Protease-Activated Receptors (PAR1 and PAR2) Contribute to Tumor Cell Motility and Metastasis

Xiaoli Shi,[1] Beena Gangadharan,[1] Lawrence F. Brass,[2] Wolfram Ruf,[3] and Barbara M. Mueller[1]

[1]Cancer Biology Division, La Jolla Institute for Molecular Medicine, San Diego, California; [2]Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania; and [3]Department of Immunology, Scripps Research Institute, La Jolla, California

Abstract

The effects of the pleiotropic serine protease thrombin on tumor cells are commonly thought to be mediated by the thrombin receptor protease-activated receptor 1 (PAR1). We demonstrate here that PAR1 activation has a role in experimental metastasis using the anti-PAR1 antibodies ATAP2 and WEDE15, which block PAR1 cleavage and activation. Thrombin also stimulates chemokinesis of human melanoma cells toward fibroblast conditioned media and soluble matrix proteins. Thrombin-enhanced migration is abolished by anti-PAR1 antibodies, demonstrating that PAR1 cleavage and activation are required. The PAR1-specific agonist peptide TFLLRNPNDK, however, does not stimulate migration, indicating that PAR1 activation is not sufficient. In contrast, a combination of TFLLRNPNDK and the PAR2 agonist peptide SLIGRL mimics the thrombin effect on migration, whereas PAR2 agonist alone has no effect. Agonist peptides for the thrombin receptors PAR3 and PAR4 used alone or with PAR1 agonist also have no effect. Similarly, activation of PAR1 and PAR2 also enhances chemokinesis of prostate cancer cells. Desensitization with PAR2 agonist abolishes thrombin-enhanced cell motility, demonstrating that thrombin acts through PAR2. PAR2 is cleaved by proteases with trypsin-like specificity but not by thrombin. Thrombin enhances migration in the presence of a cleavage-blocking anti-PAR2 antibody, suggesting that thrombin activates PAR2 indirectly and independent of receptor cleavage. Treatment of melanoma cells with trypsin or PAR2 agonist peptide enhances experimental metastasis. Together, these data confirm a role for PAR1 in migration and metastasis and demonstrate an unexpected role for PAR2 in thrombin-dependent tumor cell migration and in metastasis. (Mol Cancer Res 2004;2(7):395–402)

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Requests for reprints: Barbara M. Mueller, Cancer Biology Division, La Jolla Institute for Molecular Medicine, 4570 Executive Drive, San Diego, CA 92121. Phone: 858-587-8788; Fax: 858-587-6742. E-mail: bmueller@ljimm.org

Introduction

Tumor cell metastasis or the dissemination of a tumor from its original site to distant organs and tissues is an inherently inefficient process. The ability of tumor cells to activate the coagulation system and to generate thrombin has been shown to enhance metastatic efficiency (1-4), while anticoagulant therapies can interfere with metastatic disease in animal models and in humans (5). Thrombin can modify tumor cell behavior directly through the activation of thrombin receptors on the tumor cells or indirectly by acting on stroma cells or by the generation of fibrin matrices (1-4). The exact mechanisms, however, by which thrombin and potentially other coagulation proteases influence tumor cell metastasis remain incompletely understood.

The cellular receptors for thrombin are seven-transmembrane G protein-coupled receptors (GPCR) known as protease-activated receptors (PARs). PARs are widely expressed in vascular and extravascular tissues and are involved in responses to vascular injury and in the regulation of inflammation (6-9). The activation mechanism of PARs is unique. They are cleaved by specific serine proteases to expose a new amino terminus, which in turn serves as a tethered ligand to activate the receptor (6-9). Synthetic peptides mimicking the new amino terminus function as receptor agonists and are useful tools to dissect overlapping and distinct functions of the receptors of the PAR family (for detailed review, see ref. 8). PAR1, PAR3, and PAR4 are preferentially cleaved by thrombin, while PAR2 is cleaved by trypsin and other proteases with trypsin-like specificity but not by thrombin (7). Many cell types express multiple PARs, and physiologic responses to thrombin can depend on cross-talk between different receptors. The best understood example for cross-talk between PARs is in the activation of blood platelets, which involves PAR1 and PAR4 in human platelets and PAR3 and PAR4 in the mouse (6). Another example is the transactivation of PAR2 by cleaved PAR1. The tethered ligand sequence of PAR1 can activate PAR2 (10), and thrombin responses in endothelial cells depend in part on the transactivation of PAR2 by cleaved PAR1 (11).

