Thrombin Induces Tumor Invasion through the Induction and Association of Matrix Metalloproteinase-9 and β1-Integrin on the Cell Surface*

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The procoagulatory serine protease, thrombin, is known to induce invasion and metastasis in various cancers, but the mechanisms by which it promotes tumorigenesis are poorly understood. Because the 92-kDa gelatinase (MMP-9) is a known mediator of tumor cell invasion, we sought to determine whether and how thrombin regulates MMP-9. The thrombin receptor, PAR-1, and MMP-9 are expressed in osteosarcomas, as determined by immunohistochemistry. Stimulation of U2-OS osteosarcoma cells with thrombin and a thrombin receptor-activating peptide induced pro-MMP-9 secretion as well as cell surface-associated pro-MMP-9 expression and proteolytic activity. This was paralleled by an increase in MMP-9 mRNA and MMP-9 promoter activity. Thrombin-induced invasion of U2-OS cells through Matrigel was mediated by the phosphatidylinositol 3-kinase signaling pathway and could be inhibited with an antibody preventing binding of the MMP-9 antibody. The stimulation of MMP-9 by thrombin was paralleled by an increase in β1-integrin mRNA and β1-integrin expression on the cell surface, which was also mediated by phosphatidylinositol 3-kinase and was required for invasion. Thrombin activation induced and co-localized both β1-integrin and pro-MMP-9 on the cell membrane, as evidenced by co-immunoprecipitation, confocal microscopy, and a protein binding assay. The thrombin-mediated association of these two proteins, as well as thrombin-mediated invasion of U2-OS cells, could be blocked with a cyclic peptide and with an antibody preventing binding of the MMP-9 hemopexin domain to β1-integrin. These results suggest that thrombin induces expression and association of β1-integrin with MMP-9 and that the cell surface localization of the protease by the integrin promotes tumor cell invasion.

An increased activation of blood coagulation in cancer patients has been known since 1865, when the French physician Armand Trousseau first reported a higher incidence of clot formation in patients with cancer (1). Thrombin, a trypsin-like serine protease, is the most abundant enzyme associated with blood coagulation. It is activated from its precursor molecule, prothrombin, by the coagulation factor Xa where the extrinsic and intrinsic coagulation pathways meet. When activated during vascular injury, thrombin converts the soluble serum factor, fibrinogen, into insoluble fibrin split products, which participate in hemostasis. In addition to its role in homeostasis, thrombin also activates protease-activated receptors (PAR)3, -4, -3, -4, which belong to a group of seven transmembrane receptors on the cell surface. Cleavage of the amino-terminal exodomain of the PARs exposes a new NH2-end of the protein that serves as the tethered ligand for the receptor and leads to activation of the internal G-proteins Gi, Gq, and Gt. Upon activation, the G-proteins in turn activate cellular signaling pathways, including protein kinase C, MAPK, PI 3-kinase, and calcium signaling, and therefore, ultimately regulate gene transcription (2).

In the tumor microenvironment, thrombin is either produced by tumor cells or by tumor-associated platelets, which are avid producers of thrombin. PAR-1 is highly expressed in cultured cancer cell lines, in highly metastatic or de-differentiated human tumors, and in tumor metastases (3–5). Thrombin induced metastasis through PAR-1 has been shown in several experimental systems. Pretreatment of melanoma cells with low metastatic potential with thrombin increases the number of pulmonary metastasis in mice (6). Treatment of human and murine cancer cell lines with hirudin, a specific inhibitor of thrombin, inhibits in vivo tumor implantation, spontaneous tumor metastasis, and increases survival in mice (7). Moreover, blocking thrombin binding using PAR-1 antibodies reduces metastasis of melanoma cells to the lung (8). A clinical study prospectively examining patients with distal extremity osteosarcoma shows a high correlation between thrombin levels and the occurrence of metastasis. The authors reported that the

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The abbreviations used are: PAR, protease-activated receptors; MMP, matrix metalloproteinase; PEX, hemopexin domain; aa, amino acid(s); uPA, urokinase; Pl-5-kinase, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; TRAP, thrombin receptor-activating peptide; RT, reverse transcriptase; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PEX domain, hemopexin domain; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TIMP, tissue inhibitor of metalloproteinase; FACS, fluorescence-activated cell sorter.
Thrombin concentration in bronchoalveolar fluid at the time of initial diagnosis was 100 times higher in patients who later developed lung metastasis, when compared with patients who evidenced no manifestation of metastatic disease (9). It has also been shown that thrombin can induce the invasion of cancer cell lines through Matrigel, although the downstream mechanism(s) involved are not clearly understood (4, 10). The invasion of tumor cells after stimulation with thrombin requires PAR-1, and can be inhibited with transfection of an antisense thrombin receptor construct. This suggests that the specific binding of thrombin to its receptor is necessary for thrombin-induced invasion (3).

Invasion is a tightly regulated process. The early steps are characterized by the attachment of tumor cells to the extracellular matrix, followed by proteolysis. Subsequently, tumor cells coordinate the expression of proteases and adhesion receptors of the integrin family to cross tissue boundaries (11, 12). Among other matrix metalloproteinases (MMPs), MMP-2 and MMP-9 (72- and 92-kDa type IV collagenases) are associated with the malignant phenotype of tumor cells. The most thoroughly understood function of these MMPs is their unique ability to degrade type IV collagen, a major component of the extracellular matrix and the basement membrane (13, 14). In addition to their role in proteolysis, recent studies show that MMPs cooperate with integrins to regulate the delicate balance between adhesion and proteolysis (12, 15). Morini and colleagues (16) report that the aggressive MDA-MB-231 breast cancer cell line overexpresses αβ3-integrin on the cell surface. Inhibition of MDA-MB-231 cells with an β3-integrin antibody reduced invasion as well as MMP-9 gelatinolytic activity (16). Furthermore, activation of αβ3-integrin in MDA-MB-435 cells, or expression of constitutive active αβ3-integrin in primary human breast cancer cell lines, induces the secretion of active MMP-9, which is required for cellular migration (17). In human endothelial cells, fibronectin and collagen I induce cooperation between MT-MMP and αβ3-integrins to facilitate cell migration (18).

