Growth-Regulated Oncogene Is Pivotal in Thrombin-Induced Angiogenesis

Maresa Caunt, Liang Hu, Thomas Tang, Peter C. Brooks, Sherif Ibrahim, and Simon Karpatkin

Departments of Medicine, Radiation Oncology and Cell Biology, and Pathology, New York University School of Medicine, New York, New York

Abstract

The mechanism of thrombin-induced angiogenesis is poorly understood. Using a gene chip array to investigate the promalignant phenotype of thrombin-stimulated cells, we observed that thrombin markedly up-regulates growth-regulated oncogene-α (GRO-α) in several tumor cell lines as well as endothelial cells by mRNA and protein analysis. Thrombin enhanced the secretion of GRO-α from tumor cells 25- to 64-fold. GRO-α is a CXC chemokine with tumor-associated angiogenic as well as oncogenic activation following ligiation of its CXCR2 receptor. GRO-α enhanced angiogenesis in the chick chorioallantoic membrane assay 2.2-fold, providing direct evidence for GRO-α as an angiogenic growth factor. Anti-GRO-α antibody completely inhibited the 2.7-fold thrombin-induced up-regulation of angiogenesis, as well as the 1.5-fold thrombin-induced up-regulation of both endothelial cell cord formation in Matrigel and growth in vitro. Thrombin as well as its PAR-1 receptor activation peptide [thrombin receptor activation peptide (TRAP)] as well as GRO-α all markedly increased vascular regulatory proteins and growth factors: matrix metalloproteinase (MMP)-1, MMP-2, vascular endothelial growth factor (VEGF), angiopoietin-2 (Ang-2), CD31, and receptors KDR and CXCR2 in human umbilical vein endothelial cells. All of the thrombin/TRAP gene up-regulations were completely inhibited by anti-GRO-α antibody and unaffected by irrelevant antibody. Similar inhibition of gene up-regulation as well as thrombin-induced chemotaxis was noted with small interfering RNA (shRNA) GRO-α KD 4T1 breast tumor and B16F10 melanoma cells. In vivo tumor growth studies in wild-type mice with shRNA GRO-α KD cells revealed 2- to 4-fold impaired tumor growth, metastasis, and angiogenesis, which was not affected by endogenous thrombin. Thus, thrombin-induced angiogenesis requires the up-regulation of GRO-α. Thrombin up-regulation of GRO-α in tumor cells as well as endothelial cells contributes to tumor angiogenesis. (Cancer Res 2006; 66(8): 4125-32)

Introduction

The promalignant role of thrombin in tumor adhesion (1–4), growth (5), metastasis (1, 2, 6–9), and angiogenesis (10–15) is well recognized. However, the mechanism of thrombin-induced angiogenesis is not clear. Vascular regulatory proteins and growth factors, particularly matrix metalloproteinases (MMP-1, MMP-2, and MMP-9), vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang-1), and Ang-2, are required for the regulation of blood vessel formation.

Thrombin-induced angiogenesis in a chick chorioallantoic membrane (CAM) assay is associated with up-regulation of the major VEGF (15) as well as Ang-2 (15). Thrombin also up-regulates VEGF (10, 16) and the major VEGF receptor KDR in endothelial cells (14) and induces the secretion of VEGF (17, 18) and Ang-1 (19) from platelets. Thrombin up-regulates Ang-2, MMP-1, and MMP-2 in endothelial cells (11, 20). However, the cellular mechanisms responsible for thrombin-induced up-regulation of these genes have not been established.

The chemokine growth-regulated oncogene-α (GRO-α) has been implicated in blood vessel formation. GRO-α is a CXC chemokine with oncogenic activity. GRO-α-induced metastasis is associated with increased angiogenesis (21–23). GRO-α binds to a seven transmembrane receptor, CXCR2 on endothelial cells and neutrophils, and promotes their chemotaxis (24). It is required for maintenance of wound repair and is necessary for neutrophil recruitment (25). GRO-α enhances growth, chemotaxis, and metastasis of several tumor cell lines (22, 23, 26–28).

Using an Affymetrix gene chip array to investigate the promalignant phenotype of thrombin-stimulated cells, we observed that thrombin up-regulates GRO-α 168-fold in an undifferentiated mouse tumor cell line (UMCL) and confirmed by reverse transcription-PCR (RT-PCR). Thrombin-induced up-regulation of GRO-α mRNA and protein was also noted in several other tumor cell lines, as well as human umbilical vein endothelial cells (HUVEC).

