, incubated with 0.25 μg Tie-2–Fc for 3 hours at 4°C and then precipitated with protein A beads and run on 12% SDS–polyacrylamide gel electrophoresis for autoradiography as described.

Results
Effect of thrombin on the expression of Ang-2 mRNA
Because thrombin stimulates tumor growth and metastasis and enhanced angiogenesis is required for both, we investigated the effect of thrombin on the induction of Ang-2 mRNA, an angiogenesis growth factor required for blood vessel remodeling. Figure 1 demonstrates that the expression of Ang-2 mRNA was upregulated about 4-fold by thrombin in HUVE cells with peak at 4 hours. Thrombin specificity was demonstrated by the more than 3-fold inhibition of Ang-2 mRNA with hirudin in HUVE cells (Figure 2).

Thrombin increases the production and secretion of Ang-2 protein
To define whether the up-regulation of Ang-2 mRNA was accompanied by an increase in protein synthesis, cells were cultured in media without or with thrombin (1 U/mL) for 8 hours. The cells were extracted with lysis buffer and the media collected and concentrated. Both were analyzed for Ang-2 production by Western blot. The production (Figure 3A) and

Figure 1. Thrombin-induced increased expression of Ang-2 mRNA in HUVE cells.

Figure 2. Thrombin specificity of Ang-2 mRNA up-regulation. Cells were treated without (C) or with 1 U/mL thrombin (T) or with thrombin plus hirudin (T + H) for 3 hours and then analyzed by Northern blot.

Figure 3. Effect of thrombin on Ang-2 protein production and secretion. FS4 and HUVE cells were cultured in media with or without thrombin, 1 U/mL, for 8 hours at 37°C. The media were collected and concentrated and the cells extracted with lysis buffer. Both were then analyzed for Ang-2 production by Western blot. (A) Ang-2 in HUVE cells, not in FS4 cells. (B) Ang-2 in supernatant of HUVE cells.
secretion (Figure 3B) of Ang-2 was increased 2.5- and 3.5-fold, respectively, in HUVE cells.

Thrombin induces increased transcription of Ang-2 mRNA

Thrombin-induced increased expression of Ang-2 mRNA could reflect increased gene transcription, increased mRNA stability, or both. To determine whether the increased Ang-2 mRNA was a result of increased gene transcription, cells were examined by nuclear run-on experiments. As shown in Figure 4, the rate of transcription of Ang-2 was increased by 3-fold in HUVE cells stimulated with thrombin.

As shown in Figure 5, the mRNA stability of Ang-2 was not increased with thrombin in HUVE cells, although the level of expression of Ang-2 was clearly enhanced upon thrombin stimulation.

Thrombin promotes the expression of Ang-2 via the serine/threonine kinase and MAPK pathways

To define the signaling pathways responsible for the increased expression of Ang-2 by thrombin, 3 major cellular transduction mechanisms involved in thrombin receptor activation\(^\text{28-30}\) were studied (phosphatidylinositol-3 [PI-3] kinase, serine/threonine kinases, and mitogen-activated protein kinase [MAPK]). As shown in Figure 6, the increased expression of Ang-2 by thrombin in HUVE cells is regulated by 2 of the 3 pathways examined. Ang-2 induction was totally inhibited by H7 (serine/threonine kinase inhibitor) and by PD98059 (MAPK kinase inhibitor) (3-fold less for both) but not inhibited by wortmannin (PI-3 kinase inhibitor). These data indicate that the mechanism of thrombin-induced up-regulation of Ang-2 in HUVE cells is associated with activation of serine/threonine kinases and the MAPK pathway.

Thrombin-induced Ang-2 inhibits the binding of Ang-1 to its receptor, Tie-2. Functional evidence that the thrombin-induced Ang-2 secretion is biologically active is provided by the following experiment. Recombinant \(^3\)S-labeled Ang-1 produced from an in vitro transcription/translation ribosomal system was shown to bind to its recombinant fusion protein receptor Tie-2–Fc (Figure 7, lane 1). Ang-2 derived from the supernatants of thrombin-treated HUVE cells was then preincubated with \(^3\)S–Ang-1 to incubation with Tie-2–Fc by Ang-2. Figure 7, lane 3, demonstrates inhibition of binding of Ang-1 to Tie-2–Fc by Ang-2. Figure 7, lane 2, demonstrates reversibility of the thrombin effect with hirudin.

Discussion

The requirement of angiogenesis for tumor growth and metastasis is well documented.\(^1\) The effect of thrombin and activated platelets on the promotion of tumor growth and metastasis is similarly well documented.\(^21-25\) The role of thrombin in the induction of angiogenesis has recently been explored by several groups and can now be attributed to its effects on VEGF, Ang-1, and Ang-2.

