Thrombin Up-regulates Cathepsin D which Enhances Angiogenesis, Growth, and Metastasis

Liang Hu, Jennifer M. Roth, Peter Brooks, Joanna Luty, and Simon Karpatkin

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

Abstract
Cathepsin D (CD) up-regulation has been associated with human malignancy and poor prognosis. Thrombin up-regulated CD mRNA and protein in eight tumor cell lines as well as in human umbilical vascular endothelial cells (HUVEC). Thrombin increased the secretion of CD by 3- to 8-fold and enhanced chemotaxis (~2-fold) in 4T1 murine mammary CA cells, which was completely inhibited with the knockdown of CD. Secreted 4T1 CD induced neoangiogenesis by 2.4-fold on a chick chorioallantoic membrane, which was blocked in CD-KD cells. The addition of pure CD (2 ng) to the chick chorioallantoic membrane increased angiogenesis by 2.1-fold, which was completely inhibited by Pepstatin A (Pep A). CD enhanced human HUVEC chemotaxis and Matrigel tube formation by 2-fold, which was then blocked by Pep A. CD enhanced HUVEC matrix metalloproteinase 9 (MMP-9) activity by ~2-fold, which was completely inhibited by Pep A as well as a generic MMP inhibitor, GM6001. The injection of CD-KD 4T1 cells into syngeneic mice increased tumor growth by 3- to 4-fold compared with empty vector (EV) cells. Hirudin, a specific thrombin inhibitor, inhibited the growth of wild-type and EV cells by 2- to 3-fold, compatible with thrombin up-regulation of CD. CD and thrombin also contributed to spontaneous pulmonary metastasis; 4-fold nodule inhibition with CD versus EV and 4.6-fold inhibition with hirudin versus EV (P < 0.02). Thus, thrombin-induced CD contributes to the malignant phenotype by inducing tumor cell migration, nodule growth, metastasis, and angiogenesis. CD-induced angiogenesis requires the proteolytic activation of MMP-9. [Cancer Res 2008;68(12):4666–73]

Introduction
Although the association of thrombosis and occult cancer has been recognized for >140 years (1–4), the role of thrombin in enhancing the malignant phenotype has only been recently appreciated. Various studies using washed platelets treated with exogenous thrombin and/or experimental pulmonary metastasis (induced by tail vein injection of tumor cells grown in culture) have been performed. These revealed enhanced tumor adhesion to platelets, endothelial cells, fibronectin and von Willebrand’s factor (5–8), growth (9), experimental metastasis (5, 6, 10–12), and angiogenesis (13–18). Recent studies have tested the effect of endogenous thrombin (hirudin treatment) on spontaneously metastasizing 4T1 mammary tumor injected into the flank of syngeneic mice. Hirudin inhibition of tumor seeding into blood and spontaneous pulmonary metastasis has provided more pathophysiologic relevance to these earlier studies (19).

Despite these observations, the precise mechanisms for these reactions are poorly understood, except for angiogenesis, in which the role of thrombin has been shown to be due to the up-regulation of various vascular growth factors and receptors from tumor cells as well as platelets and endothelial cells: vascular endothelial growth factor (VEGF; refs. 13, 20–22), KDR (17, 23, 24), angiopoietin-2 (14), metalloproteinases 1 (25) and 2 (24), and most recently, growth-regulated oncogene-α and its receptor (CXCRI; ref. 24). Growth-regulated oncogene-α induces angiogenesis following thrombin-induced synthesis and secretion from both tumor cells and primary endothelial cells (24).

To better understand the mechanisms involved in the thrombin-induced malignant phenotype with respect to tumor growth and metastasis, we performed an Affymetrix gene chip array on two tumor cell lines, murine B16F10 melanoma and UMCL (undifferentiated murine cell line), to look for genes which could activate or contribute to the promalignant phenotype. Numerous genes were up-regulated or down-regulated over a 24-h exposure with 0.5 units/mL of thrombin. Of particular interest was the 2- to 4-fold up-regulation of cathepsin D (CD), confirmed by mRNA and protein analysis. We focused on CD because its up-regulation has been associated with human malignancy and poor prognosis, particularly with breast cancer (26–31). CD elevation is also a poor prognostic marker for breast, ovary, prostate, bladder, and melanoma cancer, and has been previously associated with increased risk of relapse and metastasis (32, 33). A large-scale study of 2,810 node-negative breast cancer patients with a median follow-up of 88 months revealed that patients with a high or moderate CD level in a primary tumor have a poor prognosis and relapse-free or overall survival, independent of histologic grade, hormone receptor, or tumor size (30).

