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Plasminogen activator inhibitor-1 regulates macrophage-dependent postoperative adhesion by enhancing EGF-HER1 signaling in mice

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ABSTRACT: Adhesive small bowel obstruction remains a common problem for surgeons. After surgery, platelet aggregation contributes to coagulation cascade and fibrin clot formation. With clotting, fibrin degradation is simultaneously enhanced, driven by tissue plasminogen activator–mediated cleavage of plasminogen to form plasmin. The aim of this study was to investigate the cellular events and proteolytic responses that surround plasminogen activator inhibitor (PAI-1; *Serpine1*) inhibition of postoperative adhesion. Peritoneal adhesion was induced by gauze deposition in the abdominal cavity in C57BL/6 mice and those that were deficient in fibrinolytic factors, such as $Plat^{-/-}$ and $Serpine1^{-/-}$. In addition, C57BL/6 mice were treated with the novel PAI-1 inhibitor, TM5275. Some animals were treated with clodronate to deplete macrophages. Epidermal growth factor (EGF) experiments were performed to understand the role of macrophages and how EGF contributes to adhesion. In the early phase of adhesive small bowel obstruction, increased PAI-1 activity was observed in the peritoneal cavity. Genetic and pharmacologic PAI-1 inhibition prevented progression of adhesion and increased circulating plasmin. Whereas $Serpine1^{-/-}$ mice showed intra-abdominal bleeding, mice that were treated with TM5275 did not. Mechanistically, PAI-1, in combination with tissue plasminogen activator, served as a chemoattractant for macrophages that, in turn, secreted EGF and up-regulated the receptor, HER1, on peritoneal mesothelial cells, which led to PAI-1 secretion, further fueling the vicious cycle of impaired fibrinolysis at the adhesive site. Controlled inhibition of PAI-1 not only enhanced activation of the fibrinolytic system, but also prevented recruitment of EGF-secreting macrophages. Pharmacologic PAI-1 inhibition ameliorated adhesion formation in a macrophage-dependent manner.—Honjo, K., Munakata, S., Tashiro, Y., Salama, Y., Shimazu, H., Eiamboonsert, S., Dhahri, D., Ichimura, A., Dan, T., Miyata, T., Takeda, K., Sakamoto, K., Hattori, K., Heissig, B. Plasminogen activator inhibitor-1 regulates macrophage-dependent postoperative adhesion by enhancing EGF-HER1 signaling in mice. FASEB J. 31, 000–000 (2017). www.fasebj.org

KEY WORDS: small bowel obstruction · epidermal growth factor · PAI-1 · plasminogen activator · mesothelial cell

The overshooting adhesive response potentially resulting in a postoperative adhesive small bowel obstruction is a serious problem encountered by surgeons who operate in the abdomen. At adhesive sites, the coagulation cascade that results in the generation and deposition of fibrin is simultaneously activated with the counteracting fibrinolytic system, which leads to fibrin degradation. Fibrinous exudate is an essential component of normal tissue repair, but timely resolution of the fibrin deposit is essential for proper restoration of preoperative conditions. The fibrinolytic factor, plasmin, can regulate the balance between

ABBREVIATIONS: EGF, epidermal growth factor; FACS, fluorescence-activated cell sorting; FAK, focal adhesion kinase; FGF, fibroblast growth factor; PAA, plasminogen-activating activity; PAI, plasminogen activator inhibitor; PAP, plasmin-antiplasmin; rPAI, recombinant plasminogen activator inhibitor; rtPA, recombinant tissue plasminogen activator; tPA, tissue plasminogen activator; uPA, urokinase-type plasminogen activator