Because local and systemic thrombin production is increased in cancer patients, we were motivated to investigate the mechanism by which thrombin induces invasion. We chose osteosarcomas as our model, because human osteosarcomas are locally highly aggressive tumors, which frequently metastasize and are often associated with thromboembolic events. We show here that human osteosarcomas overexpress the PAR-1 receptor and that in a human osteosarcoma cell line thrombin induces both MMP-9 and β1-integrin expression through a PI 3-kinase-dependent pathway. Thrombin stimulation leads to the association of β1-integrin and pro-MMP-9, which focuses proteolytic activity on the cell surface to mediate invasion.

EXPERIMENTAL PROCEDURES

Reagents and Cell Lines—Antibodies were obtained from the following sources: β1-integrin monoclonal antibody (P5D2), α2-integrin antibody (P1H5), PAR-1 (C18), and mouse IgG1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Akt and phospho-Akt (Ser-473) rabbit antibodies and horseradish peroxidase-conjugated secondary antibodies were obtained from Cell Signaling (Beverly, MA). MMP-9 (6-6B) antibody, which recognizes the pro- and active form of MMP-9, was from Calbiochem (Cambridge, MA) and MMP-9 antibody (2C3) against the hemopexin domain was from Abcam (Cambridge, MA). β3-(B3A), α1-(FB12), and α3-(P1B5) integrin antibodies were from Chemicon (Temecula, CA). The α5-integrin antibody (IIA1) and the CD44 antibody (515) were from BD Biosciences. The αβ5-integrin antibody (P1F6) and the MT-MMP1 antibody (AB8102) were purchased from Chemicon and the urokinase (uPA) antibody (394) from American Diagnostica (Stamford, CT). The human osteosarcoma cancer cell line U2-OS was a kind gift from Dr. M. Atkinson (Technische Universität München) and was also purchased from ATCC. The TE-85 human osteosarcoma cancer cell line was a kind gift of Dr. T. C. He (University of Chicago). Thrombin was from Calbiochem. Peptides were synthesized by Anaspec (San Jose, CA), and purified by high pressure liquid chromatography to >85% purity. The cyclic MMP-9 “LRSG”-peptide had the following sequence: H-Cys-Gln-Val-Thr-Gly-Ala-Leu-Arg-Ser-Gly-Arg-Gly-Lys-Met-Leu-Leu-Cys-NH2. The control peptide had the sequence: H-Cys-Arg-Ala-Val-Arg-Ala-Leu-Arg-Cys-OH as suggested (19).

Patients and Immunohistochemistry—Tissue blocks from 25 patients with distal extremity osteosarcoma who had undergone biopsy at the University of Chicago were selected for the study that was approved by the IRB. The slides were deparaffinized in xylene and incubated with alcohol before being placed in 3% H2O2/methanol blocking solution, followed by antigen unmasking and incubation with a 1:100 dilution of the antibody against PAR-1 and MMP-9. The slides were stained using the Envision avidin-biotin-free detection system and counterstained with hematoxylin. Corresponding negative controls were prepared using non-immune rabbit serum. The staining intensity was evaluated employing the same scoring system (20) that is currently in clinical use to grade Her2/neu staining: negative (−), weak (+), intermediate (++), and strongly positive (+++). Trichrome staining to differentiate connective tissue elements was conducted as described previously (21). The staining was evaluated by a pathologist with special expertise in orthopedic pathology (A. M.).

MMP Activity Measurement—Gelatinolytic zymography was performed as described (22). Serum-starved cultured media was concentrated (Microcon YM-10, Millipore, Bedford, MA), denatured in the absence of reducing agent, and electrophoresed in 10% SDS-PAGE containing 0.1% (w/v) gelatin. Gels were incubated in the presence of 2.5% Triton X-100 at room temperature for 2 h and subsequently at 37 °C overnight in a buffer containing 10 mM CaCl2, 0.15 M NaCl, and 50 mM Tris (pH 7.5). Gels were stained with 0.25% Coomassie Blue, and proteolysis was detected as a white zone against a dark field.

Cell surface-associated MMP activity was measured using quenched fluorescent gelatin (20 µg/96-well DQ gelatin fluorescent conjugate, Invitrogen, Carlsbad, CA) in reaction buffer (5 mM CaCl2, 0.05 M NaCl, and 50 mM Tris, pH 7.5) (23). To test the inhibitory effect of anti-MMP-9 antibody (Calbiochem 6-6B, 4 µg/ml), recombinant human 4-aminophenylmercuric acetate-activated MMP-2 (60 ng) and/or aminophenylmercuric acetate-activated MMP-9 (80 ng) were preincubated with the MMP-9 antibody or a mouse IgG (4 µg/ml) for 1 h before measuring cleaved fluorescent gelatin over 3 h. For the cell
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Based studies, 25,000 cells were plated in a 96-well plate in 10% fetal calf serum. 12 h before the experiment the cells were switched to serum-free medium. The plates were incubated at 37 °C, 5% CO₂. Substrate was added and fluorescence measured at the indicated time points using a fluorescence spectrophotometer (Synergy HT, BioTek Instruments, Winooski, VT) (excitation = 480 nm, emission = 530 nm). When thrombin, antibodies, inhibitors, or the peptide were tested, they were preincubated for 1 h before addition of the fluorogenic substrate. Matrix metalloproteinase inhibitor GM 6001 (50 μM) was preincubated for 1 h before addition of the fluorogenic substrate. Matrix metalloproteinase inhibitor GM 6001 (50 μM) and human recombinant tissue inhibitor of metalloproteinase (TIMP)-1 protein (25 μM, Chemicon) were used as inhibitors.

RNA Extraction and Quantitative Real-time Reverse Transcription PCR—TRIzol (Invitrogen) was used to extract total RNA from both cell lines and tissues. RNA content was measured and the absence of DNA contamination confirmed by a reaction that was run without reverse transcriptase. RNA was transcribed into cDNA by using the 2-Step PCR Kit from Promega (Madison WI), the RT mixture was 4 μM of random hexamers, 4 μl of 10X Taq polymerase buffer, 50 nM MgCl₂, 12 units of avian myeloblastosis virus reverse transcriptase, 1 unit of RNasin and RNA to a total volume of 40 μl. 8 μl of sample cDNA and 10 pmol of primer were added to the 2X Master Mix from the Quantitect SYBR Green PCR kit (Qiagen, Valencia, CA) and nuclease-free H₂O to a total volume of 25 μl. The MMP-9 and GAPDH mRNA was extracted from U2-OS cells and stimulated with the indicated doses of thrombin for 24 h followed by the RT step. MMP-9 and GAPDH TagMan probes (Applied Biosystems, Foster City, CA) were used to detect cycle numbers in cDNA samples. All samples were normalized to GAPDH TagMan probes. The β1-integrin and β-actin mRNA was amplified using the SYBR Green fluorescence-based detection method with the sequence-specific primer: β1-integrin forward primer, AGGAATGGTACACGGGTGCT; reverse primer, ACCAAGTTTCCCATCTCCAG. Reactions were run in duplicates on a Stratagene Sequence Detection System (Stratagene MX-3005) using the following PCR conditions: one cycle at 50 °C for 2 min followed by a 10-min activation of the polymerase at 95 °C. Subsequently 40 cycles were performed at 95 °C for 15 s and 54 °C for 60 s. Cycle threshold (Ct) values were calculated with the Stratagene MX-3005 software. Data were analyzed according to the comparative Ct method (24). The median Ct value was determined and data expressed as -fold change of relative mRNA expression. To detect the human thrombin receptor, PAR-1, in human osteosarcoma tumor tissue the following primers were used: forward primer, CCCTGCTCGAAGGCTACTAT and reverse primer, CTACAAGTCGACCTGACG. After amplification the PCR product was resolved on a 1.5% agarose gel.