In this article, we show that (a) thrombin induces the up-regulation and secretion of GRO-α from tumor cells as well as HUVECs; (b) GRO-α, like thrombin, enhances neoangiogenesis, endothelial cord formation, and endothelial cell growth; (c) GRO-α, like thrombin, up-regulates an identical series of five proangiogenic genes; (d) anti-GRO-α antibody or small interfering RNA (siRNA) GRO-α knockdown (KD) inhibits the up-regulation of these genes as well as thrombin-induced angiogenesis, endothelial cord formation, chemotaxis, and cell growth; (e) thrombin-enhanced tumor growth, angiogenesis, metastasis, and up-regulation of VEGF and Ang-2 is inhibited in wild-type mice using GRO-α KD 4T1 breast carcinoma cells. These data indicate a pivotal role for GRO-α in thrombin-induced angiogenesis.

Materials and Methods

Reagents. Thrombin and thrombin receptor activation peptide SFLLRNPDKYEPF (TRAP) were purchased from Sigma Chemical Co. (St. Louis, MO). The irrelevant control peptide, CAPESIEFPVSEARVLED, was synthesized by Quality Control Biochemicals (Hopkinton, MA). Hirudin (Refludan) was obtained from Hoechst Marion Roussel (Kansas City, MO). Antibodies to Ang-2, CD31, CD105, von Willebrand factor (VWF), KDR,
VEGF, β-actin, and tubulin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies to MMP-1 and MMP-2 were purchased from Chemicon (Temecula, CA). Human recombinant GRO-α, basic fibroblast growth factor (bFGF), and anti-GRO-α were purchased from Biosource International (Camarillo, CA). Control IgG was purchased from Pierce (Rockford, IL). Growth factor–reduced Matrigel was purchased from Becton Dickinson (Lincoln Park, NJ). The GRO-α ELISA was done with a kit from R&D Systems (Minneapolis, MN).

**Cell lines and culture conditions.** Human prostate cell line PC3 and breast carcinoma cell line MCF-7 and murine B16F10 melanoma and breast 4T1 cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in DMEM (Sigma Chemical) supplemented with 10% fetal bovine serum (FBS; Life Technologies), 2 mmol/L L-glutamine, and penicillin-streptomycin. HUVECs were obtained from Cambrex Bioscience (Walkersville, MD) and were maintained in EB-2 (Cambrex Bioscience) supplemented with 2% FBS and growth factors (singlequots) according to the instructions of the manufacturer. Human brain microvascular endothelial cells (HBMEC) were provided by Dr. Jorge Ghiso (New York University Medical Center, New York, NY). All cells were grown at 37°C in 5% CO2. Tumor cells were starved for 17 hours and HUVECs for 4 hours in the absence of FBS before incubation with agonists.

**Chick CAM angiogenesis assay.** Angiogenesis assays were done as previously described (15). Briefly, 10-day-old chick embryos were prepared by separating the CAM from the shell membrane. Filter discs were placed on the CAM and the test compound or PBS was added to the disc at 0, 24, and 48 hours for thrombin and GRO-α experiments. For bFGF experiments, the filter disc was soaked in bFGF in RPMI for 30 minutes before addition to the CAM. Embryos were sacrificed at 72 hours and the CAM was removed for analysis. Angiogenesis was quantified by counting the number of branching blood vessels within the confined area of the filter disc by stereomicroscopy. Each experiment was completed thrice, with 8 to 12 embryos per experiment. Results are mean ± SE of the angiogenic index.

**Endothelial cell cord formation.** Matrigel (100 μL) was added to a 96-well plate and left to polymerize for 1 hour at 37°C. HUVECs were trypsinized and resuspended in EB-2, 2% FBS with or without thrombin, hirudin, or anti-GRO-α antibody. One hundred microliters were added to the top of the Matrigel and incubated for 24 hours at 37°C. Branch points were analyzed with an inverted microscope by phase microscopy and counted in four random fields per well. All measurements were made in triplicate.

**Endothelial cell culture in vitro.** HUVECs were grown to confluence, starved for 4 hours in the absence of FBS, and then incubated for 4 hours in the presence of GRO-α, thrombin, or GRO-α antibody or irrelevant IgG (2 μg/mL). Growth was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (A11) with a CellTiter 96 AQueous One Solution Cell Proliferation Assay kit supplied by Roche Diagnostic Corp. (Indianapolis, IN).

**Chemotaxis assay.** Transwell plates (obtained from Costar 3422, Corning, Inc., Corning, NY) were used to measure tumor chemotaxis. Cells were grown to 75% confluence in 10% bovine serum albumin (BSA)-DMEM culture medium for 24 hours, followed by 0.1% BSA-DMEM for an additional 24 hours. Cells were then trypsinized, washed with PBS, resuspended in 0.1% BSA-DMEM, and 200 μL (5 × 10^4 cells) were added to the upper chamber. The lower chamber contained 0.1 unit/mL thrombin in 600 μL of 0.1% BSA-DMEM. Plates were incubated at 37°C, 5% CO2, for 3 hours. Inserts were removed, washed with PBS, and then stained with crystal violet for 10 minutes. Cells and solutions had been removed from the inside of the insert. Excess stain was removed from the bottom of the insert by washing with a cotton-tipped applicator and then allowed to dry. Destaining was performed in 10% acetic acid for 10 minutes. The solution was then transferred to a 96-well plate and absorbance was read at 595 nm.