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fibrin deposition and degradation at the surgical site by breaking down fibrin. Plasmin is generated from plasminogen by tissue plasminogen activator (tPA; gene name: *Plat*; secreted by mesothelial and endothelial cells and leukocytes). The local balance between fibrin production and its degradation (fibrinolysis) dictates postoperative adhesion. It has been postulated that the plasminogen-activating activity (PAA) of peritoneal mesothelium determines whether fibrin formed after peritoneal injury develops into permanent fibrous adhesions. During surgery, PAA was shown to decline in both normal and inflamed peritoneum, with tPA making up almost 95% of PAA (1). In postoperative clinical samples, a dramatically diminished fibrinolytic activity was found at the surgical site along with increased levels of plasminogen activator inhibitors (PAIs) (2, 3). Several studies have demonstrated that PAI-1 (*Serpine1*) deficiency protects lungs from excess fibrin accumulation and bleomycin-induced fibrosis (4). In a unilateral ureteral obstruction mouse model of kidney fibrosis, *Serpine1*$^{-/-}$ unilateral ureteral obstruction mice had a significantly lower number of macrophages and myofibroblasts (5). *tPA*$^{-/-}$ mice were more susceptible to adhesion formation after both surgical insult and chronic inflammation compared with *uPA*$^{-/-}$ (urokinase-type plasminogen activator) and *uPA*$^{+/+}$ mice (2). Moreover, intra-abdominal adhesion in rats was prevented by topical tPA, but not uPA (6). Although locally delivered recombinant tPA (rtPA) prevented adhesion formation after surgery (7) or after cerebral thrombosis, a major adverse effect of tPA administration, especially after surgery, has been postoperative bleeding, which has limited its clinical use (8).

After surgical manipulation, a complex immune response is initiated that leads to recruitment and activation of resident macrophages (9). Macrophages express uPA, tPA, PAI-1 and PAI-2, and cytokines that regulate fibrinolysis and inflammation. Postsurgical macrophages often have reduced fibrinolytic activity that may allow the persistence of adhesions (10).

These postsurgical macrophages secrete variable substances, such as plasminogen activator, PAI, collagenase, elastase, IL-1 and -6, TNF, leukotriene B_4, prostaglandin E_2 (11), and epidermal growth factor (EGF). EGF is mitogenic for peritoneal mesothelial cells, inducing a morphologic change toward a fibroblastic phenotype (12). EGF also enhances cell migration and adhesion to extracellular matrix molecules (13). Plasmin can alter the activity status of growth factors, thereby changing the biologic function of cells (*e.g.*, their migratory abilities) (14).

In the present study, we investigated the clinical efficiency of a newly developed PAI-1 inhibitor and its mode of action in preventing abdominal adhesion. We show that administration of a PAI-1 inhibitor suppressed macrophage-driven response during postsurgical adhesion.

MATERIALS AND METHODS

Animals

Plg$^{+/+}$ and *Plg*$^{-/-}$, *Serpine1*$^{+/+}$ and *Serpine1*$^{-/-}$, and *Plat*$^{+/+}$ and *Plat*$^{-/-}$ mice were used for experiments after >10 back-crosses onto a C57BL/6 background. C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). C57BL/6 recombinase-activating gene 2 (*Rag2*$^{-/-}$) mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan). The animal review board of Juntendo University and the Institute of Medical Science (The University of Tokyo) approved animal study protocols.

Induction of surgical adhesion

Mice were anesthetized with 2% isoflurane in oxygen. A model for postoperative adhesion was induced in mice, as previously described (9), and was modified by a method to detain gauze in the abdominal cavity. After midline incision through the abdominal wall, a round-shaped 2- × 2-cm sterilization gauze was placed on the right flank. Adhesion scores were determined as follows: 0, no adhesion; 1, mild adhesion (removable by simple pulling); 2, moderate adhesion of local site (removable with forceps); 3, moderate adhesion across a wide range (removable with forceps); 4, strong adhesion of local site (nonremovable with forceps); and 5, strong adhesion across a wide site (nonremovable with forceps; Supplemental Fig. 1). The adhesion scoring system was validated by 3 independent investigators in a blinded fashion. Thickness of adhesive lesions was measured on 5 hematoxylin and eosin–stained tissue sections.