Immunoprecipitation and Western Blot Analysis—Cells were starved in serum-free medium before stimulation with thrombin (4 units/ml) for 45 min. After lysis using ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenethylmethylsulfonyl fluoride, 1 mM Na₂VO₄, protease inhibitor mixture from Sigma at a 1:100 dilution), 250 μg of protein was added to 50 μl of anti-mouse IgG beads (eBioscence, San Diego, CA). After centrifugation the supernatant was immunoprecipitated with 5 μg/100 μl of primary antibody against β1-integrin (P5D2), MMP-9 (6-6B), or as control normal mouse IgG. The immunoprecipitation was performed at 4 °C for 1 h, and then rotated with 50 μl of anti-mouse IgG beads at 4 °C for 1 h. The beads were washed 3 times with RIPA lysis buffer and protein was eluted with 50 μl of 2X sample buffer (0.5 M Tris HCl, pH 6.8, 4% SDS, 12% β-mercaptoethanol, 20% glycerol, 0.01% bromphenol blue). Samples were boiled for 5 min and separated on a 10% SDS-PAGE. The protein was then transferred to nitrocellulose membranes and treated with Enhancer solution (eBioscence, San Diego, CA). Blocking was done for 1 h with Tris-buffered saline (10 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) containing 5% milk and 1% BSA, followed by incubation with the primary antibody overnight and the appropriate horseradish peroxidase-conjugated secondary antibody. The antigen was visualized with enhanced chemiluminescence reagents (Pierce).

Flow Cytometry—Cells suspended in Dulbecco’s modified Eagle’s medium, 10% FBS were incubated with the indicated primary monoclonal antibodies (1:250) for 30 min at 4 °C followed by incubation with phosphatidylethanolamine-labeled secondary antibody. After washing, cells were resuspended in 500 μl of 1% BSA/PBS. Isotopic mouse IgG was used as a negative control. Surface expression was measured with FACS Calibur (Becton Dickinson, San Jose, CA) and CellQuest software.

Transfections—U2-OS cells were transfected by the calcium phosphate method (25) with chloramphenicol acetyltransferase reporter constructs fused to the wild type or deleted fragments of the human MMP-9 promoter (26). To correct for transfection efficiencies all transient transfections were performed in the presence of a β-galactosidase expression vector. After normalization, chloramphenicol acetyltransferase activity was measured by incubating cell lysates at 37 °C with 4 μM [14C]chloramphenicol and 1 mg/ml acetyl coenzyme A. The mixture was separated by extraction with ethyl acetate and acetylated products separated on thin layer chromatography plates using chloroform/methanol as the mobile phase. Reactions were visualized by autoradiography and radioactivity quantified using a PhosphorImager.

Confocal Microscopy—Cells were plated on 8-well chamber slides coated with type I collagen (BD Biosciences), allowed to attach for 6 h, and then cultured under serum-free conditions for 24 h. After incubation, cells were stimulated with 2 units/ml thrombin for 30 min, fixed with 4% paraformaldehyde in PBS for 30 min, and stained with rabbit anti-human MMP-9 (Calbiochem, polyclonal, 1:50) and mouse anti-human β1-integrin (P5D2, 1:50) at 4 °C overnight. After washing, samples were incubated with Alexa Fluor 488-labeled goat anti-rabbit IgG and Alexa Fluor 555-labeled goat anti-mouse IgG (Molecular Probes, Carlsbad, CA, 1:1000) and stained with 4’,6-diamidino-2-phenylindole. The samples were observed using a Leica SP2 A OLS Laser Scanning Confocal microscope (Leica, Exton, PA).

Expression and Purification of the MMP-9 Hemopexin (PEX) Domain—The coding sequence for the hemopexin domain (amino acids 514–707 of human full-length MMP-9) was amplified using PfuTurbo polymerase from Stratagene (La Jolla, CA) and cloned into the pET28a(+) vector (Novagen, San Diego, CA) via Ncol and Xhol restriction sites. Origami (DE3)
cells from Novagen were transformed with the PEX construct, grown to an A600 of 0.6–0.8 and induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside. The His-tagged protein was expressed overnight at 22 °C. Cells were lysed using an Emulsi-flex-C5 from Avestin (Ontario, Canada) and the soluble protein was purified from the crude cell extract using Talon resin (Clontech, Mountain View, CA) and subsequent size exclusion chromatography (Superdex-75 from GE Healthcare). Protein purity was confirmed using SDS-PAGE, Western blot with Penta-His antibody from Qiagen (Valencia, CA), and MALDI-TOF.

**Purified Protein Binding Assay**—Nunc Maxisorp Immunoplates (Nalge Nunc, Rochester, NY) were coated with 10 μM MMP-9 PEX protein or BSA as reference in PBS at pH 7 at 4 °C overnight (27). After blocking for 1 h with 1% BSA at room temperature the plate was incubated with serial dilutions of α5β1-integrin-Fc fusion protein in PBS. After washing 3 times with 300 μl of PBS containing 0.05% Tween 20 bound fusion protein was detected with horseradish peroxidase-conjugated protein-A (Amersham Biosciences) and 1-Step Turbo TMB-ELISA from Pierce. Absorption from wells coated with BSA was subtracted and the average of the measurements was plotted against the subjected protein concentration. Each measurement was performed in triplicates.