**RNA analysis.** RT-PCR for human GRO-α (J03361) was done with the following primers: forward 5'-TTTACCCCAAGACATCCAAAG-3' and reverse 5'-CAAAAACATTTAGGCACATTCAGG-3', which amplifies a 291 bp fragment of GRO-α. RT-PCR for mouse GRO-α (J04956) used the following primers: forward 5'-GCACCAAAACGAGCTCATAGT-3' and reverse 5'-TTGTCAAGACCCAGGCTTCAG-3', which amplifies a 175 bp fragment.

RT-PCR for CD105 used the following primers:

- **Forward:** 5'-CTTCAAGAGACAGCAAGAAG-3'
- **Reverse:** 5'-GTGGTTGGCACTTCAAGTGG-3'

RT-PCR for VWF used the following primers:

- **Forward:** 5'-GCTTCTAGCAGGCATCTCTG-3'
- **Reverse:** 5'-CAGCTGCTTCCAGAAAGAC-3'

**Knock down of GRO-α in 4T1 cells by shRNA.** GRO-α shRNA was introduced into the shRNA-RetroQ viral vector (BD Biosciences, Clontech, San Diego, CA) at the BamHI and EcoRI ligation sites according to the instructions of the manufacturer. shRNA oligonucleotides were derived from the murine GRO-α sequence (NIH Genbank accession no. J04596) and synthesized following derivation from the computer program supplied by BD Biosciences. Successful inhibitory siRNAs include the following:

- **G2 strand sequence:** 5'-GATCCGAGAGATAGTTGTATcaagagaTACTAAACTCTTATCCTGCTTGTGTTTTG-3' plus complementary strand,
- **G8 strand sequence:** 5'-AATTCAAAAAGCAAGAGATGAGTATTATCAATccagagaTTGTA- TAGTGTGTCGAGGCTTTTGTGTTTTTGTGTTTTG-3' plus complementary strand,
- **G9 strand sequence:** 5'-GATCCGAGAGATAGTTGTATcaagagaTACTAAACTCTTATCCTGCTTGTGTTTTG-3' plus complementary strand,
- **G10 strand sequence:** 5'-AATTCAAAAAGCAAGAGATGAGTATTATCAATccagagaTTGTA-TAGTGTGTCGAGGCTTTTGTGTTTTTGTGTTTTG-3' plus complementary strand,
- **G12 strand sequence:** 5'-GATCCGAGAGATAGTTGTATcaagagaTACTAAACTCTTATCCTGCTTGTGTTTTG-3' plus complementary strand,
- **G13 strand sequence:** 5'-AATTCAAAAAGCAAGAGATGAGTATTATCAATccagagaTTGTA-TAGTGTGTCGAGGCTTTTGTGTTTTTGTGTTTTG-3' plus complementary strand,
- **G14 strand sequence:** 5'-AATTCAAAAAGCAAGAGATGAGTATTATCAATccagagaTTGTA-TAGTGTGTCGAGGCTTTTGTGTTTTTGTGTTTTG-3' plus complementary strand,
- **G15 strand sequence:** 5'-AATTCAAAAAGCAAGAGATGAGTATTATCAATccagagaTTGTA-TAGTGTGTCGAGGCTTTTGTGTTTTTGTGTTTTG-3' plus complementary strand,
- **G16 strand sequence:** 5'-AATTCAAAAAGCAAGAGATGAGTATTATCAATccagagaTTGTA-TAGTGTGTCGAGGCTTTTGTGTTTTTGTGTTTTG-3' plus complementary strand

The two paired oligonucleotides were annealed to form double strands. Verification of the inserted sequence was obtained by automated DNA sequencing from the Core Laboratory at New York University Medical Center. The plasmids were packaged into Phoenix Ampho cells (BD Biosciences) by standard calcium phosphate transfection. Virus supernatants were collected at 48 hours posttransfection, centrifuged to remove nonadherent cells and cellular debris, and frozen in small aliquots at –80°C.

4T1 cells were seeded at 20,000 per well in 24-well plates. The following day, the culture medium was aspirated and replaced with retroviral supernatant diluted 1:2 DMEM in to a final volume of 1 mL in growth medium (DMEM) plus 10% FBS and penicillin-streptomycin. Polybrene was added to a final concentration of 4 μg/mL. Twenty-four hours after infection, the cells were collected by trypsinization and resedeeded in six-well dishes in selective medium + 1 μg/mL puromycin. Single-cell colonies were picked and resedeeded with selective medium.

Traditional RT-PCR and real-time RT-PCR were done to validate KD of GRO-α mRNA as well as to measure the effect of thrombin on GRO-α KD 4T1 cells. iCycle iQ real-time PCR with SYBR green) was purchased from Bio-Rad (Hercules, CA) and done according to the instructions of the manufacturer.