CD is a lysosomal aspartyl glycoproteinase (active at acidic pH) which is found in intracellular vesicles, lysosomes, phagosomes, and late endosomes. Its major area of function seems to be the intracellular degradation of protein (regulating cell growth and tissue homeostasis; ref. 32). It is a housekeeping gene necessary for the development of newborn mice because CD(−/−) mice undergo progressive atrophy of the intestinal mucosa and destruction of lymphoid organs (34). Procathepsin D has a molecular weight of 52 kDa, which is cleaved to a 48-kDa active intermediate that is then cleaved in lysosomes to a mature two-chain 14 and 34 kDa cathepsin enzyme with critical aspartic residues, one on each chain (35, 36). Both procathepsin D and CD are overexpressed in mammary cancer by 2- to 50-fold (37) and can be secreted in the plasma of patients with breast cancer (38, 39). Secreted procathepsin D can stimulate growth and tumor cell proliferation of breast and prostate cell lines (40–43). However, this has been reported not to be related to its proteolytic function because the mutation of its active proteolytic site does not affect its mitogenic activity.
(44), suggesting that procathepsin D may be activating an unknown extracellular receptor.

In this report, we show that (a) thrombin up-regulates CD in eight different tumor cell lines as well as in a primary endothelial cell line, (b) thrombin enhances the secretion of CD from six different tumor cell lines tested, as well as in human umbilical vascular endothelial cells (HUVEC), (c) CD directly stimulates angiogenesis when applied to a chick chorioallantoic membrane (CAM), (d) knockdown of CD with short hairpin RNA (shRNA) in spontaneously metastatic breast carcinoma 4T1 cells inhibits tumor nodule growth, seeding, metastasis, and angiogenesis in vivo compared with empty vector (EV) cells.

Materials and Methods

Reagents. Thrombin, thrombin receptor activation peptide SFLRNPNDKYPF (TRAP), Pepstatin A (Pep A), and CD were purchased from Sigma. The irrelevant control peptide, CAPESIEPVEARVLED, was synthesized by Quality Control Biochemicals. Hirudin (Refludan) was obtained from Hoechst Marion Roussel. Antibody to CD was purchased from Santa Cruz Biotechnology, Inc. Matrix metalloproteinase (MMP) inhibitor GM6001 was obtained from Chemicon International.

Cell lines and culture conditions. Human prostate cell line PC3, breast carcinoma cell lines (MCF-7 and MD-MB-231), macrophage cell line THP1, murine B16F10 melanoma, and breast carcinoma 4T1 cells were purchased from American Type Culture Collection and maintained in DMEM (Sigma Chemical Co.) supplemented with 10% fetal bovine serum (FBS; Life Technologies). 25 mmol/L of l-glutamine, and penicillin-streptomycin. The undifferentiated mouse cell line (UMCL) was from our laboratory and was maintained in DMEM. HUVECs were obtained from Cambrex Bioscience and were maintained in EBM-2 (Cambrex Bioscience) supplemented with 2% FBS and growth factors according to the manufacturer's instructions. The human brain microvascular endothelial cell line was 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 overnight in the absence of FBS prior to incubation with agonists.

Endothelial cell culture in vitro. HUVECs were grown to near-confluence, starved for 4 h in the absence of FBS and then incubated for 48 h in the presence of CD, thrombin, or CD antibody or irrelevant IgG (2 μg/mL). Growth was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay with a Cell Proliferation Kit supplied by Roche Diagnostic Corp.

