Cooperation of protease-activated receptor 1 and integrin $\alpha_v\beta_5$ in thrombin-mediated lung cancer cell invasion

LINGYUN ZHU[*], XIUDONG WANG[*], JIE WU, DEKUI MAO, ZHISHAN XU, ZIYANG HE and AIPING YU

Department of Experimental Hematology, Beijing Institute of Radiation Medicine, Beijing 100850, P.R. China

Abstract. Protease-activated receptor 1 (PAR1) and integrins play an important role in thrombin-mediated tumor cell invasion. However, the role of PAR1 and integrin $\alpha_v\beta_5$ and the relationship between the two receptors in thrombin-induced lung cancer invasion remains unknown. Moreover, the mechanisms through which immobilized thrombin facilitates tumor invasion are poorly understood. In this study, both native and immobilized thrombin promoted lung cancer cell adhesion, migration and extracellular signal-regulated kinase phosphorylation. Integrin $\alpha_v\beta_5$ is involved in both native and immobilized thrombin-mediated tumor cell invasion; PAR1 had no effect on immobilized thrombin-mediated cell invasion. PAR1 and integrin $\alpha_v\beta_5$ colocalized on the surface of native thrombin-treated cells. This study suggests that targeting of integrin $\alpha_v\beta_5$ or the PAR1-integrin $\alpha_v\beta_5$ complex may present an important therapeutic opportunity to prevent lung cancer invasion.

Introduction

Overactivation of the coagulation system is a widely described pathology in cancer patients (1). Hypercoagulation of plasma in cancer patients can initiate thrombosis and also promote the progression of cancer. The risk of venous thromboembolism (VTE) is increased several-fold in cancer patients (2). Many coagulation factors are involved in the development of cancer and VTE (3-5). Thrombin, an important coagulation factor, is a trypsin-like serine protease which plays a pivotal role in both VTE and the progression of cancer (6, 7). Local and systemic thrombin production is increased in cancer patients,

and thrombin is produced by both tumor cells and tumor-associated platelets in the tumor microenvironment (8-10).

Previous research has suggested that thrombin-induced tumor invasion and metastasis are mainly mediated by protease-activated receptor 1 (PAR1). PAR1 is highly expressed in many cancer cells, especially in cancer cell lines with high metastasis potential (11-13). PAR1 belongs to a group of seven transmembrane receptors on the cell surface. The catalytic domain and exosite I domain of thrombin are both essential for PAR1 activation. The thrombin exosite I domain interacts with the hirudin (HV)-like sequence in the amino-terminal exodomain of PAR1, and cleavage of the PAR1 amino-terminal exodomain by the thrombin catalytic domain exposes a new amino-end, which serves as a tethered ligand for the PAR1 receptor and leads to activation of internal G-proteins.

It is widely recognized that integrins play a significant role in cancer cell invasion and metastasis. A relationship between thrombin and integrins has been reported in various tumor cells (14-16). Thrombin can affect the expression or distribution of integrins on the tumor cell surface (15-17). The Arg-Gly-Asp (RGD)-containing snake venom peptide and synthetic peptides can block thrombin-mediated tumor cell adhesion (15,18). However, the mechanism by which thrombin activates integrins has not yet been clearly described. To date, interactions between thrombin and integrins have mainly been reported in endothelial cells. However, there is considerable debate regarding the roles of the thrombin active site and RGD sequence during thrombin-induced integrin activation in endothelial cells. The thrombin RGD sequence is highly conserved in almost all species during evolution (19), implying that this sequence may be important for the function of thrombin. However, thrombin crystal structures show that the RGD sequence is buried within the catalytic domain. Therefore, the exposure of RGD implies a conformational change of thrombin (19-21). Tsopanoglou et al (22) reported that the catalytic site of thrombin is required for thrombin-mediated upregulation of integrin $\alpha_v\beta_3$ in human endothelial cells. However, the catalytic site of thrombin is not essential in the immobilized thrombin-mediated adhesion and migration of endothelial cells which is dependent on the interaction between thrombin and integrin $\alpha_v\beta_3$ (22). In addition, immobilized thrombin, or a synthetic thrombin peptide which lacks the catalytic site of thrombin but contains the thrombin RGD sequence can promote the attachment and migration of endo-

Correspondence to: Dr Aiping Yu, Department of Experimental Hematology, Beijing Institute of Radiation Medicine, Beijing 100850, P.R. China
E-mail: sxyap7020@yahoo.com.cn

[*]Contributed equally

thelial cells via integrin $\alpha_v\beta_3$ (23), indicating that the catalytic site of thrombin is not essential for integrin activation. Another study regarding smooth muscle cells has provided evidence that the interaction of thrombin with integrin $\alpha_v\beta_3$ in solution is inhibited by RGD mimetics. This raises the possibility that exposure of the RGD sequence may also occur independently on thrombin matrix attachment (24). In 2005, Papaconstantinou *et al* directly demonstrated that surface-absorbed thrombin promotes the attachment and migration of endothelial cells via an interaction with $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins. The RGD sequence of thrombin is exposed in crystal structures of free thrombin grown in a high salt concentration, independently of denaturation or thrombin proteolysis (25). These findings indicate that, via the RGD sequence, thrombin can directly interact with integrins in endothelial cells. However, the effect of immobilized thrombin on tumor cells remains unknown. Considering the inconsistent results observed in endothelial cells and lack of knowledge regarding the effect of immobilized thrombin on tumor cells, it is necessary to further investigate the interaction between thrombin, especially immobilized thrombin, and integrins in tumor cells. Moreover, the roles of the catalytic domain and the RGD sequence in thrombin-mediated integrin activation have not yet been revealed in tumor cells.