**In Vitro Invasion Assay**—Cell invasion was assayed by determining the ability of cells to invade through a synthetic base-membrane (Matrigel, BD Biosciences). Briefly, polycarbonate filters (8 μm pore size, 24-well insert) were coated with 100 μl of Matrigel (0.25 μg/μl) diluted with cold serum-free Dulbecco’s modified Eagle’s medium and placed in a modified Boyden chamber (28). U2-OS cells (5 × 10⁴/well) were plated in the top chamber with serum-free media and incubated with serum containing media in the bottom chamber. For the functional inhibition, anti-MMP-9 (6-6B, 2 μg/ml) and anti-β1 (PSD2, 1 and 10 μg/ml) antibodies were used. To inhibit in vitro invasion, antibodies against uPA (394, American Diagnostica, 10 μg/ml), against the hemopexin domain of MMP-9 (2C3, Abcam, 2 μg/ml), and against integrin-αvβ3 (PF6, Chemicon, 1 and 10 μg/ml) were used. All antibodies were added in the top chamber. Cells were incubated at 37 °C and allowed to invade through the Matrigel barrier for 24 h. After incubation, filters were fixed and stained with Giemsa solution. Non-invading cells were removed using a cotton swab and invading cells on the underside of the filter were enumerated using an inverted microscope.

**RESULTS**

**Human Osteosarcomas Express the PAR-1 Receptor**—As a first step to understanding the role PAR-1 plays in the invasion of human osteosarcomas, thrombin receptor expression was characterized immunohistochemically in biopsies from 25 different patients (median age 30) with distal extremity osteosarcomas. In general, the osteosarcoma cells grew in two different patterns (Fig. 1A), either as solid nests (open arrow) or as single malignant tumor cells within a cartilaginous matrix (black closed arrow). As seen in Fig. 1A, all the tumor cells show uniform reactivity with the PAR-1 antibody, whereas incubation of a serial section of the same tumor with an isotypic control IgG shows no reaction (Fig. 1B). Trichrome staining to differentiate connective tissue elements results in blue staining of the chondroid matrix (Fig. 1C, red arrow) and red staining of the collagenous matrix in which tumor cells are embedded (Fig. 1C, yellow arrow). Twenty-four of the 25 osteosarcomas expressed PAR-1, with 14 tumors showing high expression of the receptor (++) or (+++) staining (Fig. 1D). Total RNA was extracted from fresh tissue snap frozen at biopsy from two patients. Both tumors expressed PAR-1 mRNA, as did the human osteosarcoma cell line, U2-OS (Fig. 1E).
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Thrombin Stimulation of MMP-9 Expression and Invasion—To determine whether thrombin induces type IV collagenases, which were previously shown to be expressed in osteosarcomas (29), U2-OS cells, which secrete only a small amount of MMP-9 and MMP-2, were stimulated with increasing amounts of thrombin and zymography was performed. A dose-dependent increase in a gelatinolytic band identical in size to pro-MMP-9 (92 kDa) was evident in conditioned media from U2-OS cells treated with thrombin. No active MMP-9 was detected (88 kDa). In contrast, the activity of a metalloproteinase, whose size is identical to the 72-kDa MMP-2 (30), was only detected as a faint band and was therefore considered to be unaffected by thrombin stimulation (Fig. 2A, left panel). This increase in MMP-9 was not due to an increased growth rate, because we could find no evidence of augmented proliferation of U2-OS cells in the presence of thrombin (data not shown). Thrombin activates PAR-1 by cleaving its amino-terminal domain, thereby exposing a peptide sequence that folds and binds intramolecularly to the receptor and induces transmembrane signaling. A synthetic peptide (thrombin receptor-activating peptide, TRAP) consisting of the 6 amino-terminal amino acids (aa), mimics the effect of thrombin by acting as an agonist for PAR-1, activating the receptor independently of thrombin and proteolysis (2, 31, 32). U2-OS cells were stimulated with increasing concentrations of TRAP and MMP-9 secretion was measured by zymogram. TRAP mimicked thrombin activation of MMP-9 in a dose-dependent manner, whereas having only a minimal effect on MMP-2, indicating that the activation of U2-OS cells through thrombin is mediated by the thrombin receptor (Fig. 2A, right panel). The ability of thrombin to induce pro-MMP-9 secretion was confirmed in a second osteosarcoma cell line, TE85 (Fig. 2B). The dose-dependent increase in secreted 92-kDa gelatinase activity was paralleled by an increase in pro-MMP-9 expression in total cell lysates (Fig. 2C), whereas MT1-MMP, which participates in the activation of MMP-9 (33) and is correlated with MMP-9 expression in breast cancer (34), was not stimulated by thrombin (Fig. 2D). To determine whether the increase in MMP-9 protein in U2-OS cell extracts is accompanied by an increase in MMP-9 activity on the cell surface, we used fluorescent gelatin as a substrate for MMP-2/9. Given that U2-OS cell extracts do not express MMP-2 and MMP-1, gelatinolytic activity detected is predominantly from MMP-9. Whereas unstimulated U2-OS cells had no cell surface-associated MMP-9 activity, stimulation with thrombin led to a significant and persistent induction of MMP-9 cell surface activity (Fig. 2E). This induction could at least be partially inhibited with the broad spectrum MMP inhibitor GM 6001 (Ilostat) and the natural MMP-9 inhibitor, TIMP-1 suggesting that thrombin induced gelatinolytic activity is predominantly from MMP-9. Preincubation of U2-OS cells with a MT1-MMP blocking antibody resulted in no significant change in gelatinolytic activity (data not shown).

Quantitative real-time PCR indicated that the increased expression and secretion of MMP-9 by thrombin-stimulated U2-OS cells was a consequence of a higher concentration of steady-state mRNA encoding the type IV collagenase. A dose-dependent increase in MMP-9 mRNA was evident 4 h after stimulation with thrombin (Fig. 2F) and reached a plateau at 12 h, briefly preceding the increase in MMP-9 secretion (data not shown). Increased levels of MMP-9 in response to several stimuli have been ascribed to increased transcriptional activation of the gene (26, 35). To determine the role of increased MMP-9 promoter activity in the induction of metalloproteinase synthesis by thrombin, U2-OS cells were transiently transfected with a reporter gene driven by the full-length 670-bp long MMP-9 promoter. Thrombin induced a strong stimulation of the MMP-9 promoter, comparable with co-transfection of the oncogene H-ras, a known inducer of MMP-9 (Fig. 2G).