Chemotaxis assay. Transwell plates (obtained from Costar 3422; Corning, Inc.) were used to measure tumor chemotaxis. HUVEC or tumor cells were grown to 75% confluence in 10% bovine serum albumin (BSA)–DMEM culture media for 24 h, followed by 0.1% BSA-DMEM for an additional 4 or 24 h, respectively. Cells were trypsinized, washed with PBS, resuspended in 0.1% BSA-DMEM, and 200 μL (5 × 104 cells) were then added to the upper chamber. The lower chamber contained 0.1 units/mL of thrombin or 2 ng of CD in 600 μL of 0.1% BSA-DMEM. Plates were incubated at 37°C, 5% CO2 for 3 h. Inserts were removed, washed with PBS, and then stained with crystal violet for 10 min. Cells and solution were removed from the inside of the insert. Excess stain was removed from the bottom of the insert by swabbing with a cotton-tipped applicator and then allowed to dry. Destaining was performed in 10% acetic acid for 10 min. The solution was then transferred to a 96-well plate and absorbance read at 595 nm.

Angiogenesis assay with CAM. Angiogenesis assays were performed as previously described (18). Briefly, 10-day-old chick embryos were prepared by separating the CAM from the shell membrane. Filter discs were placed on the CAM and 25 μL of the test compound or PBS was added to the disc at 0, 24, and 48 h. Embryos were sacrificed at 72 h and CAMs removed for analysis. Angiogenesis was quantified by counting the number of branching blood vessels within the confined area of the filter disc using stereo-microscopy. Each experiment was completed two or three times, with four embryos per condition. The angiogenic index was calculated by subtracting the total number of blood vessel branch points derived from PBS-treated control CAMs from that of the experimental group.

Matrigel endothelial tube formation was measured as described (24). Briefly, Matrigel was added to a 96-well plate and left to polymerize for 1 h at 37°C. HUVECs (5 × 104) were then added in EBM-2 plus 2% FBS for 24 h at 37°C (with or without test agents). Branch points were measured by phase microscopy in 10 random fields per well.

Gelatin zymography. Cultures of subconfluent HUVECs were starved for 4 h in serum-free EBM-2 medium with 0.1% BSA. Cells were incubated for 24 h with various combinations of PBS, Pep A (20 μmol/L), CD (2 ng/mL), or MMP inhibitor (MPi) GM 6001 (20 μmol/L). The conditioned medium was removed and concentrated 10-fold. Fifty micrograms of protein were electrophoresed in 10% SDS-PAGE polymerized with 0.2% gelatin. The gel was then washed thrice for 1 h with 2.5% Triton X-100 and incubated for 16 h at 37°C in collagenase buffer containing 50 mmol/L of Tris, 200 mmol/L of NaCl, and 10 mmol/L of CaCl2 (pH 7.5). Gelatinolytic activity was visualized by staining with 0.5% Coomassie blue.

Knockdown of CD in B16/F10 and 4T1 cells by shRNA. CD shRNA were introduced into the shRNA-RetroQ retrovirus (BD Biosciences, Clontech) at the BamHI and EcoRI ligation sites according to the manufacturer's directions. shRNA Oligonucleotides were derived from the murine CD sequence (NIH Gene Bank accession number NM-009983) and synthesized following derivation from the computer program supplied by BD Biosciences.

Forward strand sequence: 5′ GATCCGGATGGGCTACCCTCATATTTTCCAGAGAAAGATATGAGGGTAGCCCATGCG 3′
Reverse strand sequence: 5′ AATTCCAAAAAGCATGGGCTACCCTCATA- TCTTCCTTGAAGATAGTGGAGTACCCTGAGC 3′.

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 Amphi cells (BD Biosciences) by standard calcium phosphate transfection. Virus supernatants were collected at 48 h posttransfection, centrifuged to remove nonadherent cells and cellular debris, and frozen in small aliquots at −80°C. B16F10 or 4T1 cells were seeded at 20,000 cells per well in 24-well plates. The following day, the culture medium was aspirated and replaced with retroviral supernatant–diluted 1:2 DMEM into 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 resuspended in six-well dishes in selective medium + 1 μg/mL of puromycin. Single-cell colonies were picked and reseeded with selective medium.