Expression of the thrombin receptor PAR1 has been associated with metastatic potential. In human breast cancer, PAR1 expression correlates with tumor progression (12), and in prostate cancer, it has been implicated in bone metastasis (13). Metastatic human melanoma cell lines express PAR1 (14, 15), and overexpression of PAR1 in murine and human melanoma cells leads to enhanced metastasis in mice (16, 17). Overexpression of PAR1 also increases Matrigel invasion by melanoma cells (18), and thrombin stimulates the motility of colon carcinoma cells in a PAR1-dependent manner (19, 20). PAR1

antisense markedly reduces the invasion of a metastatic breast cancer cell line through a Matrigel barrier (12), whereas thrombin and PAR1 agonist peptide inhibit chemotaxis and invasion of another breast cancer cell line (21). PAR1 activation also contributes to tumor growth by enhancing tumor cell proliferation, as has been shown for melanoma (14) and colon carcinoma cell lines (20), and by inducing the expression of proangiogenic factors such as vascular endothelial growth factor (22). PAR1 and PAR2 are coexpressed in tumor cells and in cells of the tumor microenvironment (23), but little is known about possible functions of PAR2 in the biology of malignant tumors.

We have reported previously that experimental metastasis of human melanoma cells depends on the ability of the cells to activate the coagulation proteases and to generate thrombin (14, 24). We demonstrate here that thrombin responses in melanoma cell motility and metastasis depend on PAR1. However, on investigating this further, we unexpectedly found that thrombin stimulates the motility of metastatic tumor cells by a mechanism that requires not only the activation of PAR1 but also the simultaneous activation of PAR2. We examined the role of PAR2 in tumor cell metastasis and observed that activation of PAR2 on tumor cells results in enhanced metastasis.

Results

PAR1 in Human Melanoma Metastasis

M24met melanoma cells are highly metastatic in immunodeficient mice, and M24met metastasis can be almost completely blocked by antibodies that inhibit their procoagulant function (24) or by the specific thrombin inhibitor hirudin (14). The cells express the thrombin receptor PAR1 and mobilize intracellular Ca^{2+} in response to thrombin or PAR1 agonist peptide (14). To test whether thrombin enhances M24met metastasis by activating PAR1, we employed two different function-blocking anti-PAR1 antibodies. The mouse monoclonal anti-human PAR1 antibody ATAP2 is directed against the tethered ligand domain of PAR1, and the monoclonal antibody WEDE15 is directed against the thrombin binding region in PAR1; together, these antibodies completely inhibit thrombin cleavage of PAR1 (11). Incubation of M24met cells with ATAP2 alone had a modest but not statistically significant effect on metastasis, whereas incubation with a combination of ATAP2 and WEDE15 inhibited experimental metastasis markedly (Table 1). In contrast, a control antibody of the same isotype, a non-function-blocking antibody to cell surface tissue factor (TF9-10H10), had no effect on metastasis. Overexpression of PAR1 enhances melanoma metastasis in several models (16, 17). The present results confirm a role for PAR1 in metastasis and demonstrate a requirement for receptor cleavage.

Table 1. Effect of PAR1 Antagonists on Pulmonary M24met Metastasis

	Antagonist	*n*	Pulmonary Tumor Foci, Mean (SD)	*P**	% Inhibition
Experiment 1	None	8	57 (30)		
	ATAP2 + WEDE15	8	28 (25)	0.06	50.4
	TF9-10H10	4	68 (14)	0.5	0
Experiment 2	None	8	76 (40)		
	ATAP2 + WEDE15	8	32 (13)	<0.05	57.3
	ATAP2	7	50 (27)	0.2	33.9

*Probability of no difference using the two-tailed *t* test.

Thrombin Enhances Melanoma Cell Migration

To address how PAR1 activation influences the behavior of metastatic cells, we examined thrombin-induced cell migration. Elevated cell motility is a hallmark of metastatic cells, and M24met cells migrate well toward several chemotactic stimuli, including fibroblast conditioned medium and soluble matrix proteins. Preincubation with thrombin stimulated the migration of M24met cells in Transwell migration chambers toward a gradient of either NIH/3T3CM or soluble fibronectin (FN) in the lower chamber in a dose-dependent fashion (Fig. 1A). Thrombin at a concentration of 0.5 NIH units/mL increased M24met migration toward NIH/3T3CM on average 7.0 $\pm$ 2.3-fold (six independent experiments) and to soluble FN 8.7 $\pm$ 3.5-fold (10 independent experiments). The stimulation of cell migration by thrombin was completely abolished by the specific thrombin inhibitor hirudin (data not shown), demonstrating that thrombin's proteolytic activity was required.