Reagent

PAI-1 inhibitor, TM5275 {5-chloro-2-[({2-[4-(diphenylmethyl) piperazin-1-yl]-2-oxoethoxy}acetyl)amino]benzoate}, was provided by Toshio Miyata (Tohoku University) and inhibited PAI-1 activity, with an IC_{50} value of 6.95 μM, as measured by assay of tPA-dependent hydrolysis of a peptide substrate. IC_{50} values of TM5007 and PAI-749 are 5.60 and 8.37 μM, respectively (15). The drug was resuspended in 200 μl 0.5% carboxymethylcellulose and administered orally (10 mg/kg body weight) daily for 0–6 d. Control mice received vehicle (200 μl 0.5% carboxymethylcellulose). For macrophage depletion, 200 μl of liposome-encapsulated clodronate (Clophosome; FormuMax, Palo Alto, CA, USA) was intraperitoneally injected. EGF receptor inhibitor cetuximab was injected (1 mg/mouse) on d 0, 2, 4, and 6.

Histology

Small intestine and peritoneum tissues were fixed in 10% buffered formalin, embedded in paraffin, and cut into 3-mm sections. Sections were stained with hematoxylin and eosin, Elastica van Gieson, phosphotungstic acid and hematoxylin, and azan stain. Elastica van Gieson staining identified collagen and elastin. Collagen was visualized in red and elastic fibers that were stained black. Phosphotungstic acid and hematoxylin stained connective tissues pale orange-pink to brownish red, fibrin deep blue, and coarse elastic fibers purple. Azan stained collagen blue.

Immunohistochemistry

Adherent tissues were snap-frozen in liquid nitrogen and cut into 5-μm sections. Sections were labeled with the following primary Abs: anti-Gr1 (clone RB6-8C5; R&D Systems, Minneapolis, MN, USA), anti-F4/80 (clone A3-1; Bio-Rad, Hercules, CA, USA), anti-CD3e (clone 145-2C11; BD Pharmingen, Brea, CA, USA), anti-CD45R (clone RA3-6B2; Abcam, Cambridge, MA, USA), anti-Ly49C (clone 5E6; BD Pharmingen), anti-CD11c

(clone HL3; BD Pharmingen), and anti-CD11b (clone M1-70; BD Pharmingen). Primary Abs were labeled with the following secondary Abs: goat anti-rat IgG conjugated with Alexa Fluor 594 (Thermo Fisher Scientific, Waltham, MA, USA), goat anti-rabbit IgG conjugated with Alexa Fluor 488 (Thermo Fisher Scientific), goat anti-hamster IgG conjugated with Alexa Fluor 488 (Thermo Fisher Scientific), rabbit anti-rat IgG (Vector Laboratories, Burlingame, CA, USA), and FITC-conjugated bovine anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Nuclei were stained using DAPI (Vector Laboratories).

Immunoassay

All assays were performed by using ELISA from commercially available kits. Active PAI-1 and tPA were assayed by using ELISA kits (Molecular Innovations, Novi, MI, USA). The quantitative determination of mouse plasmin-antiplasmin (PAP) complex was measured with ELISA kits (Cusabio Biotech, Newark, DE, USA).

Flow cytometry

Peritoneal cells that were responsible for adhesion were identified by using fluorescence-activated cell sorting (FACS). Cells collected after peritoneal lavage were stained with the following Abs: F4/80-PE (clone 6F12), CD11b-APC (clone M1/70), Gr-1-FITC (clone RB6-8C5), CD4-PE (clone GK1.5), CD8a-APC (clone 53-6.7), and B220-FITC (clone RA3-6B2; all from BD Pharmingen). Cells were analyzed by using FACS Caliber (Becton Dickinson, Mountain View, CA, USA).