**Immunoprecipitation and Western blotting.** HUVECs were starved for 4 hours, then medium was removed and then replaced with added 5% BSA plus agonist studied. Tumor cells were starved for 17 hours and agonist was added for an additional 24 hours. Total cell extracts were prepared after removal of medium and lysing of cells in lysis buffer containing 1% Triton X-100.
X-100, 150 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L EGTA, 2.5 mmol/L sodium PPl, 1 mm β-glycerol phosphate, 50 mmol/L Tris-HCl (pH 7.5), 10% glycerol, 1 mmol/L Na3VO4, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 μg/mL leupeptin. After centrifugation, the supernatants (1 mg/mL) were incubated with 3 μL antibody (200 μg/mL) overnight at 4°C, followed by the addition of 30 μL protein A/G beads (0.5 mL/mL; Santa Cruz Biotechnology), and further incubated at 4°C for 2 hours followed by centrifugation. The immune complexes (beads) were washed thrice with lysis buffer and then suspended in 50 μL SDS-PAGE loading buffer. The washed, suspended beads were boiled at 95°C for 5 minutes, chilled at 4°C, and centrifuged. Thirty microliters of supernatant were then run on 10% SDS-PAGE under reducing conditions and transferred to a nitrocellulose membrane. Membranes were incubated with the appropriate antibody (1 μg/mL) for 1 hour at room temperature, washed, and incubated with horsaerdase peroxidase (HRP)—conjugated secondary antibody for another hour and developed by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

**Immunostaining for GRO-α.** Cells were serum starved for 4 hours before treatment with thrombin for 4 hours and conditioned medium was collected. ELISA for human GRO-α was done using Quantikine human GRO-α Immunostain (R&D Systems).

**In vivo studies in mice.** 4T1 cells were used because they induce spontaneous metastasis in syngeneic mice without the need to sacrifice animals because of enhanced tumor burden. 4T1-green fluorescent protein (GFP) cells were injected s.c. into BALB/c mice as described here. Animals were sacrificed on day 25 and their spontaneous pulmonary metastasis was evaluated as described (8). Tumor detection in the blood was performed by injecting 10 mg/kg i.p., daily for 10 days followed by every other day for 7 days. Day 1 injection was given 5 minutes before and 4 hours after tumor inoculation. Animals were sacrificed on day 25 and their spontaneous pulmonary metastasis was evaluated as described (8). Tumor detection in the blood was performed by injecting 10 mg/kg i.p., daily for 10 days followed by every other day for 7 days. Day 1 injection was given 5 minutes before and 4 hours after tumor inoculation. Animals were sacrificed on day 25 and their spontaneous pulmonary metastasis was evaluated as described (8). Tumor detection in the blood was performed by injecting 10 mg/kg i.p., daily for 10 days followed by every other day for 7 days. Day 1 injection was given 5 minutes before and 4 hours after tumor inoculation. Animals were sacrificed on day 25 and their spontaneous pulmonary metastasis was evaluated as described (8).

**Immunohistochemical staining.** Tumor nodules were fixed in 4% formalin. Paraffin-embedded tissue sections were used for H&E as well as anti-VWF immunohistochemical staining. The stains were done on an automated autostainer (Ventana Medical Systems, Tucson, AZ) according to the instructions of the manufacturer. Primary antibody titer was determined from a dilution curve. Optimum staining was obtained at a dilution of 1:40, which gave the lowest background and best sensitivity for antigen retrieval. Rabbit polyclonal anti-VWF IgG was followed by a biotinylated goat anti–rabbit polyclonal antibody, followed by streptavidin-HRP and H2O2 substrate plus 3,3-diaminobenzidine developing chromagen, which produces a brown precipitate. The entire tissue section was examined and areas with the highest intensity of staining (i.e., hotspots) (ref. 29) were identified and further examined under high-power magnification (×400) for VWF expression. The number and intensity of staining of small vessels in the hotspots were scored in a blinded fashion. An angiogenesis index was obtained by multiplying the number of vessels by the intensity of staining (scored 1-3).