Given that thrombin induces MMP-9 (Fig. 2A–G) and that the thrombin receptor PAR-1 is expressed in human osteosarcoma tumors (Fig. 1, A, B, and D), we stained the human osteosarcomas with an MMP-9 antibody (Fig. 2H). Tumor tissues from 22 of 25 patients stained for PAR-1 were available for MMP-9 staining. The tissues of 4 patients showed weak staining for MMP-9; 8 showed intermediate staining, and 2 exhibited strong staining. Eight of the 22 patients showed no staining. It is evident that MMP-9 and PAR-1 were not correlated, because all but one patient expressed PAR-1 (97.5%), whereas only 64% of the patients expressed MMP-9.

To understand the thrombin-mediated signaling events involved in the activation of MMP-9, we first turned our attention to the MAPK signal transduction pathway, which is activated by thrombin and can regulate MMP-9 (26, 36). Unexpectedly, neither the MEK inhibitor PD98059, nor the p38 MAPK inhibitor SB203580, abrogated the thrombin-induced activation of MMP-9 (data not shown). However, preincubation of U2-OS cells with the selective PI 3-kinase inhibitor LY294002 reduced the inductive effect of thrombin on both MMP-9 expression (Fig. 2C) and secretion (Fig. 2A). Induction of MMP-9 by thrombin was paralleled by an increase in the phosphorylated form of Akt, the downstream effector of PI 3-kinase, which was abolished by pretreatment with LY294002 (Fig. 3B). Total Akt expression was not affected by thrombin stimulation or treatment with the inhibitor.

To assess the role of thrombin on invasion we used a Boyden chamber invasion assay. Matrigel was coated onto filters, and the cells invading through the membrane toward serum containing media were stained with Giemsa and counted. Whereas unstimulated U2-OS cells were poorly invasive, confirming a previous report (37), the U2-OS cells stimulated with thrombin invaded the extracellular matrix efficiently. The thrombin-stimulated invasion through Matrigel was inhibited with the PI 3-kinase inhibitor LY294002, the MMP inhibitor GM 6001 (Fig. 4A), and a MMP-9 specific antibody (23) (Fig. 4B). Treatment with the MMP-9 antibody completely abolished the induction of invasion by thrombin, and even affected the baseline invasion of U2-OS cells. To confirm the specificity of the antibody for MMP-9 (6–6B) we performed an activity assay with recombinant MMP-9 and MMP-2 protein. The MMP-9 antibody inhibited the gelatinolytic activity of aminophenylmercuric acetate-activated MMP-9 but not of aminophenylmercuric acetate-activated MMP-2 (Fig. 4C) suggesting specific inhibition of MMP-9 gelatinolytic activity supporting the notion that thrombin-induced invasion is mediated by MMP-9. Another protease known to be activated by thrombin (38), regulated by PI 3-kinase (39), and associated with tumor cell invasion, is the...
FIGURE 2. Thrombin induces MMP-9 expression. Panel A, zymography: U2-OS osteosarcoma cells were stimulated with thrombin or TRAP peptide at the indicated concentrations. HT-1080 cells were used as a positive control. For A and B conditioned media normalized for differences in cell number was subjected to gelatin zymography using a 10% SDS-PAGE containing 0.1% gelatin. Panel C, U2-OS cells were stimulated with thrombin or thrombin and the PI 3-kinase inhibitor LY294002, cells were extracted using RIPA buffer and subjected to Western blotting using a MMP-9 antibody. Panel D, the membrane was reprobed with an antibody against MT1-MMP and actin. Panel E, gelatinase assay: cell-associated gelatinolytic activity was measured in U2-OS cells using quenched fluorescent-labeled gelatin. Cells were stimulated with thrombin and where indicated human recombinant TIMP-1 protein (25 μM) or the MMP inhibitor GM 6001 (50 μM) added. Fluorescence was measured (excitation = 488 nm, emission = 528 nm) with a fluorescence spectrophotometer. Panel F, quantitative real time RT-PCR: total RNA was extracted from U2-OS cells and the relative expression of MMP-9 normalized to GAPDH was measured using TaqMan quantitative real time RT-PCR. Panel G, transfection. U2-OS were transiently transfected with the MMP-9 – 670 promoter chloramphenicol acetyltransferase construct (MMP-9) followed by stimulation with thrombin. Co-transfection with an expression plasmid for H-ras was included as a positive control. Cells were lysed 24 h after stimulation. Chloramphenicol acetyltransferase activity was measured by incubating cell lysates with 4 mM [14C]chloramphenicol and 1 mg/ml acetyl coenzyme A. The mixture was separated by extraction with ethyl acetate and acetylated products separated on thin layer chromatography plates using chloroform/methanol as the mobile phase. Reactions were visualized by autoradiography. Panel H, staining of a human osteosarcoma, fibroblastic type with a MMP-9 antibody. Pleomorphic spindle cells, organized in sheets reveal strong staining (×200 and 400).
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Thrombin induces pro-MMP-9 secretion through PI 3-kinase. U2-OS cells were stimulated with the indicated amounts of thrombin with or without the PI 3-kinase inhibitor Ly294002 and then subjected to zymography (A) or Western blotting (B) with a phosphospecific AKT (Ser-473) antibody. The membrane was reprobed with an antibody recognizing total AKT.

![Image](https://example.com/image.png)

**FIGURE 3.** Thrombin induces pro-MMP-9 secretion through PI 3-kinase. U2-OS cells were stimulated with the indicated amounts of thrombin with or without the PI 3-kinase inhibitor Ly294002 and then subjected to zymography (A) or Western blotting (B) with a phosphospecific AKT (Ser-473) antibody. The membrane was reprobed with an antibody recognizing total AKT.

We therefore sought to determine whether a uPA antibody known to block the enzymatic activity of uPA and migration (40) affects the thrombin-mediated invasion of U2-OS cells. A direct comparison with the MMP antibody shows that, whereas the uPA antibody reduces the effect of thrombin on invasion by 28% it was much less effective than treatment with the MMP-9 antibody, and the reduction did not present a significant change (Fig. 4B).

**Thrombin Stimulation of β1-Integrin Expression and Invasion**—Cellular invasion across physiologic tissue boundaries not only depends on the coordinated regulation of proteolytic mechanisms but also on cell adhesion. Recent evidence suggests that proteases are often functionally associated with receptors of the integrin family and are involved in invasion (16, 41). Indeed, it has been shown that pro-MMP-9 can bind to the cell surface and is proteolytically active, although the binding receptor and activation mechanisms involved are not clearly understood (39, 42). Because thrombin induces tumor cell invasion and metastasis and also membrane-bound MMP-9, we investigated the possibility that thrombin induces the expression of adhesion receptors that then interact with MMP-9.