Though the roles of PAR1 and integrins in thrombin-mediated tumor invasion have been well-characterized, it is still not known how thrombin can simultaneously activate PAR1 and integrins on the cell surface. Additionally, it is intriguing to investigate whether these receptors can cooperate in thrombin-mediated tumor cell function. In this study, we used human lung cancer cells to evaluate and compare the effect of native and immobilized thrombin on PAR1 and integrin $\alpha_v\beta_5$ in tumor cells.

Materials and methods

Reagents. Bovine thrombin, bovine serum albumin (BSA), and the polypeptides GRGDS and SDGRG were purchased from Sigma (St. Louis, MO, USA). The rabbit anti-human PAR1, mouse anti-p-Erk and rabbit anti-Erk antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and the mouse anti-human integrin $\alpha_v\beta_5$ antibody was obtained from R&D Systems (Minneapolis, MN, USA). Horseradish peroxidase (HRP)-labelled goat anti-mouse IgG and goat anti-rabbit IgG were purchased from Jackson ImmunoResearch (West Grove, PA, USA). HV was produced in our laboratory (purity >95%, with a specific activity of 10,000 ATU/mg).

Cell culture. The human pulmonary adenocarcinoma cell line Glc-82 was obtained from the Kunming Cell Bank of the Chinese Academy of Sciences (Kunming, China). The cells were cultured in RPMI-1640 medium containing 10% foetal bovine serum, in saturated humidity at 37°C with 5% CO_2.

Preparation of rat tail collagen. The tails of Wistar rats were soaked in 75% ethanol for 30 min, the skin was prepared and the tail was cut into 3-cm fragments. The tail tendons were drawn out, cut into small fragments, soaked in 0.1% acetic acid at 4°C for 48 h and collagen was obtained by centrifugation at 200 x g for 30 min. The collagen concentration was determined using the Lowry method. All animal studies were approved by the Chinese Animal Research Ethics Board and all animals received care in compliance with the Chinese Convention on Animal Care.

Cell adhesion assay. For the native thrombin adhesion assay, Glc-82 cells were grown for 16 h in serum-free medium, digested with 0.25% trypsin, washed with PBS, resuspended in serum-free medium and 200 μl aliquots of 5×10^4 cells were transferred to a 96-well plate coated with rat tail collagen (2 μg/well). For the antagonism studies, the anti-PAR1, anti-integrin $\alpha_v\beta_5$ antibodies (10 μg/ml) or GRGDS (400 μM) were incubated with Glc-82 cells at 37°C for 15 min, and after removal of the antagonists, thrombin (8 IU/ml) was added to the cells. HV (10 μg/ml) were pre-incubated with thrombin at 37°C for 15 min, and then the mixture was added to the Glc-82 cells.

For the immobilized thrombin adhesion assay, the 96-well plates were coated with thrombin (4 IU/ml, 40 μl/well) instead of rat tail collagen, washed three times with PBS, then blocked with 3% BSA for 1 h. The Glc-82 cells were serum-starved, resuspended in serum-free medium and transferred to the thrombin-coated 96-well plates as previously described. For the HV and thrombin co-coating experiments, HV (0, 10 or 100 μg/ml) was incubated with thrombin for 15 min, the 96-well plates were coated with the mixture, and the cells were added to the plate. For the experiments where HV was added to the media, 100 μg/ml HV was added to the thrombin-coated plate for 15 min, then the cells were added. For the other antagonists, the cells were pre-incubated with the anti-PAR1 or anti-integrin $\alpha_v\beta_5$ antibodies, GRGDS or the negative control SDGRG for 15 min. After removal of the antagonists, the cells were added to the thrombin-coated plate.

After a 1 h incubation period, non-adherent cells were removed by washing with PBS. Adherent cells were fixed in 4% paraformaldehyde for 30 min, washed in 0.1 M borate buffer (pH 8.5), stained with 1% methylene blue for 10 min, washed four times in borate buffer, incubated with 0.1 M hydrochloric acid for 40 min and absorbance was measured at 600 nm on a Multiskan Spectrum Plate Reader (Thermo Electron Corporation, Waltham, MA, USA).

Cell migration assay. Glc-82 cells were grown in serum-free medium for 16 h prior to the migration assay. For the native thrombin-induced cell migration assay, 1×10^6/ml Glc-82 cells were treated with the indicated agonists or antagonists as described in the cell adhesion assay section. Then 200 μl of the treated cells were added to the upper wells of the Transwell migration chambers (0.8 μm, Corning, NY, USA). For the immobilized thrombin induced cell migration assay, the bottom of the Transwell migration chamber was coated with thrombin (4 IU/ml) before the cell migration assay was performed. Glc-82 cells were treated with the indicated agonists or antagonists as described in the cell adhesion assay section.

The cells were allowed to migrate for 19 h, and the chambers were then fixed with 4% paraformaldehyde for 30 min and stained with 0.05% crystal violet for 10 min. After washing with PBS, cells which had not migrated were wiped off and the chambers were observed using an inverted Olympus IX70
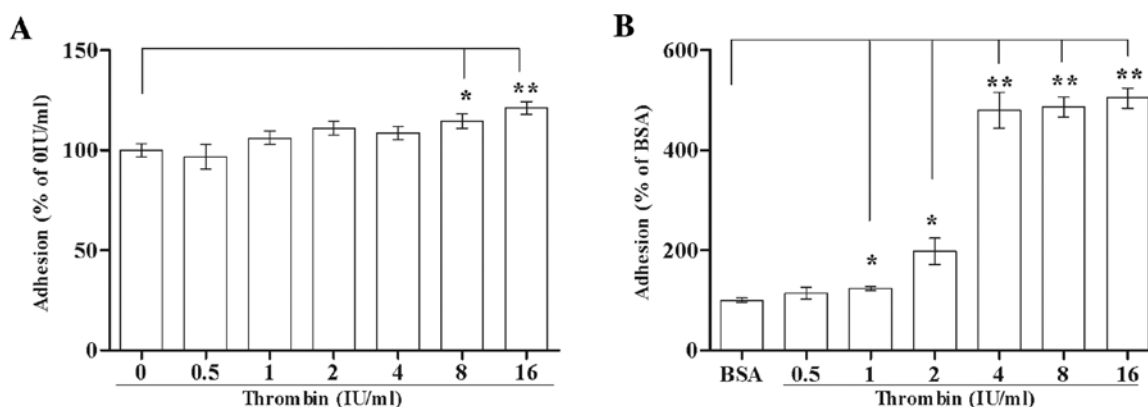
Figure 1. Native and immobilized thrombin promote adhesion of Glc-82 cells to rat tail collagen. The effect of different concentrations of native thrombin (A) and immobilized thrombin (B) on the adhesion of Glc-82 cells (*P<0.05, **P<0.01).

microscope (Olympus, Tokyo, Japan). The number of cells was counted in five fields of view.