To test whether thrombin itself is a chemoattractant for M24met cells, we performed checkerboard experiments in the absence and presence of soluble FN as chemotactic stimulus. Minimal migration (i.e., ~10 cells on an entire filter) was observed in the absence of FN in the lower chamber (Table 2). Under these conditions, high concentrations of thrombin in the lower chamber functioned as a chemoattractant, but thrombin also stimulated cell motility per se when present in the upper chamber. When soluble FN was present as a chemoattractant in the lower chamber, thrombin enhanced cell migration independent of a thrombin gradient (Table 2). These findings indicate that, in the presence of a strong chemoattractant, thrombin does not function as an additional chemoattractant but has a pronounced stimulatory effect on chemokinesis.

Chemotactic migration of M24met cells toward soluble FN depended on integrin $\alpha_5\beta_1$, because a function-blocking antibody against $\alpha_5\beta_1$, but not a function-blocking antibody against integrin $\alpha_v\beta_3$, inhibited cell migration. Stimulation of the cells with thrombin did not result in the use of a different or additional integrin for migration toward FN because the anti-$\alpha_5\beta_1$ antibody inhibited thrombin-stimulated migration to the same background level as unstimulated migration (Fig. 1B).

Melanoma Cell Migration Requires Activation of PAR1 and PAR2

To determine the role of PAR1 in thrombin-enhanced M24met motility, we used the function-blocking monoclonal anti-PAR1 antibodies in the migration assay. Preincubation of M24met cells with either ATAP2, directed against the tethered ligand domain of PAR1, or WEDE15, directed against the thrombin binding region, completely inhibited the thrombin-induced enhancement of M24met cell migration toward FN

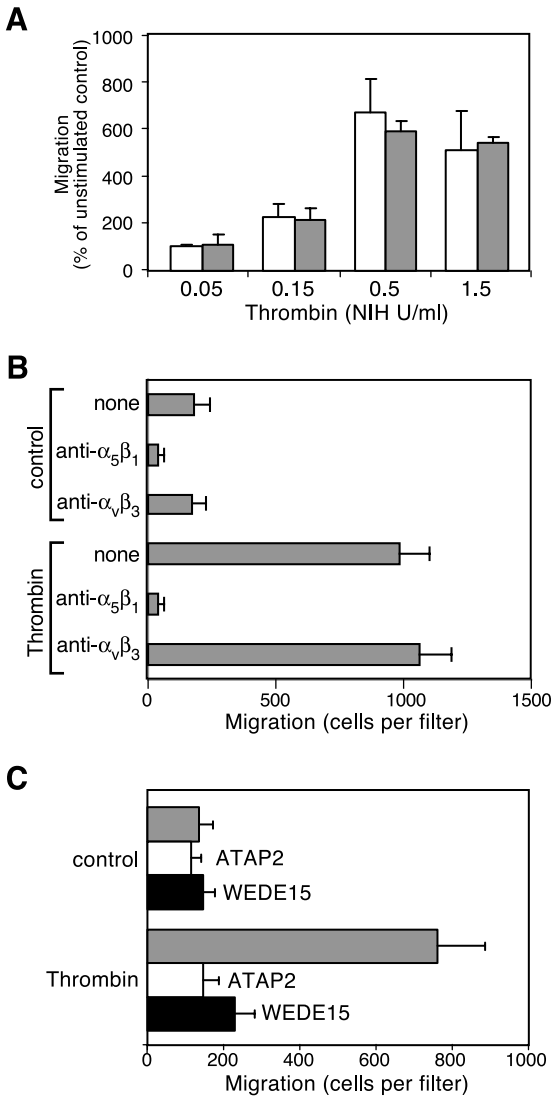

FIGURE 1. Thrombin enhances chemotactic migration of melanoma cells in a PAR1-dependent manner. **A.** M24met cells were incubated with thrombin for 90 minutes and migration toward 3-fold concentrated NIH/3T3CM (*white bars*) or soluble FN (50 μg/mL; *gray bars*) was determined in a 2-hour assay in Transwell migration chambers. **B.** Cells were incubated with function-blocking anti-integrin $\alpha_5\beta_1$ and $\alpha_v\beta_3$ antibodies (50 μg/mL) for 60 minutes, followed by thrombin (0.5 NIH units/mL) for 90 minutes and migration toward FN was determined. **C.** Cells were incubated with anti-PAR1 antibodies (25 μg/mL), followed by thrombin prior to migration. *Columns,* mean of four individual filters; *bars,* SD.

(Fig. 1C) and NIH/3T3CM (data not shown) but had no effect on cell migration in the absence of thrombin stimulation. These data demonstrate that thrombin has to bind to and cleave PAR1 to enhance M24met chemotactic migration.