Traditional reverse transcription-PCR and real-time quantitative reverse transcription-PCR were performed to validate the knock down of CD mRNA as well as to measure the effect of thrombin on CD-KD 4T1 cells. iCycle qrt real-time PCR (iScript one-step RT-PCR with SYBR Green) was purchased from Bio-Rad and performed according to the manufacturer's instructions.

Immunoprecipitation and Western blotting. HUVECs were starved for 4 h, medium was removed and then replaced with the additional 5% BSA plus agonist studied. Tumor cells were starved overnight and agonist added for an additional 24 h. Total cell extracts were prepared after the removal of medium, and after lysing cells in lysis buffer containing 1% Triton X-100, 150 mmol/L of NaCl, 5 mmol/L of EDTA, 1 mmol/L of EGTA, 2.5 mmol/L of sodium pyrophosphate, 1 mmol/L of β-glycero phosphate, 50 mmol/L of Tris-HCl (pH 7.5), 10% glycerol, 1 mmol/L of Na2VO3, 1 mmol/L of DTT, 1 mmol/L of phenylmethylsulfonyl fluoride, and 1 μg/mL of leupeptin. After centrifugation, the supernatants (1 mg/mL) were incubated with 3 μL of antibody (200 μg/mL) overnight at 4°C followed by the addition of 30 μL of protein A/G beads (0.5 μL/mL; Santa Cruz Biotechnology), and further incubated at 4°C for 2 h followed by centrifugation. The immune complexes (beads) were washed thrice with lysis buffer and then suspended in 50 μL of SDS-PAGE loading buffer. The washed, suspended beads were boiled at 95°C for 5 min, chilled at 4°C, and centrifuged. Thirty microilters of the
supernatant was 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 h at room temperature, washed and incubated with horseradish peroxidase–conjugated secondary antibody for another hour, and developed by enhanced chemiluminescence (Amersham Biosciences).

**Immunassay for CD.** Cells were serum-starved for 4 h prior to treatment with thrombin for 4 h, and conditioned media were collected and tested for CD by an ELISA kit from Calbiochem/EMD Chemicals.

**In vivo studies in mice.** A preliminary experimental pulmonary metastasis approach was performed with B16F10 CD-KD cells (1 × 10⁶) injected into the tail vein of syngeneic C57BL/6 mice to test the feasibility of an in vivo CD-KD approach. Animals were sacrificed on day 21 and their lungs examined for tumor nodules.

4T1 CD-KD cells were then used because they induce a more physiologic spontaneous metastasis in syngeneic mice, permitting measurements of cell seeding and metastasis without the need to sacrifice animals because of enhanced tumor burden. 4T1 green fluorescent protein (GFP)–labeled cells were prepared as described (19). These cells were infected with shRNA CD retrovirus or EV cells. CD-KD GFP cells (1 × 10⁶) or EV GFP cells were injected s.c. into BALB/c mice as described previously (19). Both groups were also treated with and without hirudin, 10 mg/kg ip, every day for 10 days followed by treatment every other day for 7 days. Day 1 injection was given 5 min before and 4 h after tumor inoculation. The dose and frequency was optimally determined in a previous study (19). Animals were sacrificed on day 30 and their spontaneous pulmonary metastasis evaluated as described (19). Tumor detection in the blood was determined by flow cytometry of GFP-labeled 4T1 cells on day 24 as previously described (19).

**Figure 1.** Thrombin up-regulates CD mRNA and protein in tumor cells and HUVEC. **A,** mRNA was measured by real-time quantitative PCR on five tumor cell lines and HUVEC following 24 h of stimulation at 37°C. Ctl, control; bars, SE. **B,** immunoblot of CD protein in six different tumor cell lines and HUVEC. **C,** tubulin was used as an internal “loading” control for each cell. Ctl, control; 0.1T, 0.5T, and 1T, thrombin concentration in units/mL. Numbers over immunoblots, the increase in immunoblot protein density divided by the control protein, representative of two to three experiments. **D,** ELISA assay of concentrated conditioned medium of three different tumor cell lines incubated as in A. White columns, unstimulated (PBS treatment); black columns, treatment with thrombin (0.5 units/mL); bars, SE.
Flow cytometry was gated with standard GFP cells prior to the enumeration of cells in the blood. Ten thousand events were accumulated for each sample. Approximately 80% of infected cells were GFP-positive.