Cell lines

RAW 264.7 mouse macrophage cell line was maintained at 37°C in 5% CO2 in 10-cm dishes with DMEM (Wako Pure Chemicals, Tokyo, Japan) that was supplemented with 10% fetal bovine serum. Before the start of each experiment, cells were incubated overnight in serum-free medium.

Cell migration assay

Macrophage migration assay was established by using Transwell units with a 3-μm pore size (Corning Costar, Corning, NY, USA) in 24-well plates. Cells were loaded into the top chamber that contained 100 μl of serum-free RAW 264.7 cells that contained DMEM (16), with or without neutralizing CD11b Abs (BioLegend, San Diego, CA, USA). Cells migrated toward the lower chamber that contained recombinant PAI-1 (rPAI-1; 1 μg/ml; Millipore, Billerica, MA, USA), rtPA (1 μg/ml; Molecular Innovations), or peritoneal tissue culture supernatants. Migrated macrophages were quantified by counting 5 random regions within each culture dish of transmigrated cells. Peritoneum supernatants were prepared as described previously (17). In brief, peritoneum tissue culture supernatant was prepared by culturing 2- × 2-cm pieces of peritoneum from *Serpine1*$^{+/+}$ and *Serpine1*$^{-/-}$ mice on d 0 and 1 after surgery for 24 h with DMEM.

In another set of experiments, supernatants from mesothelial cells cultured overnight with or without recombinant EGF were subjected to a migration assay that was supplemented with or without rtPA and with or without TM5275 in the lower migration chamber.

Growth factor expression in macrophages and mesothelial cells

Total RNA was isolated from primary mesothelial cells, as previously described (18), by using a Nucleospin RNA plus kit (TaKaRa Bio, Otsu, Japan). Total RNA was isolated from peritoneal F4/80^{+}CD11b^{+} macrophages by MoFlo (Cytomation, Fort Collins, CO, USA) by using a single-shot cell lysis quantitative RT-PCR direct SYBR Green kit (Bio-Rad). Real-time PCR was performed by using SYBR Green PCR Master Mix (Toyobo, Osaka, Japan) on a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific). Relative mRNA expression was calculated by using the $2^{-\Delta\Delta Ct}$ method. PCR was performed using the following specific forward and reverse primer pairs, respectively: *Egf*: 5′-GTTGTTAGCACCATCCCTCATCCC-3′ and 5′-GCAAGGCCTGCAGGTGACTGAT-3′; *Tgfb*: 5′-TGACGTCACTGGAGTTGTACGG-3′ and 5′-GGTTCATGTCATGGATGGTGC-3′; *Fgf2* (fibroblast growth factor 2): 5′-GTCCTTGAAGTGGCCTGGTGGG-3′ and 5′-TTCAGGAAGAGTCCGGCTGCACT-3′; *Egfr*: 5′-CCACGCCAACTGTACCTATG-3′ and 5′-ATCCACTGCCATTGAACGTA-3′; *Erbb2*: 5′-CCCAGATCTCCACTGGCTCC-3′ and 5′-TTCAGGGTTCTCCACAGCACC-3′; *Erbb3*: 5′-TACCCATGACCACCTCACACT-3′ and 5′-ATATCTGGCAGTCTTCTGGTC-3′; *Erbb4*: 5′-GAAATGTCCAGATGGCCTACAGGG-3′ and 5′-CTTTTTGATGCTCTTTCTTCTGAC-3′; *Rac-1*: 5′-GAGACGGAGCTGTTGGTAAAA-3′ and 5′-ATAGGCCCAGATTCACTGGTT-3′; *FAK* (focal adhesion kinase): 5′-CCATGCCCTCGAAAAGCTATG-3′ and 5′-TCCAATACAGCGTCCAAGTTCTA-3′; *CD11b*: 5′-ATGGACGCTGATGGCAATACC-3′ and 5′-TCCCCATTCACGTCTCCCA-3′; *Gapdh*: 5′-ACGGCCGCATCTTCTTGTGCA-3′ and 5′-AATGGCAGCCCTGGTGACCA-3′; and *Actb*: 5′-GGCTGTATTCCCCTCCATCG-3′ and 5′-CCAGTTGGTAACAATGCCATGT-3′.