Western blotting. To investigate the effect of native thrombin on Erk phosphorylation, Glc-82 cells were serum-starved for 24 h before the addition of agonists or antagonists, as described in the cell adhesion assay section. To investigate the effect of immobilized thrombin on Erk phosphorylation, Glc-82 cells were plated on the thrombin-coated culture flasks and treated as described in the cell adhesion assay section.

After 1 h, the cells were washed twice with ice-cold PBS, lysed in RIPA buffer and subjected to SDS-PAGE electrophoresis. The proteins were transferred to PVDF membranes (Millipore, MA, USA), and the membranes were washed and blocked with 10% non-fat milk in TBS containing 0.1% Tween (TBS-T). The blots were incubated with primary antibodies to Erk or p-Erk in 10% non-fat milk/TBS-T. After washing with TBS-T, the membranes were incubated with HRP-conjugated secondary antibodies for 45 min at room temperature, washed with TBS-T and the bands were visualized using the Pro-light HRP Chemiluminescence kit (Tiangen Biotech, Beijing, China).

Rat tail collagen contraction assay. Serum-starved Glc-82 cells (250 μl/1x10^6/ml) were treated with antagonists mixed with 500 μl 2.4 mg/ml rat tail collagen and carefully plated in 6-well plates containing 3 ml serum-free medium/well. The mixture was allowed to polymerize at room temperature for 1 h, and was then incubated at 37°C for 2-3 weeks and the diameter of the collagen was measured.

Immunofluorescence. Glc-82 cells were seeded on sterile 22-mm glass coverslips in 6-well plates, allowed to attach overnight, cultured in serum-free medium for 24 h, treated with 1 IU/ml thrombin for 1 h at 37°C and then fixed in ice-cold methanol for 10 min. After washing with PBS for 3 times, the cells were permeabilized in 0.1% Triton X-100 for 15 min and blocked in 3% BSA for 30 min at room temperature. The cells were incubated overnight with an anti-integrin $\alpha_v\beta_5$ (1:100) and anti-PAR1 (1:50) primary antibodies, washed in PBS, then incubated for 60 min with FITC-conjugated goat

anti-mouse (1:100) and PE-conjugated goat anti-rabbit (1:50) secondary antibodies, and cell nuclei were stained using 4,6-diamidino-2-phenylindole (DAPI).

Immunofluorescence staining was viewed and analyzed using a LSM 510 META confocal microscope (Carl Zeiss, Inc., Oberkochen, Germany).

For the immunofluorescence analysis of immobilized thrombin-treated cells, the cells were plated on thrombin-coated glass coverslips and allowed to attach for 24 h in serum-free medium before immunofluorescent staining.

Statistical analysis. Numerical data are presented as mean ± SE. Each experiment was repeated at least two times. Data were analyzed using the Student's t-test and one-way analysis of variance. P<0.05 was considered significant and all statistical tests were two-sided.

Results

Role of PAR1 and integrin $\alpha_v\beta_5$ in thrombin-mediated Glc-82 adhesion. Different thrombin concentrations were used to analyze the effect of native thrombin on the adhesion of Glc-82 cells to rat tail collagen. Native thrombin at high concentrations (8 IU/ml, P<0.05, 16 IU/ml, P<0.01) significantly promoted the adhesion of Glc-82 cells to rat tail collagen (Fig. 1A).

Immobilized thrombin could significantly stimulate Glc-82 cell adhesion at low concentrations (1 or 2 IU/ml, P<0.05), with the maximal effect observed at high concentrations (4, 8 or 16 IU/ml, P<0.01; Fig. 1B).

To evaluate the roles of PAR1 and integrin $\alpha_v\beta_5$ in the effects of native thrombin or immobilized thrombin-induced Glc-82 cell adhesion, PAR1 antagonists (HV and anti-PAR1 antibody) and integrin $\alpha_v\beta_5$ antagonists (GRGDS and anti-integrin $\alpha_v\beta_5$ antibody) were used. As shown in Fig. 2A, the ability of native thrombin to stimulate Glc-82 cell adhesion was significantly attenuated by all of the PAR1 and $\alpha_v\beta_5$ antagonists.