Results

Thrombin up-regulates CD mRNA. To verify the results of our Affymetrix gene chip display as well as the general biological response to thrombin, we examined the effect of thrombin (0.5 units/mL for 24 hours) on CD mRNA up-regulation in six different cell lines (human MCF7 and MD-MB-231 mammary carcinomas, PC3 prostate carcinoma, primary HUVECs as well as murine B16F10 melanoma, and 4T1 mammary cell lines). mRNA increased 1.5- to 2.1-fold in all six cell lines examined as determined by real-time PCR (Fig. 1A).

Thrombin up-regulates CD protein synthesis and secretion. We next examined the effect of thrombin on intracellular protein levels (Fig. 1B) under similar conditions. The same cell lines, as well as two additional cell lines, human THP1 (macrophage) and murine UMCL (undifferentiated murine cell line), were used. Protein increased 1.8- to 4.2-fold.

We also examined the effect of the PAR-1 thrombin receptor activation peptide as well as thrombin in the same cell lines as well as in two additional cell lines, human HeLa cervical carcinoma and BMEC (brain microvascular endothelial cells). Figure 1C reveals that both thrombin and thrombin receptor activation peptides increased the secretion of CD by 3.1- to 8.2-fold compared with control buffer, as measured by immunoblots. Similar increases were noted with MCF7, HeLa, and PC3 cells when measured by ELISA ($P < 0.01$; Fig. 1D). Thus, tumor cells secrete CD.

Requirement of CD for thrombin-induced chemotaxis of 4T1 mammary tumor cells. In order to study the effect of CD on spontaneous tumor metastasis of 4T1 cells, we knocked down CD with CD-shRNA using an shRNA-Retro Q viral vector. Figure 2A shows the successful knockdown clone for 4T1 cells.

We first examined whether thrombin could induce the chemotaxis of 4T1 cells. Figure 2B shows that this was the case, and that this was dependent on the presence of CD in 4T1 cells because CD-KD 4T1 cells were not stimulated to migrate with thrombin stimulation ($P < 0.006$, $n = 3$). Similar results were noted with FBS as a stimulant (data not shown).

CD induces angiogenesis in the CAM assay. We have successfully used the chick CAM system to measure increased angiogenesis induced by thrombin (18). We therefore used the same system to test the effect of CD on this process. Figure 3A depicts the effect of conditioned media from wild-type, EV, scrambled vector (SV), or CD-KD on angiogenesis at 72 hours. Blood vessel density significantly increased with conditioned media (46 ± 4) compared with PBS treatment (22 ± 2, $P = 0.03$; Fig. 3B). However, blood vessel density significantly decreased with CD-KD medium (19 ± 2.8) versus wild-type (46 ± 4.4), EV (49 ± 4.5), and SV (45 ± 2.1; $P = 0.03$, $n = 3$; Fig. 3A). We also tested the effect of anti-CD versus control IgG on conditioned medium. Anti-CD inhibited wild-type conditioned medium by ~2-fold (31.3 ± 1.5 versus 63.7 ± 2.6, respectively; $n = 3$, $P = 0.001$). CD-KD medium was 28.7 ± 3.3 ($P = 0.001$). Thus, tumor-secreted CD contributes to angiogenesis. To confirm this conclusion, we next tested the effect of purified CD applied directly to the CAM. Figure 3B shows enhanced cathepsin-induced angiogenesis compared with buffer (PBS): 47 ± 5 versus 22 ± 2, respectively ($P = 0.005$, $n = 6$). This requires proteolytically active CD because it was blocked by the selective CD inhibitor, Pep A, an inhibitor of acid aspartyl proteases.