**Statistical analysis.** This was done by Student’s t test.

**Results**

Thrombin up-regulates GRO-α mRNA and protein in tumor and endothelial cells. RT-PCR, Northern blot analysis, and immunoblotting were done on thrombin-stimulated tumor cell lines and HUVECs to confirm the gene chip array data and test for a generic effect in multiple cell lines. Figure 1A shows thrombin-induced mRNA up-regulation of GRO-α as measured by semiquantitative RT-PCR in UMCL, B16F10, and MCF7 cells at 24 hours. Figure 1B shows that thrombin or TRAP also induce up-regulation of GRO-α protein in PC3 cells 3.4- and 3.1-fold, respectively, by immunoblot at 24 hours. Similar up-regulation of 2.0- and 1.8-fold was noted with HUVECs at 0.1 and 0.5 unit/mL, respectively. Similar up-regulation of 2.4-fold was also noted with primary endothelial cells obtained from a second source, HBMECs treated with 0.5 unit/mL thrombin. Figure 1C shows concentration-dependent, thrombin-induced up-regulation of GRO-α secretion with 0.5 and 1 unit/mL thrombin by ELISA assay in MCF7 cells at 24 hours of 9- and 25-fold, respectively, as well as in PC3 cells of 36- and 64-fold, respectively. These data indicate that thrombin can induce various tumor cells to produce GRO-α.

GRO-α and thrombin-induced angiogenesis and endothelial cell growth is inhibited by anti-GRO-α antibody. We next focused our attention on the possible association of GRO-α with thrombin-induced angiogenesis. Because GRO-α has been associated with enhanced tumor metastasis and angiogenesis, we tested whether GRO-α per se was capable of enhancing angiogenesis in a CAM assay. Figure 2A shows a 2.2-fold enhanced angiogenesis (increased branching) with GRO-α at 50 ng/egg/d for 3 days (n = 19, P = 0.005), providing direct proof of GRO-α as an angiogenic factor. The increased branching was essentially...
identical to the pattern that we published for thrombin (15). Because thrombin markedly up-regulates GRO-α, we determined whether GRO-α is necessary for thrombin-induced angiogenesis. We therefore used a CAM assay to measure thrombin-induced angiogenesis in the presence of anti-GRO-α antibody. Figure 2B shows that thrombin enhanced angiogenesis 2.7-fold (n = 9, P < 0.01), which was completely inhibited by anti-GRO-α antibody, not by irrelevant IgG. Similar results were obtained with thrombin-induced endothelial cell cord formation in Matrigel (Fig. 2C), a required mechanism for neoangiogenesis. In these experiments, thrombin (0.25 unit/mL) enhanced cord formation 1.5-fold (n = 20, P < 0.0001) at 24 hours, which was inhibited by hirudin, a direct inhibitor of thrombin, as well as anti-GRO-α antibody (n = 7, P < 0.01). Control cell cord formation was not inhibited by anti-GRO-α antibody (n = 7, P > 0.2), suggesting that the GRO-α effect was on neoangiogenesis. These observations are supported by the effect of GRO-α and thrombin on HUVEC growth in culture (Fig. 2D). In these experiments, both GRO-α (0.25-0.5 μg/mL) and thrombin (0.5 unit/mL) increased HUVEC growth 1.5-fold (n = 4, P = 0.0005) and 2.6-fold (P = 0.0001), respectively. The thrombin effect was inhibited 80% by anti-GRO-α antibody, not with irrelevant IgG (n = 4, P = 0.0003), suggesting that thrombin may have an additional effect on enhancement of HUVEC growth not requiring GRO-α up-regulation.

GRO-α and thrombin up-regulate the same vascular regulatory proteins and growth factors in HUVECs. Thus, GRO-α and thrombin both induce angiogenesis in the CAM, endothelial cord, and endothelial growth assays, and anti-GRO-α antibody inhibits the thrombin effect. We therefore hypothesized that thrombin may be operating through the up-regulation of GRO-α. This hypothesis was tested by comparing the effects of both thrombin and GRO-α on vascular growth factors known to be up-regulated by thrombin, as well as other vascular factors with an unknown thrombin effect. Thrombin had no effect on up-regulation of Ang-1, MMP-9, epidermal growth factor, or bFGF in HUVECs (data not shown). However, vascular regulatory protein up-regulation by both GRO-α and thrombin was noted for MMP-1 (2.3- and 2.1-fold, respectively), MMP-2 (3.7- and 2.3-fold), CD31 (2.5- and 2.3-fold), Ang-2 (2.5- and 3.7-fold), and KDR (2.1- and 2.5-fold) in HUVECs (Figs. 3 and 4). Thrombin also up-regulated CXCR2, the receptor for GRO-α, which could be inhibited by hirudin (data not shown). TRAP also up-regulated CXCR2 in HUVECs (1.8- to 2-fold; Fig. 4D and E), indicating activation by PAR-1.