HV in the culture media, and the anti-integrin $\alpha_v\beta_5$ and anti-PAR1 antibodies did not affect the ability of immobilized thrombin to induce Glc-82 cell adhesion (Fig. 2B and 2C). When thrombin was pre-incubated with HV and this mixture was coated on the plate, the ability of immobilized thrombin
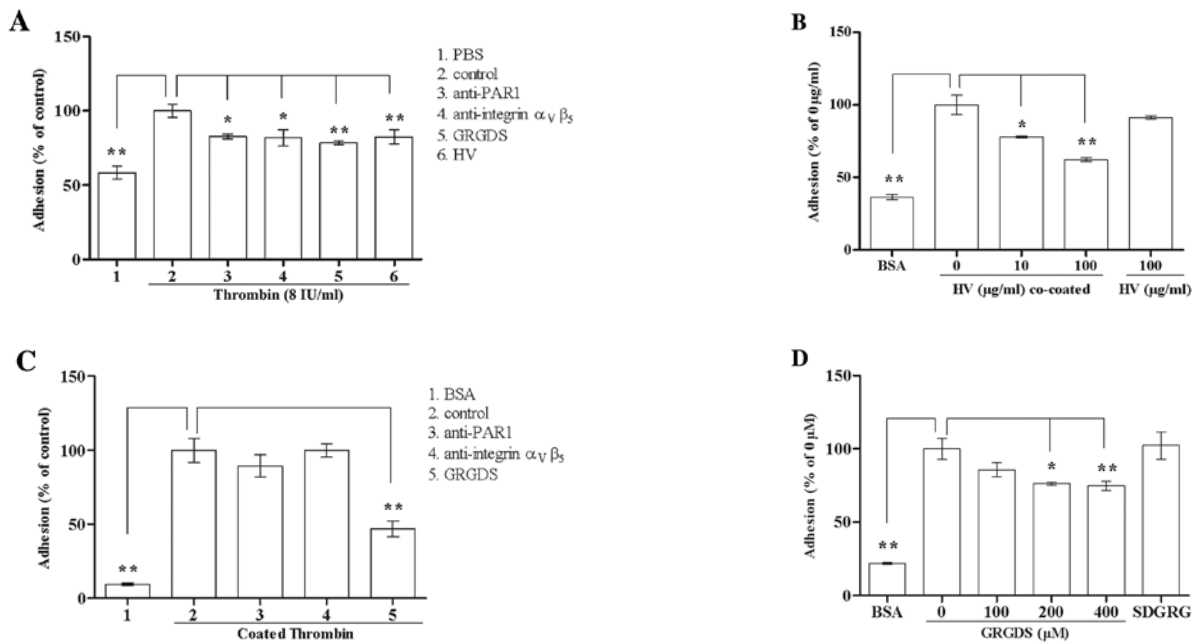
Figure 2. The effect of PAR1 and integrin $\alpha_v\beta_5$ antagonists on thrombin-mediated Glc-82 cell adhesion. (A) The roles of PAR1 and integrin $\alpha_v\beta_5$ in native thrombin-mediated Glc-82 cell adhesion. Anti-PAR1, anti-integrin $\alpha_v\beta_5$ antibodies (10 μg/ml) or GRGDS (400 μM) were pre-incubated with Glc-82 cells, then the antibodies were removed and 8 IU/ml thrombin was added to the cells. HV (10 μg/ml) were pre-incubated with thrombin, and the mixture was added to Glc-82 cells. The treated cells from all groups were allowed to attach for 1 h. (B) The role of HV in immobilized thrombin-mediated Glc-82 cell adhesion. HV (0, 10 or 100 μg/ml) was incubated with thrombin, the mixture was coated on the plate or HV (100 μg/ml) in the media was added to the thrombin-coated plate, and then the cells were added and allowed to attach for 1 h. (C) The roles of PAR1 and integrin $\alpha_v\beta_5$ in immobilized thrombin-mediated Glc-82 cell adhesion. The cells were pre-incubated with the anti-PAR1 antibody, anti-integrin $\alpha_v\beta_5$ antibody or GRGDS, and then added to the thrombin-coated plate and allowed to attach for 1 h. (D) The role of GRGDS in immobilized thrombin-mediated Glc-82 cell adhesion. The cells were pretreated with different concentrations of GRGDS or the negative control SDGRG for 15 min, and then added to the thrombin-coated plate and allowed to attach for 1 h (*P<0.05, **P<0.01).
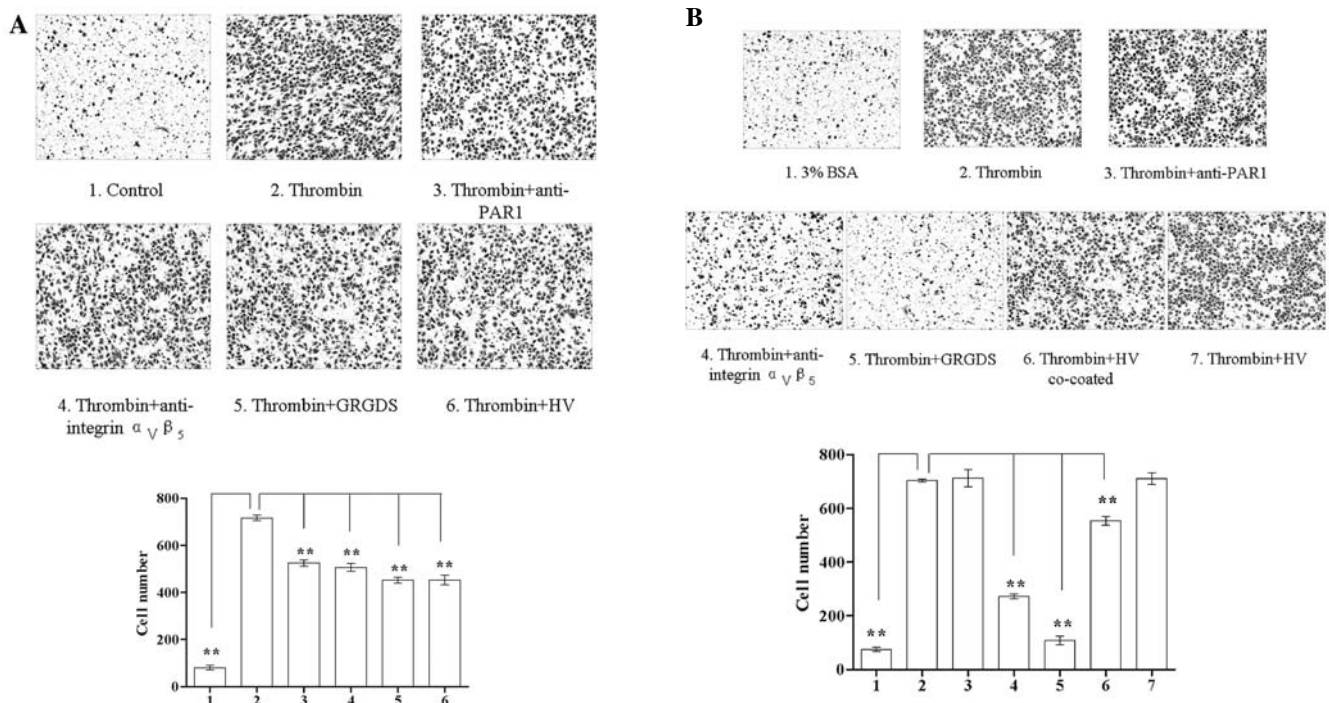