Endothelial cell growth. Because endothelial cells are necessary for angiogenesis, and thrombin enhances HUVEC growth by ~2-fold in culture (24), as well as enhancing CD production, we determined whether CD has a direct effect. Such proved to be the case (Fig. 3C). CD-KD decreased HUVEC growth by 1.8-fold (EV versus KD; $P < 0.02$, $n = 3$).
Endothelial cell chemotaxis. An early step in angiogenesis is the migration of endothelial cells within the subendothelial matrix. We therefore examined HUVEC chemotaxis. Figure 3D shows a 2.3-fold increase in HUVEC chemotaxis elicited by proteolytically active pure CD (Pep A inhibition) as well as VEGF (positive control), (CD versus Pep A + CD, \( P = 0.02, n = 4 \)). Figure 3D is a photomicrograph of the migrated HUVEC on the underside of the insert polycarbonate membrane.

Endothelial cell tube formation in Matrigel. We next tested the ability of pure CD to enhance Matrigel tube formation. Figure 3E shows a 1.5-fold increase with CD (\( P = 0.03 \); compared with 1.8-fold VEGF effect) which was inhibited by Pep A (\( P = 0.001, n = 3 \)). A photomicrograph of Matrigel tube formation is shown.

Activation of MMP-9 in HUVEC. Because CD protease activity is required for the positive effect of CD on angiogenesis, we hypothesized that it may be activating MMPs which facilitate endothelial cell migration. We therefore applied pure CD directly to the CAM which enhanced angiogenesis ~2-fold. We then used the generic MPI GM6001 in the CAM assay stimulated by CD. Figure 4A shows inhibition by both Pep A as well as MPI (\( P = 0.03, n = 3 \)).
To confirm that MPi inhibited the enhanced angiogenesis induced by CD, we examined its effect in HUVECs by measuring the proteolytic activity of MMPs with zymography. Figure 4B shows enhanced levels of inactive and active MMP-9, which is inhibited by MPi as well as Pep A. Thus, CD activates MMP-9 in HUVEC.

**Effect of CD-KD on *in vivo* tumor growth and metastasis.** This encouraged us to study the effect of CD-KD as well as the requirement of thrombin for spontaneous 4T1 growth, seeding, and metastasis. A preliminary experimental pulmonary metastasis study was first performed with B16F10 cells to test the feasibility of an *in vivo* effect with CD-KD cells. CD-KD inhibited metastasis (data not shown). We therefore used a more pathophysiologic model in which tumor implantation lead to tumor seeding into the blood and spontaneous metastasis (rather than tail vein injection of a huge tumor burden). This can be performed with 4T1 syngenic mammary carcinoma cells which have these properties (19).

Figure 5 shows the exquisite requirement of CD for tumor growth following injection of 4T1 tumor into the flank of C57BL/6 mice. Tumor growth was inhibited 3- to 4-fold on days 10 and 15, and by 6.1-fold on day 28 compared with EV cells (*P* < 0.01, *n* = 8).

The effect of the simultaneous addition of hirudin, a specific thrombin inhibitor, was examined to test the contribution of thrombin to this process. Note that hirudin also significantly inhibited tumor growth of wild-type EV 4T1 cells (*P* < 0.01, as previously reported; ref. 19), but not to the same extent as CD-KD. The CD-KD cells had ~2-fold less tumor growth than hirudin-treated animals. This would suggest that CD up-regulation by thrombin contributed to tumor growth, but that CD may also have an effect independent of thrombin.

**Effect of CD-KD and hirudin on 4T1 tumor, seeding, and spontaneous metastasis.** GFP-labeled 4T1 tumor cells have the capacity to spontaneously metastasize to the lung. Therefore, seeding into the blood as well as pulmonary metastasis could be quantitated. Tumor seeding into the blood was decreased by 6.2-fold in CD-KD 4T1 cells compared with EV wild-type cells on days 23 to 30, with a mean of 31 cells versus 5 cells (*P* < 0.01; Table 1).

### Discussion

These data clearly show a general up-regulation of CD mRNA, protein, and protein secretion by thrombin in eight different tumor cell lines tested, as well as in HUVEC. This observation, as well as the recognition that CD up-regulation is associated with human tumor malignancy, poor prognosis, and increased risk of relapse and metastasis, strongly supports the evidence that thrombin up-regulation of CD contributes to the malignant tumor phenotype.