Anti-GRO-α antibody or shRNA versus GRO-α inhibit the effect of thrombin on up-regulation of MMP-1, MMP-2, CD31, Ang-2, KDR, VEGF, and CXCR2. If thrombin or TRAP-induced angiogenesis requires up-regulation of a series of vascular regulatory genes by GRO-α, then antibody against GRO-α should inhibit the same pattern of thrombin-induced genes. Figure 4 shows that TRAP induced up-regulation of CD31 (2.6-fold; A), KDR (2.3-fold; B), Ang-2 (5.9-fold; C), CXCR2 (1.8- to 2.0-fold; D and E), MMP-1 (2.9-fold; F), and MMP-2 (3.7-fold; G) in HUVECs are all inhibited by anti-GRO-α antibody, whereas irrelevant antibody had no effect. To prove that TRAP/GRO-α was operating via the CXCR2 receptor, we did similar experiments with anti-CXCR2 antibody in HUVECs (E). The effect of TRAP

Figure 2. Effect of GRO-α, thrombin, and anti-GRO-α antibody on angiogenesis in the CAM, endothelial cord formation, and endothelial cell growth assays. A, GRO-α activation of angiogenesis at 72 hours and 37°C in the CAM assay (25 and 50 ng/egg, n = 19). B, inhibition of thrombin-induced angiogenesis (Thr) by anti-GRO-α antibody (Thr+GROAb, 5 μg/egg) or irrelevant IgG (Thr+IgG), n = 9. C, effect of thrombin (0.25 unit/mL), thrombin plus 1 unit/mL hirudin (Thr+H), or hirudin (H) on thrombin-induced cord formation of endothelial cells (n = 20). Effect of GRO-α (2 μg/mL), thrombin plus anti-GRO-α antibody (Thr+Ab, 5 μg/mL), or control plus anti-GRO-α antibody (C+Ab, 5 μg/mL) on thrombin-induced cord formation of endothelial cells in Matrigel at 24 hours and 37°C (n = 7). D, effect of GRO-α (0.025-0.5 μg/mL; Gro) or thrombin (Thr, 0.5 unit/mL) on HUVEC growth in vitro at 48 hours and 37°C. Inhibition of thrombin-induced HUVEC growth in the presence of 5 μg/mL anti-GRO-α antibody. Absence of inhibition in the presence of irrelevant IgG (n = 4).
(2.0-fold) was inhibited by anti-CXCR2 to the same extent as with anti-GRO-α antibody.

The specificity of this reaction was further shown by using an shRNA knockdown of GRO-α in 4T1, B16F10, and HUVECs (Fig. 5A). Successful KD was obtained with cell lines G2, G3, G2/8, and hG2. As found with other tumor cell lines (10, 11), thrombin up-regulated VEGF and Ang-2 mRNA levels in cells transfected with empty vector. Preliminary data using semiquantitative PCR revealed inhibition of thrombin-induced up-regulation of VEGF in G2/G8 and G3 KD 4T1 cells (data not shown).

A more thorough analysis was done using real-time RT-PCR in 4T1, B16F10, and HUVEC GRO-α KD cells. This showed inhibition of thrombin-induced up-regulation of VEGF and Ang-2 (Fig. 5B), confirming the requirement of GRO-α for up-regulation of VEGF and Ang-2. Similar results were obtained with GRO-α KD HUVEC in which thrombin up-regulation of Ang-2, CD31, CXCR2, and KDR was inhibited (Fig. 5C).

A functional chemotaxis analysis was also used for GRO-α KD cells to confirm operational GRO-α KD. Figure 5D shows that GRO-α KD successfully inhibits thrombin-induced chemotaxis in 4T1 and B16F10 cells. Empty vector had no effect on baseline chemotaxis. Scrambled siRNA had no effect on thrombin-induced chemotaxis.

**Effect of GRO-α and hirudin on in vivo angiogenesis, tumor growth, seeding, and metastasis.** We next designed studies to test whether thrombin-induced up-regulation and secretion of GRO-α in tumor cells was contributing to tumor angiogenesis, growth, and metastasis in vivo. GRO-α KD 4T1 tumor cells were injected into syngeneic mice to follow tumor angiogenesis, growth, and metastasis. We hypothesized that tumor incapable of synthesizing and secreting GRO-α would have fewer blood vessels, smaller tumor nodules, and less metastasis. We also hypothesized that the inhibitory effect of the potent thrombin inhibitor, hirudin, on 4T1 tumor growth (8) would be diminished to absent in GRO-α KD tumors. Such proved to be the case. Figure 6A shows decreased 4T1 tumor growth in vivo, which varied from 4- to 3-fold during earlier time points (1-13 days) to 2-fold at later time points (14-25 days). Of particular note is the observation that hirudin had no effect on tumor growth in GRO-α KD cells, suggesting that the hirudin-inhibited endogenous thrombin effect was operating through GRO-α.