Figure 3. The effect of PAR1 and integrin $\alpha_v\beta_5$ antagonists on thrombin-mediated Glc-82 cell migration. (A) The roles of PAR1 and integrin $\alpha_v\beta_5$ in native thrombin-mediated Glc-82 cell migration: 1, PBS control; 2, 0.5 IU/ml thrombin; 3-5, Glc-82 cells were pre-incubated with 10μg/ml anti-PAR1, anti-integrin $\alpha_v\beta_5$ antibody or 400 μM GRGDS, the antibodies were removed and 0.5 IU/ml thrombin was added; 6, thrombin was pre-incubated with 10 μg/ml HV and the mixture was added to Glc-82 cells; magnification, x100. (B) The roles of PAR1 and integrin $\alpha_v\beta_5$ in immobilized thrombin-mediated Glc-82 cell migration: 1, Glc-82 cells were added to a control BSA-coated Transwell migration chamber; 2, 4 IU/ml thrombin-coated Transwell migration chamber; 3-5, the cells were pre-treated with 10 μg/ml anti-PAR1, anti-integrin $\alpha_v\beta_5$ antibody or 400 μM GRGDS and then added to a thrombin-coated Transwell migration chamber; 6, 4 IU/ml thrombin and 10 μg/ml HV were co-coated on the Transwell migration chamber before Glc-82 cells were added; 7, 10 μg/ml HV was pre-incubated in the thrombin-coated migration chamber before Glc-82 cells were added. The cells were allowed to migrate for 19 h, then stained with crystal violet and observed using a inverted microscope; magnification, x100 (**P<0.01).
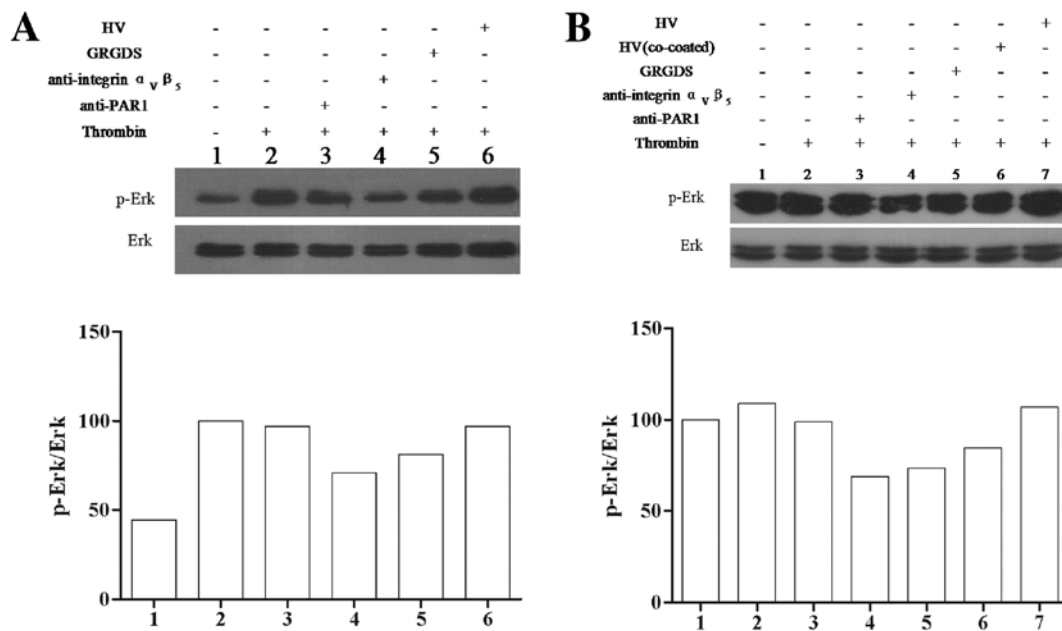
Figure 4. The effect of PAR1 and integrin $\alpha_v\beta_5$ antagonists on thrombin-mediated Erk phosphorylation in Glc-82 cells. (A) The effect of PAR1 and integrin $\alpha_v\beta_5$ on native thrombin-mediated Erk phosphorylation. Western blot and quantification of the Erk phosphorylation/total Erk ratio in lane 1, Glc-82 cells treated with PBS; lane 2, Glc-82 cells treated with 0.5 IU/ml thrombin; lanes 3-5, Glc-82 cells pre-incubated with 10 μg/ml anti-PAR1, anti-integrin $\alpha_v\beta_5$ antibody or 400 μM GRGDS, then treated with 0.5 IU/ml thrombin; lane 6, Glc-82 cells treated with thrombin which was pre-incubated with 10 μg/ml HV. (B) The effect of PAR1 and integrin $\alpha_v\beta_5$ on immobilized thrombin-mediated Erk phosphorylation. Western blot and quantification of the Erk phosphorylation/total Erk ratio in Glc-82 cells treated with lane 1, BSA; lane 2, 4 IU/ml thrombin or lanes 3-5, incubated with 10 μg/ml anti-PAR1 or anti-integrin $\alpha_v\beta_5$ antibody or 400 μM GRGDS and then plated into 6-well plates coated with thrombin; lane 6, Glc-82 cells were plated into wells co-coated with 4 IU/ml thrombin and 10 μ/ml HV; or lane 7 10 μg/ml HV was pre-incubated in the thrombin-coated plate before the cells were added to the plate. All data shown are from a single representative experiment.

to promote Glc-82 cell adhesion was significantly attenuated (Fig. 2B). The integrin $\alpha_v\beta_5$ antagonist GRGDS also markedly inhibited the ability of immobilized thrombin to induce Glc-82 cell adhesion, in a dose-dependent and specific manner (Fig. 2C and D).