The surface expression of several integrins, previously found to be involved in osteosarcoma invasion, was determined by flow cytometry analysis. Although CD44 and several α- and β-integrins are expressed on U2-OS cells, only β1-integrin expression was significantly induced by thrombin. Upon stimulation of the cells with thrombin, expression of β1-integrin increased 10-fold (Fig. 5A). The activation of β1-integrin expression in U2-OS cells was confirmed by Western blotting and was inhibited with the PI 3-kinase inhibitor (Fig. 5B). Moreover, stimulation of U2-OS cells with the TRAP peptide also induced β1-integrin expression (not shown). To determine whether β1-integrin expression is transcriptionally controlled, mRNA expression levels of β1-integrin were examined by quantitative real-time PCR following treatment of U2-OS cells with thrombin (Fig. 5C). The addition of thrombin resulted in a strong up-regulation of β1-integrin mRNA. Treatment with the PI 3-kinase inhibitor likewise abolished the effect of thrombin on β1-integrin mRNA, suggesting that thrombin regulates β1-integrin expression through a PI 3-kinase dependent signaling pathway. The inhibition of β1-integrin mRNA was more substantial than the inhibition of β1-integrin protein, possibly reflecting low protein turnover. The thrombin-mediated induction of β1-integrin is clearly of functional importance for U2-OS invasion, because the pro-invasive effect of thrombin can be dose dependently inhibited with a β1-integrin antibody (76% with 10 μg/ml), whereas the isotype IgG control did not influence the effect of thrombin on invasion (Fig. 5D). Because of the partial inhibition of invasion by the β1-integrin antibody we asked if other integrins contribute to the effect of thrombin on invasion. One thrombin-regulated integrin is αvβ5-integrin, which regulates vascular permeability and the cytoskeleton (43). A low concentration of αvβ5-integrin antibody (1 μg) did not significantly inhibit invasion but 10 μg blocked thrombin-mediated invasion by 68%. The inhibition by the αvβ5-integrin antibody is not a result of increased cell surface expression of αvβ5-integrin in response to thrombin stimulation, because thrombin did not affect expression of this integrin in U2-OS cells (Fig. 5A) and could be caused by clustering or a conformational change (43).

Thrombin Stimulates the Association of MMP-9 with β1-Integrin—Our results show that both MMP-9 and β1-integrin are co-regulated by thrombin through the PI 3-kinase pathway. We therefore considered the possibility that the induction of pro-MMP-9 by thrombin leads to the association of β1-integrin with MMP-9. To address this we first determined whether MMP-9 is expressed on the cell surface and found, using FACS analysis, that thrombin induces MMP-9 cell surface expression (Fig. 6A). This was mediated by the thrombin receptor as the TRAP peptide also induced MMP-9 and was comparable with the induction seen with 12-O-tetradecanoylphorbol 13-acetate (not shown). Then we determined whether the two proteins co-immunoprecipitate by comparing unstimulated and thrombin-stimulated U2-OS cell lysates. In the unstimulated cells, MMP-9 co-immunoprecipitated with β1-integrin at a low level, but upon activation of the cells with thrombin, there was a significant induction in the association of MMP-9 with β1-integrin. When the cells were preincubated with the PI 3-kinase inhibitor, however, the association was significantly lower (Fig. 6B). The results were analogous when immunoprecipitation was first performed with a β1-integrin antibody followed by blotting and detection with MMP-9 antibody. Immunoprecipitating and blotting with the MMP-9 or β1-integrin antibody showed a specific band, whereas immunoprecipitation with the isotype-specific IgG did not precipitate any proteins that could be detected with the MMP-9 or β1-integrin antibody (Fig. 6B, lower panel). To substantiate the finding that MMP-9 and β1-integrin bind to each other, we visualized the localization of MMP-9 and β1-integrin by confocal microscopy. As shown in Fig. 6C the expression of both MMP-9 and β1-integrin in U2-OS cells is enhanced in the presence of thrombin. The merged images show that the β1-inte-
Thrombin Regulates MMP-9 and β1-Integrin

FIGURE 4. Thrombin induces cell invasion via MMP-9 and a PI 3-kinase dependent pathway. Panel A, invasion assay: U2-OS cells were plated in a modified Boyden chamber coated with Matrigel and incubated for 24 h. Cells were stimulated with thrombin followed by treatment with the PI 3-kinase inhibitor LY294002 or the MMP inhibitor GM 6001 or in panel B with either a blocking antibody against MMP-9 (#6-6B, 2 μg/ml) or with a blocking antibody against urokinase (uPA) (394, 10 μg/ml). Experiments were performed in triplicate with a minimum of 10 grids (×40 magnification) per filter counted (*** indicates p < 0.001). Panel C, the inhibitory effect of the anti-MMP-9 antibody on recombinant human MMP-9 (squares) and MMP-2 (triangles) was tested using quenched gelatin as described in the legend to Fig. 2E.

MMP-9 PEX domain. To determine whether thrombin-induced association of MMP-9 with β1-integrin is, at least in part, mediated through the LRSG region in the MMP-9 PEX domain, we performed a data base search, using the computer program MOLMOL, which compared the structure of MMP-9 and β1-integrin, and evaluated the three-dimensional structures for potential binding sites between the two proteins. Because currently no high-resolution data is available for β1 integrin, the coordinates of the highly homologous αVβ3-integrin were used instead (1JV2). Inspection of the structures revealed that the hemopexin domain of MMP-9 could potentially bind the I-like domain of β1-integrin, and that this activity might be mediated through a LSRG sequence in the PEX domain of MMP-9 (aa 617–621).

We therefore examined if the PEX domain of MMP-9 can bind β1-integrin using purified proteins.