We next examined the effects on tumor seeding into the blood as well as spontaneous pulmonary metastasis in mice injected with GRO-α KD 4T1 cells with and without hirudin treatment. Animals treated with hirudin decreased their 4T1 seeding into the blood from 47 ± 7 cells with empty vector to 2.2 ± 1.2 cells with hirudin treatment (P = 0.0005), confirming our previously reported observations (8). However, GRO-α KD had no effect on seeding (55 ± 3.1 with GRO-α KD), whereas GRO-α KD + hirudin treatment decreased seeding to 6.8 ± 3.1 cells (P = 0.0013). Hirudin also inhibited spontaneous tumor metastasis, as previously reported [10.8 ± 1.4 nodules with empty vector versus 6.0 ± 0.8 (P < 0.001) with addition of hirudin]. GRO-α KD also inhibited metastasis [10.8 ± 1.4 with empty vector versus 6.8 ± 1.4 (P < 0.04) with GRO-α KD cells] and GRO-α KD plus hirudin inhibited metastasis to 4.6 ± 0.8 nodules (P = 0.02). Thus, although GRO-α KD impairs the development of spontaneous pulmonary metastasis, it has no effect on tumor seeding, suggesting that GRO-α is required for tumor nodule development once tumor cells are already seeded in the blood.

We next focused our attention on angiogenesis, using real-time RT-PCR for CD105 and VWF in tumor nodules of 4T1 cells as surrogate markers for angiogenesis. Inhibition of tumor nodule CD105 mRNA was observed when using shRNA GRO-α KD cells, hirudin alone, or both of 40% (P = 0.02), 36% (P = 0.002), and 51% (P = 0.002), respectively, when compared with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) internal control mRNA (n = 3). Similar results were obtained with VWF as marker of GRO-α KD cells, hirudin alone, or both of 40% (P = 0.02), 36% (P = 0.002), and 55% (P = 0.002), respectively, for GRO-α, hirudin alone, or both (n = 3). This was confirmed by immunohistochemistry with the VWF marker (Fig. 6B). Control nodules had higher numbers and intensity of staining than the experimental samples. The angiogenesis index for control nodule (270 ± 33) was decreased 38% in GRO-α KD nodules (P = 0.002), 51% with hirudin alone (P = 0.030), and 47% in both (P = 0.042; n = 5). It should be pointed out that functional antiangiogenesis effects may also occur without or with minimal reduction in increased density, as discussed by Hlatky et al. (29).

**Thrombin up-regulation of vascular genes is different from bFGF or VEGF.** To examine the specificity of GRO-α up-regulation by thrombin/TRAP, we analyzed the effect of two other vascular growth factors, bFGF and VEGF, on GRO-α and angiogenesis. bFGF (1-2 μg/filter disc on egg) enhanced angiogenesis 2.6-fold at 72 hours (n = 7, P < 0.001) in the CAM assay. However, bFGF had no effect on GRO-α up-regulation by immunoblot in HUVECs, yet it
up-regulated VEGF, KDR, CD31, CXCR2, and Ang-2 (data not shown). VEGF similarly had no effect on GRo-α, yet it up-regulated CD31 and Ang-2 in HUVEC and had no effect on KDR (Fig. 3) or CXCR2 (data not shown). Thus, thrombin-induced angiogenesis specifically requires the up-regulation of GRo-α.

**Discussion**

These data clearly show a pivotal role for the chemokine GRo-α and its endothelial cell CXCR2 receptor in thrombin-induced neo-angiogenesis. This was documented by analyzing the effect of both thrombin and GRo-α on the up-regulation of vascular regulatory genes as well as angiogenesis in the CAM, endothelial cell cord–forming, and endothelial growth assays. Anti-GRo-α antibody inhibited both GRo-α as well as thrombin-induced angiogenesis in the CAM, endothelial cell cord formation, and endothelial cell growth assays.

Both thrombin and GRo-α up-regulate MMP-1, MMP-2, VEGF, KDR, Ang-2, and CD31 and thrombin up-regulates the GRo-α receptor CXCR2. Up-regulation of MMP-1 (20), VEGF (10), KDR (14, 30), and Ang-2 (11) has been reported for thrombin but not for GRo-α. Up-regulation of the GRo-α receptor CXCR2 has not been reported for thrombin or GRo-α. Up-regulation of GRo-α by thrombin has been reported for HUVEC (31). Our data indicate that this is likely to be a general response because it is also up-regulated by thrombin in several tumor cell lines, murine B16F10 and UMCL, and human MCF7 breast and PC3 prostate cell lines.

The similar pattern of up-regulation of these vascular regulatory genes in HUVEC and tumor cells by both thrombin and GRo-α as agonists, as well as the marked thrombin-induced up-regulation of GRo-α, suggested that thrombin could be having its effect through the up-regulation of GRo-α. Such proved to be the case because (a) anti-GRo-α antibody inhibited thrombin-induced angiogenesis in both the CAM and endothelial cell cord–forming assay; (b) anti-GRo-α antibody inhibited thrombin-induced up-regulation of MMP-1, MMP-2, VEGF, KDR, Ang-2, CD31, and CXCR2; (c) VEGF, Ang-2, KDR, and CXCR2 were not up-regulated by thrombin in...
GRO-α KD cells. Further proof that thrombin is operating through GRO-α ligation of its CXCR2 receptor was obtained by demonstrating that an antibody against CXCR2 also inhibited the effect of thrombin on up-regulation of the vascular regulatory genes tested.