Role of PAR1 and integrin $\alpha_v\beta_5$ in thrombin-mediated Glc-82 migration. Both native thrombin (0.5 IU/ml) and immobilized thrombin (4 IU/ml) significantly promoted Glc-82 cell migration (P<0.01, Fig. 3). All of the PAR1 and integrin $\alpha_v\beta_5$ antagonists significantly inhibited the ability of native thrombin to induce cell migration (P<0.01, Fig. 3A). The anti-integrin $\alpha_v\beta_5$ antibody (10 μg/ml) and GRGDS (400 μM) significantly inhibited the ability of immobilized thrombin to induce cell migration (P<0.01). The anti-PAR1 antibody (10 μg/ml) and HV in the culture media had no effect on the ability of immobilized thrombin to induce cell migration; however, when HV was co-coated with thrombin on the plate, it significantly decreased the ability of immobilized thrombin to induce Glc-82 cell migration (P<0.01, Fig. 3B).

Role of PAR1 and integrin $\alpha_v\beta_5$ in thrombin-mediated Erk phosphorylation in Glc-82 cells. Native thrombin markedly stimulated Erk phosphorylation in Glc-82 cells. Both the anti-integrin $\alpha_v\beta_5$ antibody (10 μg/ml) and GRGDS (400 μM) attenuated the ability of native thrombin to induce Erk phosphorylation. The anti-PAR1 antibody and HV had no significant effect on the ability of native thrombin to induce Erk phosphorylation (Fig. 4A).
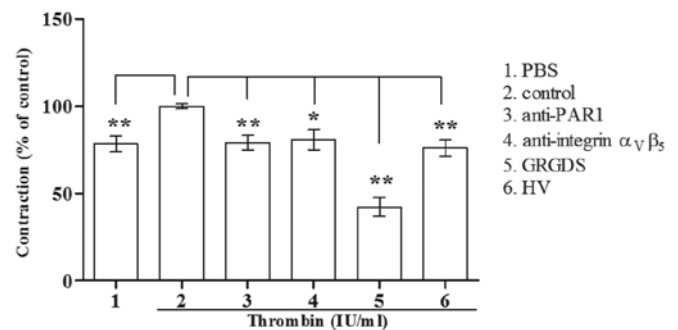

Figure 5. The effect of PAR1 and integrin $\alpha_v\beta_5$ antagonists on thrombin-treated Glc-82 cell-induced rat tail collagen contraction. Glc-82 cells were pre-incubated with 10 μg/ml anti-PAR1 or anti-integrin $\alpha_v\beta_5$ antibody or 400 μM GRGDS and then 1 IU/ml thrombin was added. HV was pre-incubated with 1 IU/ml thrombin, and the mixture was added to Glc-82 cells. After treatment, the cells were added carefully to rat tail collagen, incubated for 2-3 weeks, and the collagen size was determined (*P<0.05; **P<0.01).

Immobilized thrombin also increased Erk phosphorylation in Glc-82 cells. The anti-integrin $\alpha_v\beta_5$ antibody (10 μg/ml) and GRGDS (400 μM) significantly inhibited the ability of immobilized thrombin to induce Erk phosphorylation. The anti-PAR1 antibody and HV in the culture media had no significant effect on the ability of immobilized thrombin to induce Erk phosphorylation. When the culture plates were co-coated with HV and thrombin, HV significantly inhibited the ability of immobilized thrombin to induce Erk phosphorylation (Fig. 4B).
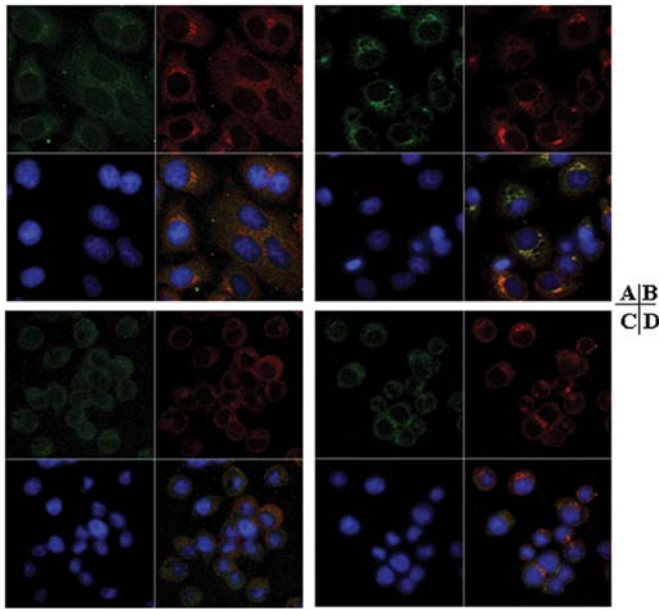
A|B
C|D

Figure 6. Immunohistochemical localization of PAR1 and integrin α$_v$β$_5$ on the cell surface of Glc-82 cells after thrombin stimulation. Confocal images of Glc-82 cells treated with (A) PBS control or (B) native thrombin, which induced specific colocalization of PAR1 and integrin α$_v$β$_5$ on the cell surface. Confocal images of Glc-82 cells treated with (C) BSA and (D) immobilized thrombin, which induced a clustered distribution of PAR1 on the cell surface. Red, PAR1; green, integrin α$_v$β$_5$, blue, DAPI nuclear staining. Data shown are from a single representative experiment (magnification, x400).