The PEX domain of MMP-9 was expressed in Escherichia coli origami cells. Purity and size (23.5 kDa) was confirmed using SDS-PAGE, Western blot, and MALDI-TOF. The secondary structure of the protein containing predominantly β-sheets was determined using circular dichroism (data not shown). An α5β1-integrin heterodimer (27, 46) was used for a protein binding assay to evaluate binding of the MMP-9 hemopexin domain to α5β1-integrin. The MMP-9 PEX protein was immobilized, and binding to the Fc fusion protein of α5β1-integrin was measured using protein A-horseradish peroxidase. Fig. 7A shows that α5β1-integrin binds the MMP-9 PEX in a dose-dependent manner. Using data analysis software a binding constant of ~8 nm was calculated (correlation coefficient, r = 0.998) suggesting high affinity of the

MMP-9 PEX domain for the integrin. In view of these results, as well as the bioinformatic analysis that suggested that the LSRG region may be the interaction site between MMP-9 and β1-integrin, a cyclic peptide was designed (aa 615–622) spanning the LRSG region in the PEX domain of MMP-9. The LRSG region of the MMP-9 PEX protein was immobilized, and binding to the Fc fusion protein of α5β1-integrin was measured using protein A-horseradish peroxidase. Fig. 7A shows that α5β1-integrin binds the MMP-9 PEX in a dose-dependent manner.

Using data analysis software a binding constant of ~8 nm was calculated (correlation coefficient, r = 0.998) suggesting high affinity of the

MMP-9 PEX domain for the integrin. In view of these results, as well as the bioinformatic analysis that suggested that the LSRG region may be the interaction site between MMP-9 and β1-integrin, a cyclic peptide was designed (aa 615–622) spanning the LRSG region in the MMP-9 PEX domain. Indeed, the thrombin-induced association of β1-integrin with MMP-9 was inhibited with the LRSRG peptide, whereas the scrambled version had no effect (Fig. 7B). Moreover, an antibody raised against a sequence (aa 603–614) adjacent to the LRSG sequence (aa 617–621) within the MMP-9 PEX domain was able to nearly abolish the association of the two proteins. These results suggest that the thrombin-induced association of MMP-9 with β1-integrin is, at least in part, mediated through the LRSG region in the MMP-9 PEX domain. To determine whether thrombin-induced cell surface gelatinolytic activity is affected by the association of MMP-9 and β1-integrin, U2-OS cells were preincubated with the LRSRG-peptide and cell surface-associated

**Grin receptors co-localize with MMP-9 (yellow overlay).** Together with the immunoprecipitation results this suggests that MMP-9 and β1-integrin do associate upon thrombin stimulation.

In view of these results, we asked which protein domains are important for the thrombin-stimulated association of β1-integrin with MMP-9, and whether interference with the association of the two proteins affects cellular invasion. Several reports show that the COOH-terminal hemopexin domain of type IV collagenases can directly bind the I-like domain of β-integrins, raising the possibility that MMP-9 and β1-integrin interact through these domains. For example, MMP-9 binds directly the I-like domain of β5-integrin (44), and the hemopexin domain of MMP-2 mediates interactions with αvβ3-integrin (45). Therefore, we performed a data base search, using the computer program MOLMOL, which compared the structure of MMP-9 and β1-integrin, and evaluated the three-dimensional structures for
digelatinolytic activity measured (Fig. 7C). Unstimulated U2-OS cells showed no change of cell surface gelatinolytic activity during the experiment. Upon thrombin stimulation gelatinolytic activity increased to a $V_{\text{max}}$ of 155 units/min, but addition of the LRG5-peptide could slow down gelatin cleavage on the cell surface (Fig. 7C; $V_{\text{max}} = 95$ units/min).

We then assessed whether the association of $\beta_1$-integrin with MMP-9 is of functional importance and performed invasion assays to determine whether thrombin-mediated invasion can be inhibited with the LRG5-peptide (Fig. 7D) or with the MMP-9 hemopexin antibody (Fig. 7E). Incubation with the LRG5-peptide reduced the thrombin-mediated induction of U2-OS invasion by 53%, whereas the scrambled control peptide had only a small effect on invasion that was not significant (Fig. 7D). These results were confirmed when we added the antibody against the MMP-9 PEX domain. Invasion after thrombin stimulation was inhibited by 52% (Fig. 7E), the same degree the LRG5 peptide was able to inhibit invasion. Taken together, these data suggest that the stimulation of invasion by thrombin in U2-OS osteosarcoma cells is at least partially mediated by induction and association of MMP-9 and $\beta_1$-integrin through the MMP-9 hemopexin domain. Last we evaluated if the stimulation of invasion by thrombin is limited to U2-OS cells. Fig. 7F shows that stimulation of another osteosarcoma cell line (TE-85) with thrombin induces invasion consistent with the secretion of MMP-9 in this cell line (Fig. 2B) after thrombin stimulation.

**DISCUSSION**

Cellular adhesion and invasion, which occurs in several physiologic and pathological conditions, depends on the tightly coordinated regulation of proteolysis and adhesion at the cell surface. Thrombin, a protease in the coagulation cascade, is known to induce invasion and metastasis, but the mechanisms of its action are only poorly understood.

Several of our observations suggest that thrombin and its receptor, PAR-1, play a pivotal role in the regulation of MMP-9 and $\beta_1$-integrin expression. Stimulation of an osteosarcoma cell line with thrombin induces cell surface association and secretion of enzymatically active pro-MMP-9, but not the 88-kDa form of MMP-9. Thrombin induces MMP-9 mRNA, at least in part, through the transcriptional activation of the MMP-9 promoter. Of equal importance is that thrombin strongly induces $\beta_1$-integrin mRNA transcription and protein expression, but not CD44 or other integrins. Upon overexpression, $\beta_1$-integrin and MMP-9 associate, as shown by co-immunoprecipitation, protein binding assay, and immunofluorescence, where the confocal picture suggests that all $\beta_1$-integrin receptors are associated with MMP-9. The association of MMP-9 and $\beta_1$-integrin, which is required for thrombin-mediated invasion, can be inhibited by preincubation with an antibody against, or a specific peptide binding to the hemopexin domain of MMP-9. These data support a model in which the stimulation of tumor cells by thrombin induces the transcription of both the $\beta_1$-integrin and the MMP-9 gene through PI 3-kinase, followed by protein expression, co-localization on the cell membrane, and
the enzymatic activation of pro-MMP-9, which can mediate cellular invasion (Fig. 8).