To further support a role for PAR1 in our migration model, we tested whether a peptide that mimics the tethered ligand of PAR1 had a chemokinetic effect on M24met cells. Unexpectedly, we found that preincubation of M24met cells with the PAR1 agonist peptide TFLLRNPNDK (8) at concentrations ranging from 5 to 100 μmol/L had no effect on cell motility. We considered the possibility that binding to and cleaving of PAR1 by thrombin is necessary but not sufficient for the stimulation of cell migration in our model. Therefore, we tested whether synthetic peptide agonists for other known PARs could enhance cell migration. M24met cells express PAR2 on the cell surface, albeit at lower levels than PAR1 (Table 3). Preincubation of M24met cells with the PAR2 agonist peptide SLIGRL or with agonist peptides for the thrombin receptors PAR3 or PAR4 had no influence on cell motility. However, simultaneous addition of the PAR1 and PAR2 agonist peptides to M24met cells resulted in a marked enhancement of migration toward FN, comparable with stimulation by thrombin (Fig. 2A). In contrast, costimulation with PAR1 and PAR3 or PAR1 and PAR4 agonist peptides had no effect (Fig. 2A). Checkerboard analysis demonstrated that, like thrombin, the combination of the PAR1 and PAR2 agonist peptides had a strong chemokinetic and a weak chemotactic effect on M24met migration (data not shown).

Cells used in the migration assay are routinely harvested from tissue culture with trypsin, and we considered that exposure to trypsin might influence the activation of PAR2. When M24met cells were harvested with an EDTA solution (Versene), their migration was still significantly stimulated by thrombin or by a combination of PAR1 and PAR2 agonist peptides (Fig. 2B), indicating that PAR2 on the cells remained responsive to the agonist peptide with or without prior exposure to trypsin.

To determine whether activation of PAR1 and PAR2 enhances cell migration because of unique properties of melanoma cells or perhaps the M24met cell line, we examined the response of metastatic cell lines of epithelial origin. The prostate carcinoma cell lines PC-3 and DU-145 express PAR1 and PAR2 (Table 3). Thrombin stimulated the migration of PC-3 cells toward soluble FN (Fig. 2C). Simultaneous addition of PAR1 and PAR2 agonist peptides stimulated PC-3 migration similar to thrombin, whereas the PAR1 agonist peptide alone had no effect and the PAR2 agonist peptide enhanced PC-3 migration marginally (Fig. 2C). Thrombin and the combination of PAR1 and PAR2 agonists also stimulated migration of DU-145 cells but resulted only in a 2-fold enhancement of migration to FN (data not shown).

Table 2. Checkerboard Analysis of Thrombin-Stimulated Migration

		Thrombin (mU/mL)			
		Upper Well			
	Lower Well	0	50	150	500
Lower well no FN	0	16 ± 5*	20 ± 7	14 ± 2	140 ± 76
	50	8 ± 5	3 ± 3	4 ± 3	79 ± 12
	150	11 ± 4	13 ± 10	7 ± 7	56 ± 38
	500	129 ± 28	110 ± 25	87 ± 57	53 ± 21
FN	0	426 ± 90	858 ± 34	1,358 ± 180	1,126 ± 30
	50	362 ± 77	573 ± 75	872 ± 46	1,059 ± 110
	150	404 ± 61	675 ± 17	966 ± 12	936 ± 60
	500	840 ± 20	944 ± 17	1,167 ± 350	788 ± 150

*Cell migration as cells per filter. Mean ± SD of triplicate samples.

Table 3. Cell Surface Expression of PAR1 and PAR2

Primary Antibody	None	Anti-PAR1	Anti-PAR2
M24met	7.0 ± 1.1*	51.1 ± 8.2	26.5 ± 6.9
PC-3	7.5 ± 1.6	30.1 ± 1.2	10.7 ± 3.5
DU-145	5.2 ± 1.1	26.7 ± 2.6	9.1 ± 1.8

*Mean ± SD fluorescence intensity for three independent experiments.

Thrombin Activates PAR2 by a Cleavage-Independent Mechanism

The chemokinetic effect of thrombin on tumor cells is reproduced by the simultaneous activation of PAR1 and PAR2. Thus, in this context, thrombin may activate PAR1 and PAR2; alternatively, thrombin may only activate PAR1 and have additional PAR-independent effects on cell migration that are also induced by PAR2 activation with agonist peptide. To test whether thrombin activates PAR2 in cell migration, we performed desensitization experiments. Preincubation of cells with the PAR2 agonist SLIGRL had no direct effect on migration but abolished the effect of thrombin on M24met chemotactic migration (Fig. 3A), demonstrating that PAR2 is required for thrombin-enhanced cell migration. Desensitization with PAR2 agonist also inhibited the effect of PAR1/PAR2 agonist peptide stimulation, and as expected, desensitization with the PAR1 agonist TFLLRNPNDK abolished the response to thrombin and PAR1/PAR2 (Fig. 3A).