Effect of PAR1 and integrin α$_v$β$_5$ on rat tail collagen contraction induced by thrombin-treated Glc-82 cells. Thrombin-treated (1 IU/ml) Glc-82 cells significantly promoted the contraction of rat tail collagen. The PAR1 antagonists (HV and anti-PAR1 antibody) and integrin α$_v$β$_5$ antagonists (GRGDS and anti-integrin α$_v$β$_5$ antibody) significantly attenuated the ability of thrombin-treated Glc-82 cells to induce rat tail collagen contraction (Fig. 5).

Effect of thrombin on the cell surface localization of PAR1 and integrin α$_v$β$_5$ in Glc-82 cells. Native thrombin treatment affected the cell-surface distribution of integrin α$_v$β$_5$ and PAR1 in Glc-82 cells. After stimulation with native thrombin, both integrin α$_v$β$_5$ and PAR1 clustering localized to the cell surface. Moreover, specific colocalization of PAR1 and integrin α$_v$β$_5$ was observed (Fig. 6B). In cells cultured on slides coated with thrombin, integrin α$_v$β$_5$ was diffusely distributed over the cell surface, and PAR1 was clustered on the cell surface. Colocalization of integrin α$_v$β$_5$ and PAR1 was not observed (Fig. 6D).

Discussion

Few reports have described the relationship between PAR1 and integrins in thrombin-mediated tumor cell functional responses. In human melanoma cells, PAR1 has been reported to cooperate with integrin α$_v$β$_5$ via its cytoplasmic tail, and PAR1 activation affects integrin α$_v$β$_5$ distribution on the cell surface without altering integrin α$_v$β$_5$ expression levels. The specific activation of PAR1 by the activation polypeptide TRAP induces co-precipitation of integrin α$_v$β$_5$ and paxillin

(16). However, the specific activation mechanism of PAR1 and integrins by thrombin, especially immobilized thrombin, and the potential relationship between PAR1 and integrin in thrombin-induced tumor cell functions have not been characterized.

In this study, we evaluated the effect of immobilized thrombin on lung cancer cell invasion. Immobilized thrombin could promote tumor cell invasion in a similar manner to native thrombin, and lead to increased cell adhesion and migration, activation of the Erk signaling pathway and collagen contraction (Fig. 1-5). These experiments provide a strong model to elucidate the effects of ECM-immobilized thrombin on tumor invasion. It is known that there is a high concentration of thrombin in the stromal tissue surrounding tumor cells or the metastasis environment. The thrombin stored in the fibrin around the tumor tissue or bound to subendothelial ECM is protected from inactivation by circulating thrombin inhibitors (26-29). This results in a prolonging stimulation of the thrombin receptor and transduction of tumor-promoting signals, and eventually facilitates tumor invasion. However, it is not known how immobilized thrombin promotes tumor cell invasion, or whether immobilized thrombin can activate PAR1 and the integrins.

Antagonists of PAR1 and integrin α$_v$β$_5$ were used to evaluate the role of PAR1 and integrin α$_v$β$_5$ on immobilized thrombin-mediated tumor cell invasion. We compared the results with the effects mediated by native thrombin. In view of the inconsistent results in previous studies on the activation of PAR1 and integrins by thrombin in endothelial cells or tumor cells, we presume that the illustration for the roles of PAR1 and integrin α$_v$β$_5$ would provide evidence to describe the activation mechanism of the two receptors by thrombin, and simultaneously evaluate the function of the thrombin catalytic domain and RGD motif during PAR1 and integrin α$_v$β$_5$ activation by native and immobilized thrombin.

In the cell adhesion assay, PAR1 antagonists (anti-PAR1 antibody and HV) and integrin α$_v$β$_5$ antagonists (anti-integrin α$_v$β$_5$ antibody and GRGDS) significantly attenuated the ability of native thrombin to stimulate Glc-82 cell adhesion (Fig. 2A). These results indicate that PAR1 and integrin α$_v$β$_5$, as well as the catalytic domain of thrombin are required for native thrombin-mediated tumor cell function. However, HV in the culture media had no effect on the ability of immobilized thrombin to stimulate Glc-82 cell adhesion, and intriguingly, when the plates were co-coated with HV and thrombin, thrombin-mediated cell adhesion was dramatically attenuated (Fig. 2B). We hypothesize that the promotion effect of immobilized thrombin on Glc-82 cell adhesion is not dependent on the thrombin catalytic domain. The conformation of thrombin changes when it is immobilized, thus blocking the ability of HV to bind thrombin. The inhibitory effect of co-coated HV on cell adhesion in response to immobilized thrombin suggests pre-incubation of HV with thrombin prevents, at least in part, a conformational change in thrombin, resulting in a reduced ability to promote cell adhesion. Thus, we investigated whether the conformation change in immobilized thrombin exposed the RGD sequence and destroyed the catalytic domain. An anti-PAR1 antibody had no effect on the ability of immobilized thrombin to stimulate cell adhesion (Fig. 2C), indicating that immobilized thrombin-mediated cell adhesion is not dependent on the thrombin catalytic domain. The anti-integrin α$_v$β$_5$ anti-

body also had no effect on immobilized thrombin-mediated cell adhesion. However, the integrin antagonist GRGDS significantly, specifically and dose-dependently inhibited immobilized thrombin-mediated cell adhesion (Fig. 2C and D). Collectively, these results indicate that integrins, but not integrin $\alpha_v\beta_5$ participate in immobilized thrombin-mediated tumor cell adhesion, and that immobilization of thrombin may induce a conformational change in the catalytic domain of thrombin and expose the RGD sequence.