The mechanism of how a lysosomal, acidic pH-optimum CD contributes to malignancy is not clearly understood. Conflicting and puzzling studies have been published. It has been reported that CD supports breast tumor metastasis by a mechanism unrelated to its proteolytic activity, and that CD both enhances and inhibits the apoptosis of tumor cells. CD correlates weakly with *in vivo* tumor angiogenesis in association with its up-regulation of angiogenesis inhibitors, angiostatin, and 16K-like prolactin fragments (36, 45). The weak correlation of CD with angiogenesis could not
be ruled out on the basis of increased tumor size (46). It has also been reported that CD releases basic fibroblast growth factor from heparin sulfate sites in the extracellular matrix (47).

Perhaps the best evidence for CD being a stimulator of tumor growth rather than an associative finding comes from studies performed with transgenically elevated CD (both proteolytically active or inactive) in 3Y1-Ad12 rat tumor xenografts injected s.c. into athymic mice (48), as well as from studies with antisense CD in MDA-MB-231 mammary tumor cells similarly injected into nude mice (49). Transfected rat tumors had an enhanced tumor surface area, proliferating cell nuclear antigens, and apparent enhanced angiogenesis with proteolytically inactive as well as active CD. In addition, apoptosis was only noted in proteolytically active transgenics. Antisense CD MDA-MB-231 cells showed decreased experimental pulmonary metastasis.

Our data extend these observations by using a more relevant syngeneic mouse model in which tumor seeding as well as spontaneous tumor metastasis were measured with a 4T1 tumor short hairpin knockdown system. In our system, surface tumor size differences were noted between wild-type and CD-KD tumor models. However, unlike other studies, we also measured tumor seeding and more physiologically relevant spontaneous pulmonary metastasis. Both were decreased in CD-KD mice. Indeed, in support of our hypothesis, we also showed that CD was required for thrombin-induced tumor chemotaxis.

We next examined the possible direct effect of CD on angiogenesis. We found that proteolytically active CD directly stimulated angiogenesis in the CAM model and that angiogenesis could be specifically inhibited with anti-CD antibody as well as with CD-KD conditioned medium. An explanation for the mechanism behind CD-induced angiogenesis revealed increased endothelial cell growth, chemotaxis, Matrigel formation, and activation of MMP-9.

We took our observations one step further by comparing the effect of CD-KD with hirudin-treated mice in which thrombin is inactive. Both the hirudin-treated wild-type mouse model as well as the CD-KD mouse model gave similar reduced surface tumor size, spontaneous blood seeding of tumor, and spontaneous metastasis. These observations support the concept that CD in tumor is regulated by thrombin activity, and that patients with higher CD tumor content and poor prognosis are likely to have higher tumor burden and thrombin activity. This is supported by older observations on tumor burden and hypercoagulativity in which it was shown that low-grade intravascular coagulation with the generation of thrombin had been observed in most patients with solid tumors (3, 50–52), as well as in 60% of patients with cancer at the time of diagnosis. It progresses with greater tumor burden, and is associated with a poor prognosis (52).

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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### Table 1. Effect of CD-KD and hirudin treatment on pulmonary spontaneous metastasis of 4T1 mammary carcinoma cells

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NOTE: 4T1 cells (1 × 10⁵) were injected into the flank of syngeneic BALB/c mice and pulmonary nodules enumerated at day 30 following animal sacrifice. Two-tailed t tests revealed statistically significant P values for EV vs. CD-KD (0.018), EV vs. EV + hirudin (0.001), and EV vs. CD-KD + hirudin (0.001). No significance was noted between EV + hirudin vs. CD-KD + hirudin.

### References

1. Troussseau A. Plegmasie alba dolens. Clinique Medical
Thrombin–Up-regulated Cathepsin D Enhances Angiogenesis

46. Gonzalez-Vela MC, Garjio MF, Fernandez F, Buete L, Val-Bernal JE. Cathepsin D in host stromal cells is associated with more highly vascular and aggressive invasive breast carcinoma. Histopathology 1999;34:35–42.
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