PAR2 is not a thrombin substrate (7). Therefore, we speculated that thrombin may activate PAR2 here by an indirect mechanism that does not require cleavage of PAR2. To test this idea, we incubated M24met cells with an antibody that blocks cleavage of PAR2 but leaves the receptor sensitive to PAR2 agonist peptide stimulation (9). The cleavage-blocking anti-PAR2 antibody had only a minimal inhibitory effect on thrombin-enhanced migration (Fig. 3B) and, as expected, did not inhibit the stimulation of cell migration by the combination of PAR1 and PAR2 agonist peptide. Together, these data suggest that, in the context of cell motility, thrombin can activate PAR2 by an indirect cleavage-independent mechanism.

PAR2 in Metastasis

For tumor cell motility, we have shown that thrombin cleaves and activates PAR1 and also indirectly activates PAR2. This raises the unexpected possibility that PAR2 activity also contributes to metastasis. Because there are currently no validated specific inhibitors for PAR2 available, we tested whether direct activation of PAR2 can further enhance metastasis. Direct stimulation of PAR2 may enhance metastasis if PAR2 in the metastatic process, as in cell migration, is indirectly activated by thrombin, because indirectly activated PARs may not signal with optimal efficiency (25). To test the effect of direct PAR2 activation, M24met cells were harvested from tissue culture using a nonenzymatic method and exposed to trypsin or PAR2 agonist peptide under defined conditions. As shown in Table 4, pretreatment of cells with either 500 nmol/L trypsin or 100 μmol/L PAR2 agonist peptide SLIGRL significantly enhanced metastasis of M24met cells, demonstrating that PAR2 signaling can contribute to experimental metastasis.

Discussion

Thrombin generation downstream of tissue factor plays a central role in the metastatic process (3, 4), and thrombin's effects on tumor cells are generally assumed to be mediated by PAR1 (12-22). In this study, we show that thrombin markedly enhances migration of metastatic tumor cells and that PAR1 activation is necessary but not sufficient. Rather, the simultaneous activation of PAR1 and PAR2 by thrombin is required to cause the chemokinetic effect. A role for thrombin and PAR1 activation in cell motility has been implicated previously in several different cell types, including chemotaxis of a murine hematopoetic cell line (26) and smooth muscle cells (27) as well as random migration of colon carcinoma cell lines (19, 20). Thrombin and PAR1 agonist peptide also inhibit migration and

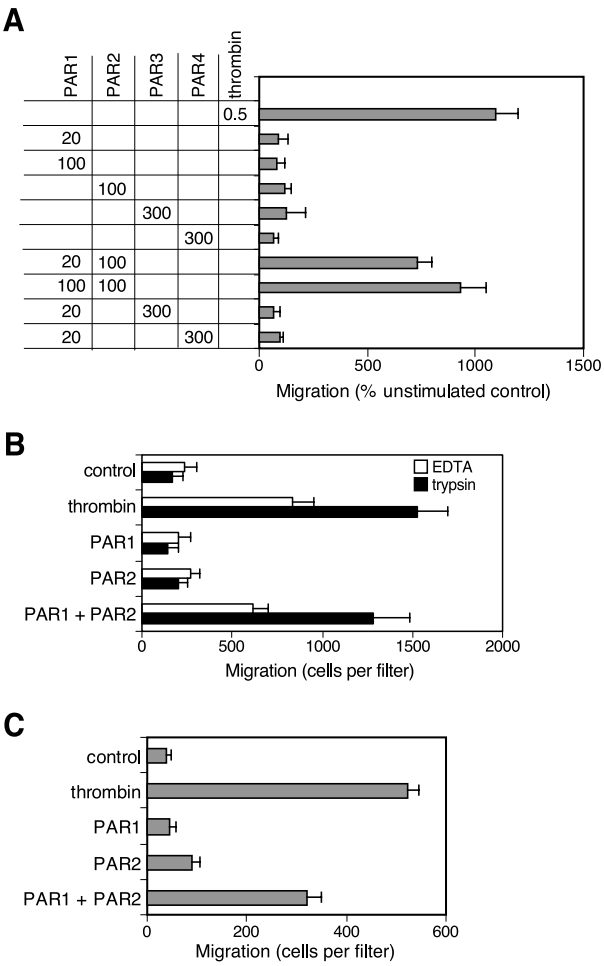

FIGURE 2. Stimulation with PAR1 and PAR2 agonist peptides reproduces the thrombin effect on migration. **A.** M24met cells were incubated with thrombin (NIH units/mL) or PAR agonist peptides (μmol/L) for 90 minutes and migration toward FN was determined. See Materials and Methods for peptide sequences. **B.** M24met cells were harvested from tissue culture using trypsin or EDTA and incubated with thrombin (0.5 NIH units/mL) or PAR1 (20 μmol/L) and/or PAR2 (100 μmol/L) agonist peptide prior to migration toward FN. **C.** PC-3 prostate cancer cells were incubated with thrombin (0.05 NIH units/mL) or PAR1 (20 μmol/L) and/or PAR2 (100 μmol/L) agonist peptides for 90 minutes prior to 2-hour migration in Transwell chambers toward soluble FN (50 μg/mL). *Columns,* mean of four individual filters; *bars,* SD.