The signaling pathway connecting thrombin stimulation with the transcriptional control of MMP-9 expression merits discussion. In some models, thrombin stimulation of tumor growth requires the participation of Ras proteins and the MAPK pathway (47). However, the stimulation of MMP-9 and β1-integrin by thrombin does not require MAPK signaling, but is heavily dependent on the PI 3-kinase pathway. Thrombin stimulation of U2-OS cells induces Akt phosphorylation, and the inhibition of PI 3-kinase completely abolishes thrombin-mediated invasion. Inhibition of PI 3-kinase abrogates thrombin-mediated induction of both β1-integrin and MMP-9 protein, as well as mRNA expression, suggesting that they are co-regulated by thrombin through the same signaling pathway. Our data are supported by a study with ovarian cancer cells, showing that epidermal growth factor promotes PI 3-kinase-dependent induction of pro-MMP-9 binding on the cell surface. Inhibition of PI 3-kinase signaling with LY294002 was shown to reduce cell surface-associated pro-MMP-9 gelatinolytic activity, but the mechanism of MMP-9 regulation was not addressed further (39). We found that thrombin induces MMP-9 mRNA and promoter activity, suggesting that thrombin affects MMP-9 transcription, which is consistent with the data from us and others, showing that MMP-9 is regulated transcriptionally (22, 26, 48, 49).

Our data also suggest that MMP-9 and β1-integrin interact through the carboxyl-terminal hemopexin domain of MMP-9. This protein domain is functionally important, because it includes the binding region for TIMP-1, and because, in the absence of TIMP-1, it allows the formation of MMP-9 homodimers (50, 51). A cyclic peptide spanning the tetrapeptide, LRSG, within the MMP-9 hemopexin domain, inhibited association of β1-integrin with MMP-9, reduced invasion of osteosarcoma cells through Matrigel, and inhibited thrombin-mediated gelatinolytic activity on the cell surface of U2-OS cells. The importance of this sequence for MMP-9/β1-integrin binding is also supported by the fact that a monoclonal antibody raised against a peptide within the MMP-9 protein (aa 603–614), which is only 2 amino acids adjacent to the MMP-9616LRSG621 sequence, also inhibited association of the two proteins. It has been shown that in β3-integrin, the three-dimensional structure of which has been resolved at high resolution (52), the I-like domain is exposed on the protein surface, making it a candidate region for interaction with MMP-9. Indeed, a peptide with sequence similarity to the I-like domain of β5-integrin binds the MMP-9 hemopexin domain with high specificity (44). This peptide inhibits the interaction of MMP-9 with αvβ5-integrin in HT1080 cells and therefore inhibits tumor cell migration and tumor growth (44).
Stimulation of U2-OS cells with thrombin induced secretion of pro-MMP-9 (92 kDa), which was detected by Western blot and zymogram. Notably, we never detected the 88-kDa form, which has been identified as enzymatically active (53). Nevertheless, thrombin stimulation induced MMP-9 expression on the cell surface as detected by immunofluorescence and membrane associated enzymatic activity that cleaved quenched gelatin and stimulated invasion through Matrigel. This activity was inhibited with a broad spectrum MMP inhibitor (GM 6001), recombinant TIMP-1, an MMP-9 antibody, or with the LRG-S peptide, which was found to inhibit β1-integrin/MMP-9 interaction in immunoprecipitation experiments. Whereas allowing enzymatic activation of MMP-9 without cleavage of the proenzyme.

These results strongly suggest cell surface-associated MMP-9 activity we cannot rule out that there is transient association of pro-MMP-9 on the cell surface and generation of active protease after MMP-9 release.

Several known inducers of MMP-9 have been shown to stimulate both secreted and membrane-associated pro-MMP-9 expression, but not the 88-kDa MMP-9 form. In the breast epithelial MCF10A cell line, stimulation with phorbol ester resulted in a portion of the enzyme becoming associated with the cell surface. Surface-bound MMP-9 was only detected in its proform, which, in contrast to the secreted form, is not associated with TIMP-1. No other binding partner for MMP-9 was identified at that time (42). Stimulation with epidermal growth factor (39) and insulin growth factor (54) also induced surface-bound pro-MMP-9, and again, processed MMP-9 was not detected. Last Ban-nikov et al. (55) found that binding of pro-MMP-9 to type IV collagen induces proteolytic activity of the enzyme without cleavage of the amino-terminal pro-peptide. The authors suggest that binding to extracellular matrix might activate pro-MMP-9 through a conformational change. In view of these reports and our results, it is possible that β1-integrin acts as a receptor for pro-MMP-9 on the cell surface. It is tempting to speculate that binding of MMP-9 to β1-integrin indeed induces a conformational change in the MMP-9 protein that reduces binding of the cysteine in the propeptide domain to the active site zinc in the catalytic domain, thus allowing enzymatic activation of MMP-9 without cleavage of the proenzyme.

There is increasing evidence that thrombin plays a prominent role in cancer cell invasion and metastasis, and that some of its effects are mediated by the activation of proteases and integrins. Whereas we have focused on the regulation of MMP-9 and β1-integrin by thrombin, there are certainly other integrins and proteases targeted by thrombin. Our results show that PAR-1 is expressed on almost all of the human osteosarcoma tumors stained (24 of 25) in agreement with expression patterns found in breast and ovarian cancer (3–5). However, MMP-9 was only expressed in two-thirds of all patients, sug-
suggesting that the presence of PAR-1 does not necessarily express that of MMP-9. Indeed, thrombin activates MMP-2, the other type IV collagenase, in vascular endothelial cells (56) and can induce angiogenesis through a MMP-2-dependent mechanism (57). In addition, stimulation of prostate cancer cells by thrombin and TRAP activates the serine protease urokinase (58). From these reports, it seems that MMP-2 and urokinase are alternative proteases that can be activated by thrombin, and in future studies, it will be interesting to learn what determines the thrombin induced activation of one protease versus the other. β1-Integrin is, similarly, not the only integrin effector of thrombin. Thrombin can induce pulmonary vascular permeability through αβ5-integrin, which can be inhibited with a blocking antibody (43). In extension of these results, we report here that thrombin-mediated invasion can be inhibited with an antibody against αβ5-integrin, although this inhibition is not as effective as that seen with the β1-integrin blocking antibody.

In this study we have identified two new effectors of thrombin in tumor cells, MMP-9 and β1-integrin, adding to our mechanistic understanding of how thrombin stimulates cells to overcome tissue boundaries. Given that this, and other reports, show that one of the mechanisms by which thrombin induces tumor cell invasion is activation of cell surface-associated proteolysis, inhibition of thrombin or its receptor can be seen as an attractive therapeutic target. Indeed, recent clinical studies have shown that cancer patients receiving heparin therapy (which inhibits thrombin) have a better prognosis (58).

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FIGURE 8. Hypothesis of thrombin regulation of MMP-9 and β1-integrin.
See text for details.
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