In the cell migration assay, both native and immobilized thrombin promoted Glc-82 cell migration (Fig. 3). In a similar manner to the cell adhesion assay, all of the PAR1 and integrin $\alpha_v\beta_5$ antagonists attenuated the ability of native thrombin to induce cell migration (Fig. 3A), providing further evidence for a role of both PAR1 and integrin $\alpha_v\beta_5$ in native thrombin-stimulated tumor cell function. PAR1 antagonists (anti-PAR1 antibody and HV in the culture media) had no effect on immobilized thrombin-mediated cell migration, suggesting that the catalytic domain is not required for immobilized thrombin to stimulate cell migration. Integrin $\alpha_v\beta_5$ antagonists (anti-integrin $\alpha_v\beta_5$ antibody and GRGDS) significantly inhibited the ability of immobilized thrombin to stimulate cell migration, indicating that integrin $\alpha_v\beta_5$ is required for immobilized thrombin-mediated cell migration. When HV was co-coated with thrombin, the ability of immobilized thrombin to stimulate cell migration was attenuated (Fig. 3B), in a similar manner to cell adhesion. We presume that binding of HV to thrombin inhibits the catalytic activity, and represses the conformation change and an attenuated ability to stimulate tumor cell invasion.

Previous research has indicated that the Erk signaling pathway plays an important role in thrombin/PAR1-mediated platelet (30-32) or cancer cell function (33,34). The Erk signaling pathway is reported to participate in integrin function (35,36). However, it is not known if Erk signaling plays a role in thrombin-mediated integrin function. We evaluated the relationship between Erk signaling and activation of PAR1 and integrin $\alpha_v\beta_5$ in thrombin-treated lung cancer cells. Phosphorylation of Erk occurred independently of thrombin-induced PAR1 activation (Fig. 4), suggesting that the thrombin catalytic domain is not necessary for the activation of Erk signaling. The interaction of both native and immobilized thrombin with integrin $\alpha_v\beta_5$ significantly induced Erk phosphorylation, which could be inhibited by the anti-integrin $\alpha_v\beta_5$ antibody, GRGDS and co-coated HV (Fig. 4). As the anti-PAR1 antibody and HV in the culture media had no effect on thrombin-induced Erk phosphorylation, activation of PAR1 and the thrombin catalytic domain may not be necessary for Erk activation. These results indicate that activation of the Erk signaling pathway by native or immobilized thrombin is due to activation of integrin $\alpha_v\beta_5$, suggesting that Erk phosphorylation results from outside-in activation of the integrin $\alpha_v\beta_5$ signaling pathway which may involve the thrombin RGD sequence. These observations are not consistent with the interactions between thrombin and integrins reported by others, where the activation of integrins by thrombin is PAR1 dependent on inside-out signaling (14,16).

Tumor invasion and metastasis depend on the ability of tumor cells to invade beyond the primary site and establish at remote sites. Tumor cell invasion is characterized by remod-eling of the tumor microenvironment, and ECM remodeling by tumor cells can be evaluated using the collagen contraction assay (37,38). Native thrombin-treated cells significantly enhanced rat tail collagen contraction, resulting in a smaller diameter of the collagen mass. PAR1 and integrin $\alpha_v\beta_5$ antagonists attenuated the ability of native thrombin-treated cells to mediate collagen contraction (Fig. 5), in agreement with the cell adhesion and migration assays results, indicating that both PAR1 and integrin $\alpha_v\beta_5$ are required for thrombin to induce ECM remodeling, which may facilitate tumor cell invasion.

Aside from the effects on Erk signaling, our experiments demonstrate that both PAR1 and integrin $\alpha_v\beta_5$ play an important role in the ability of native thrombin to affect Glc-82 cell function. Therefore, we assessed if potential interactions exist between PAR1 and integrin $\alpha_v\beta_5$ during thrombin-induced tumor cell invasion using a dual immunofluorescent colocaliza-tion assay. After treatment with native thrombin, PAR1 and integrin $\alpha_v\beta_5$ were clustering distributed and colocalized on the cell surface of Glc-82 cells (Fig. 6B). We assume that the thrombin exosite I domain combines with PAR1, leading to a conformational change in thrombin, which exposes the RGD sequence allowing integrin $\alpha_v\beta_5$ to interact with thrombin. Under this situation, thrombin, at least transiently, acts as a bridge between PAR1 and integrin $\alpha_v\beta_5$. Therefore, PAR1 and integrin $\alpha_v\beta_5$ are simultaneously required for native thrombin-mediated Glc-82 cell function. This hypothesis is in compliance with studies in other cells. Thrombin can simultaneously bind to and activate PAR1 and PAR4 in platelets, possibly via binding of the thrombin exosite I to the HV-like motif of cleaved PAR1, allowing the active site of thrombin free to potentially interact with PAR4. This indicates that thrombin may remain tethered to the surface of platelets via its association with cleaved PAR1. PAR1 and PAR4 form a stable heterodimer which enables thrombin to act as a bivalent functional agonist (39). Another study has indicated that transient binding of thrombin to PAR1 prior to receptor cleavage may serve as an RGD-exposing event, which enables integrin binding during PAR1 activation (20). Thus, it is possible, at least theoretically, that the thrombin exosite I domain remains bound to PAR1 while the thrombin catalytic domain is liberated from PAR1, followed by a confor-mation change in thrombin and exposure of the RGD sequence to allow integrin $\alpha_v\beta_5$ binding.

In conclusion, we studied the effects of native and immobi-lized thrombin on tumor cell invasion, and evaluated the role and relationship between PAR1 and integrin $\alpha_v\beta_5$. The results of this study suggest that immobilized thrombin can induce tumor cell invasion in a similar manner to native thrombin. Integrin $\alpha_v\beta_5$ play a pivotal role in both immobilized thrombin and native thrombin-mediated tumor cell invasion. Thus, inhi-bition of thrombin or its receptors, especially integrin $\alpha_v\beta_5$, may provide an attractive therapeutic target. The ability of PAR1 and integrin $\alpha_v\beta_5$ to cooperate in native thrombin-induced cell invasion suggests that targeting of the PAR1-integrin complex may present an important therapeutic opportunity to prevent tumor invasion.

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