Statistical analysis

All data are presented as means ± SEM. Statistical significance was determined by using Student's *t* test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Genetic and pharmacologic PAI-1 inhibition suppresses postoperative adhesion

A new simple postoperative adhesion model was established by depositing gauze tissue on the right flank in the abdominal cavity. Seven days after gauze deposition, the degree of adhesion was scored (Supplemental Fig. 1).

Confirming results by others (19), plasma active PAI-1 levels increased in mice with a developing intra-abdominal postoperative adhesion, peaking at d 1 after surgery as determined by ELISA (**Fig. 1*A***). To further study the consequences of early increases in circulating PAI-1, intra-abdominal adhesion was induced in wild-type mice, *Serpine1*$^{-/-}$ mice, or mice that were treated daily for 0–6 d with PAI-1 inhibitor, TM5275, a novel drug that blocks the binding site between tPA and PAI-1 (15). Decreased active PAI-1 levels were found in mice that were treated with TM5275 (Fig. 1*B*). Macroscopic and microscopic evaluation of the adhesive area after surgery showed impaired intra-abdominal adhesion after 7 d, a lower adhesion score, and a reduced number of histologically detectable fibrotic changes, as well as microscopically

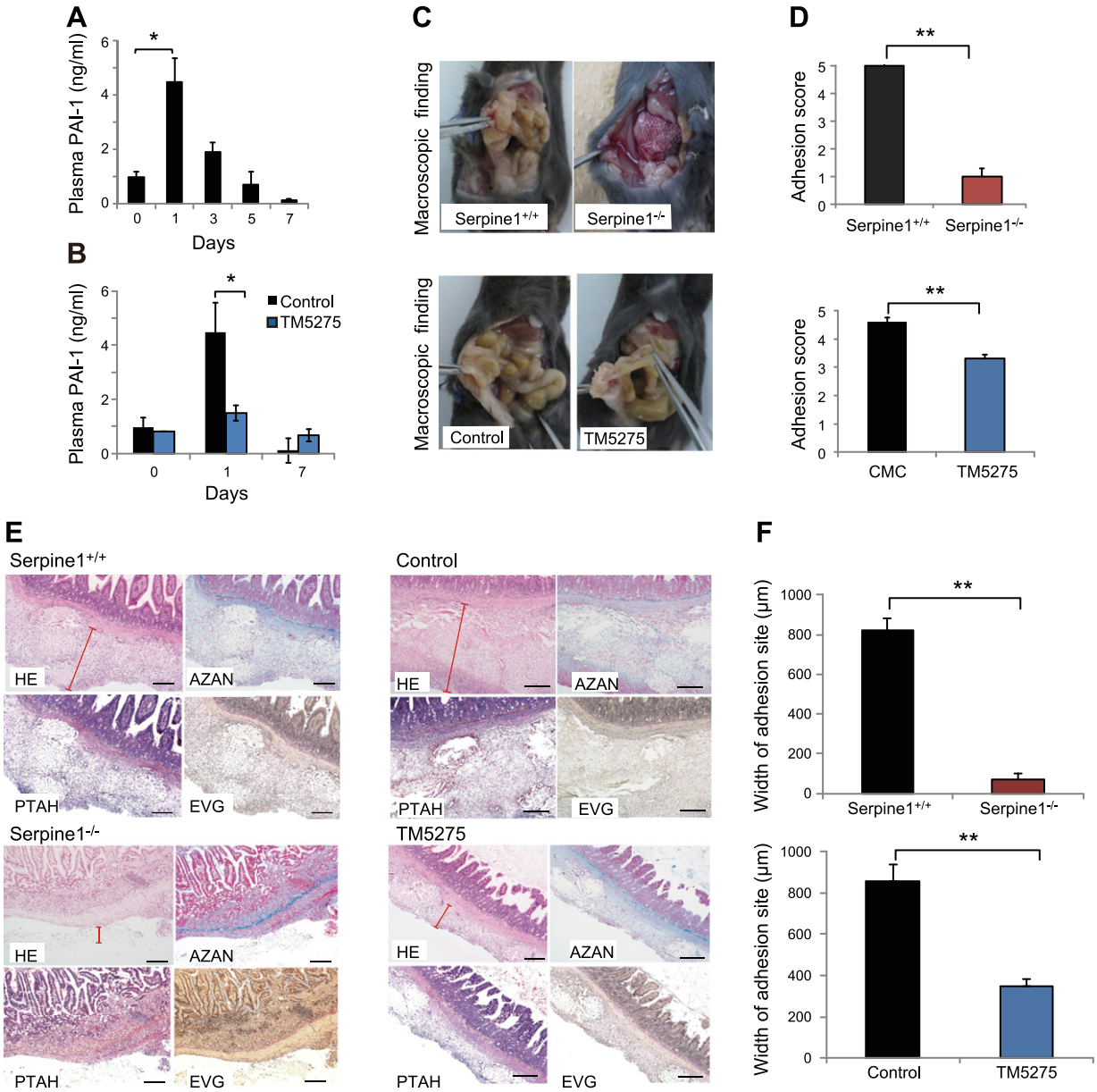

Figure 1. Genetic and pharmacologic PAI-1 inhibition prevents gauze-induced adhesion. Adhesion was induced by surgically placed gauze. *A*, *B*) Plasma derived from C57BL/6 mice was treated without (*A*) or with (*B*) PAI-1 inhibitor, TM5275, and analyzed for active PAI-1 by ELISA ($n = 3$/group). *C*, *D*) Representative macroscopic pictures of adhesive areas are shown (*C*) and adhesion scores