Thrombin-induced up-regulation of GRO-α in tumor cells is likely to contribute to the effect of thrombin on endothelial cells by providing an additional source of GRO-α production. This is supported by in vivo studies on endogenous thrombin and tumor growth in wild-type mice, injected with GRO-α KD 4T1 cells in the presence and absence of hirudin. Hirudin had no effect on the impaired tumor growth of GRO-α KD cells. Thus, tumor cells can then be contributing to angiogenesis by the production of GRO-α as is the case with VEGF (10), and thrombin can be contributing to the malignant tumor phenotype by enhancing tumor angiogenesis. Indeed, it has been reported that angiogenesis contributes to the activation of dormant tumor cells (32).

The mechanism of thrombin-induced up-regulation of angiogenesis (in association with the seven vascular regulatory genes tested) operates through the ligation of its PAR-1 receptor. This was documented with the protein-activated receptor agonist SFLLRNPDYKYPFF whose gene up-regulation effect was also inhibited by anti-GRO-α antibody. MMP-1 has similarly been reported to be up-regulated by ligation of PAR-1 in human endothelial cells (20).

The requirement of GRO-α up-regulation for angiogenesis is specific for thrombin. The angiogenic growth factor bFGF does not up-regulate GRO-α, whereas bFGF up-regulates KDR, Ang-2, CXCR2, and CD31 in HUVEC. VEGF does not up-regulate GRO-α or KDR in HUVEC, whereas it up-regulates Ang-2 and CD31 in HUVEC. In addition, we have recently observed that thrombin-induced up-regulation of cathepsin D and α-synuclein does not require GRO-α,4 further supporting GRO-α specificity for thrombin-induced angiogenesis.

Stimulation of angiogenesis by GRO-α has been suggested from histochemical studies by the inhibitory effect of anti-GRO-α antibody on angiogenesis associated with DU145 prostate tumor growth in severe combined immunodeficient mice as well as the use of tumor homogenates applied to a rat corneal micropocket assay, before and after in vivo treatment of mice with anti-GRO antibody (27). It also has been suggested by experiments in which GRO-α has been transfected into a squamous cell carcinoma cell line with low levels of GRO-α, which resulted in increased CD31 blood vessels as well as tumor growth (22). Our work provides data for the first direct evidence of GRO-α-induced angiogenesis as documented by the CAM, endothelial cord formation, and endothelial growth assays as well as the up-regulation of seven vascular regulatory proteins and growth factors. Our in vivo studies provide further evidence that both GRO-α and thrombin are capable of regulating tumor angiogenesis. Stimulation of angiogenesis by thrombin is well documented in the CAM and endothelial cell cord formation assays (12, 15). Our data report the first evidence for the pivotal role of GRO-α in thrombin-induced angiogenesis in HUVEC as well as tumor cells.

Exclusive of its effect on angiogenesis, both thrombin (2, 4, 5, 7, 8) and GRO-α are pro-oncogenic (22, 23, 27, 33). GRO-α is an autocrine growth factor first identified in melanoma. Overexpression in immortalized mouse melanocytes enabled the cells to form large colonies in soft agar as well as tumors in nude mice (33). In melanoma, GRO-α has been shown to have mitogenic as well as angiogenic effects on tumor growth (33). A DU145 human prostate cancer cell line had in vivo growth, which correlated with tumor-derived GRO-α and could be inhibited with anti-GRO-α antibody (31). GRO-α promotes murine squamous cell carcinoma growth, metastasis, and leukocyte infiltration by a host CXCR2-dependent mechanism. An autocrine increase in growth was also noted in vivo (22). Variants of murine squamous cell carcinoma that grow and metastasize more rapidly in vivo express increased levels of GRO-α compared with those that grow and metastasize more slowly (22). Transfection of GRO-α into a cell line with low levels of GRO-α showed an increased rate of growth and spontaneous metastasis in BALB/c mice in association with increased infiltration of host leukocytes. This increase in growth was inhibited in CXCR2(-/-) mice (22). S.c. growth of Lewis lung carcinoma as well as spontaneous pulmonary metastasis is significantly

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4 L. Hu and S. Karpatkin, unpublished data.
decreased in CXCR2−/− mice; however, this was attributed to decreased angiogenesis because GRO-α had no effect on Lewis lung carcinoma in vitro (23).

The above data clearly describe a new mechanism for thrombin induction of the malignant phenotype in numerous tumor cell lines. Its effect on tumor angiogenesis requires up-regulation of the pro-oncogenic/angiogenic gene GRO-α. Thus, thrombin serves a dual purpose in enhancing angiogenesis as well as tumor growth and metastasis.

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Growth-Regulated Oncogene Is Pivotal in Thrombin-Induced Angiogenesis

Maresa Caunt, Liang Hu, Thomas Tang, et al.


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