Platelets contain VEGF\(^21,32\) and Ang-1, which are released following platelet activation with thrombin. Tumor specimens are surrounded by platelets and when removed at surgery have VEGF and thrombin localized on their surface.\(^34-37\) Tumor growth is inhibited by VEGF antibody.\(^38,39\) The recombinant Tie-2 receptor, AdExTek, capable of blocking Tie-2 activation by Ang-1, inhibits the growth and metastasis of murine mammary carcinoma (4T1) and melanoma (B16F10.9) cells.\(^40\) Ang-2 has been found in hypervascular human hepatocarcinomas as well as in an animal model in which it was highly expressed only in tumor tissue. Ectopic expression of Ang-2 in nonexpressing human hepatocellular cells promoted hepatomas in nude mice.\(^41\) Ang-2 has been found in advanced-stage neuroblastoma compared with low-stage tumors as well as in neuroblastoma cell lines,\(^42\) in increased intensity in blood vessels of non–small cell lung carcinoma,\(^43\) uveal melanoma...
cell lines, thyroid tumor progression, and endothelial cells of human gliomas. However, these associations do not define the mechanism by which thrombin promotes angiogenesis. Recent publications contribute to our understanding of this mechanism. Maragoudakis and coworkers have reported that thrombin promotes endothelial cell alignment in vitro in matrigel and angiogenesis in vivo, 12 that angiogenesis is independent of fibron formation, 15 and that thrombin potentiates VEGF by upregulating its receptor, KDR. 14 Herbert et al have demonstrated up-regulation of endothelial cell growth by thrombin in the autocrine release of basic fibroblast growth factor. 13 Our group and Ollivier et al have recently demonstrated up-regulation of VEGF mRNA and protein in prostate DU145 cells 47 and fibroblasts. 37,48 We have recently demonstrated the presence of Ang-1 in platelets and its release by thrombin. 33 The role of Ang-2 in tumorigenesis and metastasis has recently been more clearly defined by Yanopoulos and coworkers. 50 They have observed that tumor cells do not initially require vascular support but then proceed to co-opt existing host endothelial cells in which Ang-2 is highly induced prior to VEGF induction. The co-opted vessels then regress via disruption of endothelial cell interactions and undergo apoptosis, resulting in central necrosis of the tumor. Angiogenesis is then induced at the tumor margin associated with the induction of VEGF and Ang-2, supporting further growth.

Our study indicates, for the first time, up-regulation of functional Ang-2 by thrombin. Our current report on the induction of Ang-2 protein synthesis and secretion contributes to an understanding of the mechanism of thrombin-induced angiogenesis, tumor growth, and metastasis. Unlike the effect of thrombin on up-regulation of VEGF via enhanced stabilization of VEGF mRNA, 47 Ang-2 upregulates via enhanced transcription, with absence of enhanced stabilization of mRNA. It has recently been reported by Oh et al 11 that both hypoxia and VEGF up-regulate Ang-2 mRNA in bovine microvascular endothelial cells and that VEGF is capable of upregulating Ang-2. One should therefore consider the possibility that thrombin-induced up-regulation of Ang-2 may be secondary to its up-regulation of VEGF. However, this was shown not to be the case by these authors, 32 who demonstrated that neutralizing anti-VEGF antibody had no effect on anoxia-induced up-regulation of Ang-2. Our studies similarly do not support this possibility because up-regulation of VEGF mRNA by thrombin is inhibited by wortmannin (PI-3 kinase inhibitor), 47 whereas up-regulation of Ang-2 mRNA is not and HUVE cells have no detectable VEGF mRNA before or after thrombin stimulation.

Up-regulation of Ang-2 mRNA was inhibited by both serine/threonine kinase and a highly specific MAPK kinase inhibitor. Both signaling pathways are involved in thrombin stimulation of cells. Thrombin-induced cell protection of astrocytes is inhibited by the serine/threonine kinase inhibitor, H7. 28 Thrombin-stimulated platelet activation and aggregation requires the activation of a MAPK kinase and the phosphorylation of a serine/threonine kinase. 30 Thus, phosphorylated MAPK kinases and serine/threonine kinases are required for the up-regulation of Ang-2 in HUVE cells, whereas the PI-3 kinase pathway does not appear to be involved.

It is generally accepted that VEGF, Ang-1, and Ang-2 are necessary for efficient blood vessel growth and development. It has been proposed that Ang-2, a natural antagonist of Ang-1, may be an important proangiogenic factor in that it may counteract Ang-1–mediated blood vessel stability, thus maintaining the endothelium in a more plastic state and promoting the response of endothelial cells to angiogenesis growth factors. 50 Up-regulation of both VEGF and Ang-2 by thrombin indicates that angiogenesis might be facilitated by thrombosis. Thus, the well-described association of thrombosis with cancer may be contributing to tumorigenesis by the initiation of thrombin-stimulated angiogenesis, which could explain, at least in part, the enhancement of experimental tumorigenesis by thrombin. 21,25

References


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