invasion of a breast cancer cell line (28), and thrombin is a PAR4-dependent chemoattractant for another breast cancer cell line (21). PAR2 activation has also been implicated in cell motility and leads to chemotaxis of PAR2-transfected fibroblasts (28) and breast cancer cells (21, 28). While these studies have implicated a role for thrombin, PAR1, and PAR2 in cell motility, a requirement for thrombin to activate PAR1 and PAR2 has not been described. In certain cell types, PAR1 activation may be sufficient to modulate cell motility. However, several previous studies have used the PAR1 agonist peptide SFLLRN, which likely also functions as a PAR2 agonist (8), rather than the PAR1 specific TFLLRN and therefore may have overlooked a requirement for PAR2 activation in thrombin-dependent cell migration.

Our data indicate that PAR2 is activated by thrombin in an indirect cleavage-independent manner. GPCRs are known to form hetero-oligomers, and the constitutive or ligand-induced oligomerization of GPCRs can lead to signal amplification and transactivation (29, 30). PAR1 and PAR2 may form hetero-oligomers, and in such complexes, thrombin-activated PAR1 may mediate the indirect activation of PAR2. One potential scenario involves the transactivation of PAR2 by the tethered ligand of cleaved PAR1, which has been described to contribute to thrombin responses in endothelial cells (11). Activities of

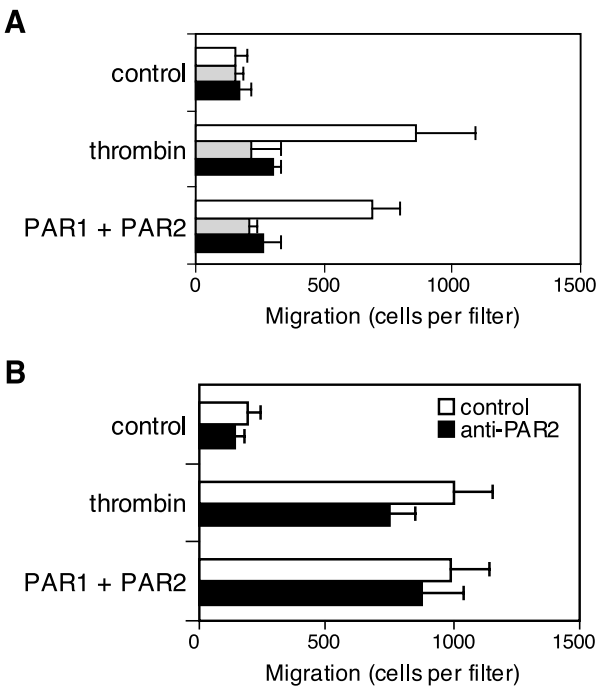

FIGURE 3. Thrombin activates PAR2 in a cleavage-independent manner. **A.** M24met cells were incubated for 10 minutes at 37°C in migration buffer (*white bars*) with 100 μmol/L PAR1 agonist peptide (*gray bars*) or 100 μmol/L PAR2 agonist peptide (*black bars*) followed by 30-minute stimulation with thrombin (0.15 NIH units/mL) or the combination of PAR1 (20 μmol/L) and PAR2 (100 μmol/L) agonist peptides. Migration toward FN was determined as described in Materials and Methods. **B.** M24met cells were incubated for 60 minutes with a cleavage-blocking polyclonal anti-PAR2 antibody (500 μg/mL), stimulated for 90 minutes with thrombin (0.5 NIH units/mL) or PAR1 (20 μmol/L) and/or PAR2 (100 μmol/L) agonist peptides, and migrated toward FN. *Columns,* mean of four individual filters; *bars,* SD.

Table 4. PAR2 Activation in M24met Experimental Metastasis

Activation	*n*	Pulmonary Tumor Foci Mean (SD)	*P**	Fold Stimulation
None	6	151 (83)		
Trypsin	6	300 (104)	0.02	1.99
SLIGRL	6	325 (87)	0.008	2.15

*Probability of no difference using the two-tailed *t* test.

GPCRs are also regulated by post-translational modifications and second messenger activated proteins (31). For example, receptor phosphorylation has been implicated in GPCR cross-talk (31). Thus, an alternate mechanism by which thrombin can indirectly activate PAR2 may involve the modification of PAR2 by activated PAR1.

Our finding that thrombin stimulates chemokinetic motility of melanoma and prostate cancer cells by a mechanism that requires PAR1 and PAR2 activation suggests that PAR1 and PAR2 have distinct nonoverlapping functions in cell migration. Recent evidence points to different roles for PAR1 and PAR2 in the reorganization of the cytoskeleton in endothelial cells. PAR1 signals predominantly through RhoA and leads to cell rounding, disruption of intercellular junctions, and increased permeability of the endothelium (32, 33). In contrast, PAR2 signaling has only a modest effect on RhoA but, unlike PAR1, leads to activation of the Rac/p21-activated kinase pathway (32), which is involved in cytoskeletal changes associated with cell motility. PAR2 activation also leads to the formation of a scaffolding complex containing β-arrestin and extracellular signal-regulated kinase 1/2. This complex is required for PAR2-dependent extracellular signal-regulated kinase 1/2 activation (34) and has been implicated in the PAR2-dependent reorganization of the actin cytoskeleton, the extension of polarized pseudopodia and chemotaxis (28).

PAR1 and PAR2 signaling has also been shown to affect integrin activation differentially. PAR1 preferentially influences the activity state and subcellular localization of α_v integrins (18, 19, 35), whereas PAR2, but not PAR1, promotes integrin $\alpha_5\beta_1$-dependent cell adhesion (35). In M24met melanoma cells, migration toward FN depends on $\alpha_5\beta_1$ in the absence of thrombin and also after thrombin stimulation. While there appears to be no PAR-dependent switch to the use of a α_v integrin in our model, PAR2 signaling may affect the activation state of $\alpha_5\beta_1$ or its coupling to downstream signaling transduction molecules.

Many cellular responses to PAR activation depend on gene transcription, and PAR signaling may induce the expression of motility factors, their receptors or activators. For example, signaling of PAR1 and other GPCRs has been shown to lead to the transactivation of tyrosine kinase receptors, such as the epidermal growth factor receptor, in a process that involves the up-regulation of a matrix metalloproteinase and the activation of latent heparin binding epidermal growth factor-like growth factor (36). In fact, GPCR-mediated release of heparin binding epidermal growth factor and transactivation of the epidermal growth factor receptor have been shown to enhance migration

of a squamous cell carcinoma cell line (37) and rat smooth muscle cells (27). Thrombin-enhanced cell migration in the M24met model is not inhibited by epidermal growth factor receptor antagonists but may involve the transactivation of a different growth factor receptor.

We present here evidence for a role of PAR2 in tumor cell metastasis and we confirm a role for PAR1. This suggests that, similar to cell migration, hematogenous metastasis is most efficient when tumor cells activate both PAR1 and PAR2. Circulating tumor cells generate thrombin, and thrombin most likely activates PAR1 in the context of metastasis directly. It remains to be determined whether PAR2 in metastasis is activated indirectly by thrombin or directly by a protease with trypsin-like specificity.

How can PAR signaling enhance tumor cell metastasis? We demonstrate that treating tumor cells prior to injection with a single dose of PAR1 antagonist or PAR2 agonist has significant effects on metastasis. Thus, while PAR1 signaling has been shown to induce tumor cell proliferation (14, 38), it is unlikely that, under our experimental conditions, modulation of PAR activity directly influences the growth of pulmonary metastases. Instead, PAR activity appears to support early events in the metastatic process. *In vivo* videomicroscopy demonstrates that postextravasation cell motility is critical for metastatic success in the liver and the chicken embryo chorioallantoic membrane (39, 40) but appears to be less relevant in the lung (41). It remains to be seen whether the activation of PAR1 and PAR2 contributes to metastatic success through enhanced cell motility. Another important determinant of metastatic efficiency is the rate of apoptosis of tumor cells in the early phase of metastatic arrest (42). PAR1 signaling is a survival factor for several cell types including neuronal cells (reviewed in ref. 7), endothelial cells (43, 44), and fibroblasts (45) and can prevent apoptosis under conditions that otherwise induce cell death (44, 45). PAR activation may contribute to metastatic success by preventing apoptosis. Finally, it should be noted that high doses of function-blocking anti-PAR1 antibodies did not inhibit metastasis in the M24met model by more than 50%. In contrast, the inhibition of coagulation initiation or thrombin activity leads to >90% inhibition of M24met metastasis (14, 24). This difference indicates that thrombin enhances metastasis not only by activating PAR1 and possibly PAR2 on the tumor cells but also contributes to metastatic efficiency by increasing fibrin generation (46, 47) and potentially the activation of thrombin receptors on platelets and endothelial cells.

Materials and Methods

Materials

The monoclonal anti-PAR1 antibodies ATAP2 and WEDE15 have been described (11). The monoclonal anti-PAR2 antibody SAM-11 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The cleavage-blocking polyclonal rabbit anti-human PAR2 antibody has been described (9). PAR1 agonist peptide TFLLRNPNDK and PAR2 agonist peptide SLIGRL were generated as described (48). PAR3 agonist peptide TFRGAP and PAR4 agonist peptide GYPGQV were from Bachem (King of Prussia, PA). The anti-tissue factor antibody TF9-10H10 has been described (24), the anti-integrin $\alpha_5\beta_1$ antibody JBS5 was from Chemicon (Temecula, CA), and the anti-integrin $\alpha_v\beta_3$ antibody LM609 was a kind gift from Dr. David Cheresh (Scripps Research Institute, La Jolla, CA). Human FN was from BD Bioscience (Bedford, MA), bovine thrombin was from Calbiochem (La Jolla, CA), and porcine trypsin was from Invitrogen (Grand Island, NY).

Cell Culture

M24met human melanoma cells were cultured as described (14). The human prostate carcinoma cell lines PC-3 and DU-145 were obtained from the American Type Culture Collection (Rockville, MD). PC-3 was cultured in 1:1 mixture of DMEM and Ham's F-12 supplemented with 10% fetal bovine serum, and DU-145 was cultured in Eagle's MEM with 10% fetal bovine serum. Mouse NIH/3T3 fibroblasts were obtained from the American Type Culture Collection and grown in DMEM with 10% fetal bovine serum. Semiconfluent NIH/3T3 cultures were rinsed and incubated in serum-free medium for 24 hours to generate NIH/3T3CM. NIH/3T3CM was concentrated and stored at −80°C.

Indirect Immunofluorescence

Cell surface expression of PAR1 and PAR2 was determined using flow cytometry as described (24). Briefly, cells were suspended in PBS and stained with WEDE15 or SAM-11 as primary antibody and fluorescein-labeled goat anti-mouse IgG as secondary antibody.

Migration Assay

Cell migration assays were performed using modified Boyden chambers with a 6.5 mm diameter, porous (8.0 μm) polycarbonate membrane separating the two chambers (Transwell, Corning, Inc., Corning, NY). Unless indicated otherwise, cells were harvested by brief exposure to trypsin/EDTA (Invitrogen) followed by soybean trypsin inhibitor (Calbiochem). Cells were washed and resuspended in fibroblast basal medium (Cambrex Bioscience, Walkersville, MD) containing 0.5% bovine serum albumin, 2 mmol/L $CaCl_2$, 1.8 mmol/L $MgCl_2$, and 0.2 mmol/L $MnCl_2$. Cells (2.5×10^6 per milliliter) were stimulated for 90 minutes at 37°C with thrombin or PAR agonist peptides. When indicated, cells were incubated with anti-integrin antibodies (50 μg/mL), anti-PAR1 antibodies (25 μg/mL), or anti-PAR2 antibodies (500 μg/mL) for 60 minutes prior to incubation with thrombin. FN (50 μg/mL) in migration medium or 3-fold concentrated NIH/3T3CM was placed in the lower compartment of the migration chamber, and 2.5×10^5 cells in 100 μL were placed in the upper compartment. When function-blocking antibodies were tested, antibodies were present at the indicated concentration in the upper and lower compartments. For checkerboard analysis, cells were placed in the upper compartment of the migration chamber with thrombin at the indicated concentration in the upper and/or lower compartment. Cells were allowed to migrate for 2 hours at 37°C in 5% CO_2. At the end of the assay, the

upper surface of the membrane was wiped with a cotton tip applicator to remove nonmigratory cells. Cells on the lower surface were fixed in 1% paraformaldehyde, stained with 1% crystal violet, and counted.

Metastasis Assay

For the experiments using function-blocking antibodies, cells were harvested from tissue culture using trypsin/EDTA and resuspended in serum-free tissue culture medium. Cells (2×10^5 in 200 μL per mouse) were mixed with antibodies (1.5 mg per mouse) and injected i.v. into the tail vein of 6-week-old female CB-17 SCID/Beige mice (Taconic, Germantown, NY). For PAR2 experiments, cells were harvested with EDTA (Versene, Invitrogen), resuspended in serum-free medium, and incubated with trypsin (500 nmol/L) or PAR2 agonist peptide (100 μmol/L) for 10 minutes followed by soybean trypsin inhibitor (BD Bioscience). Cells were then washed and injected (1×10^6 in 200 μL) i.v. into SCID mice. Mice were killed 3 weeks after injection, lungs were fixed in Bouin's fixative, and tumor foci on the surface of the lungs were counted.

Acknowledgments

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Xiaoli Shi, Beena Gangadharan, Lawrence F. Brass, et al.

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