were determined on d 7 in *Serpine1*$^{+/+}$ and *Serpine1*$^{-/-}$ mice ($n = 20$/group), and in C57BL/6 mice treated with or without TM5275 ($n = 10$/group; *D*). Evaluation of antiadhesion activity by 3 independent investigators. *E*, *F*) Adhesive tissues retrieved at d 7 after surgery from *Serpine1*$^{-/-}$ or *Serpine1*$^{+/+}$ mice or mice that were treated with or without PAI-1 inhibitor, TM5275. Representative hematoxylin and eosin (HE)–, Elastica van Gieson (EVG)–, phosphotungstic acid hematoxylin (PTAH)–, and azan-stained sections of adhesive tissue are shown. Scale bar, 500 μm. Red arrows indicate thickness of adhesion tissues. Width of adhesive tissues was measured in 5 tissue sections per treatment group $n = 5$/group). CMC, carboxymethylcellulose. Values represent means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, determined by 2-tailed Student's *t* test.

adhesive areas in mice with genetic or pharmacologic PAI-1 inhibition (Fig. 1*C*–*F*). These data indicate that genetic and pharmacologic PAI-1 inhibition ameliorates gauze-induced postoperative adhesion.

TM5275-induced endogenous tPA prevents adhesion

Although increased fibrinolytic activity after PAI-1 inhibition enhances bleeding, TM5275, the PAI-1 inhibitor used in our study, has demonstrated fewer bleeding complications compared with conventional PAI-1 inhibitors (15). Intra-abdominal bleeding as reflected by the appearance of blood ascites after intra-abdominal adhesion was prevented in PAI-1 inhibitor–treated, but not *Serpine1*$^{-/-}$, mice (**Fig. 2*A*, *B***). Genetic and pharmacologic PAI-1 inhibition augmented circulating plasminogen anti-PAP complex, as a measure of plasmin (Fig. 2*C*), and tPA plasma levels 1 d after surgery (Fig. 2*D*).

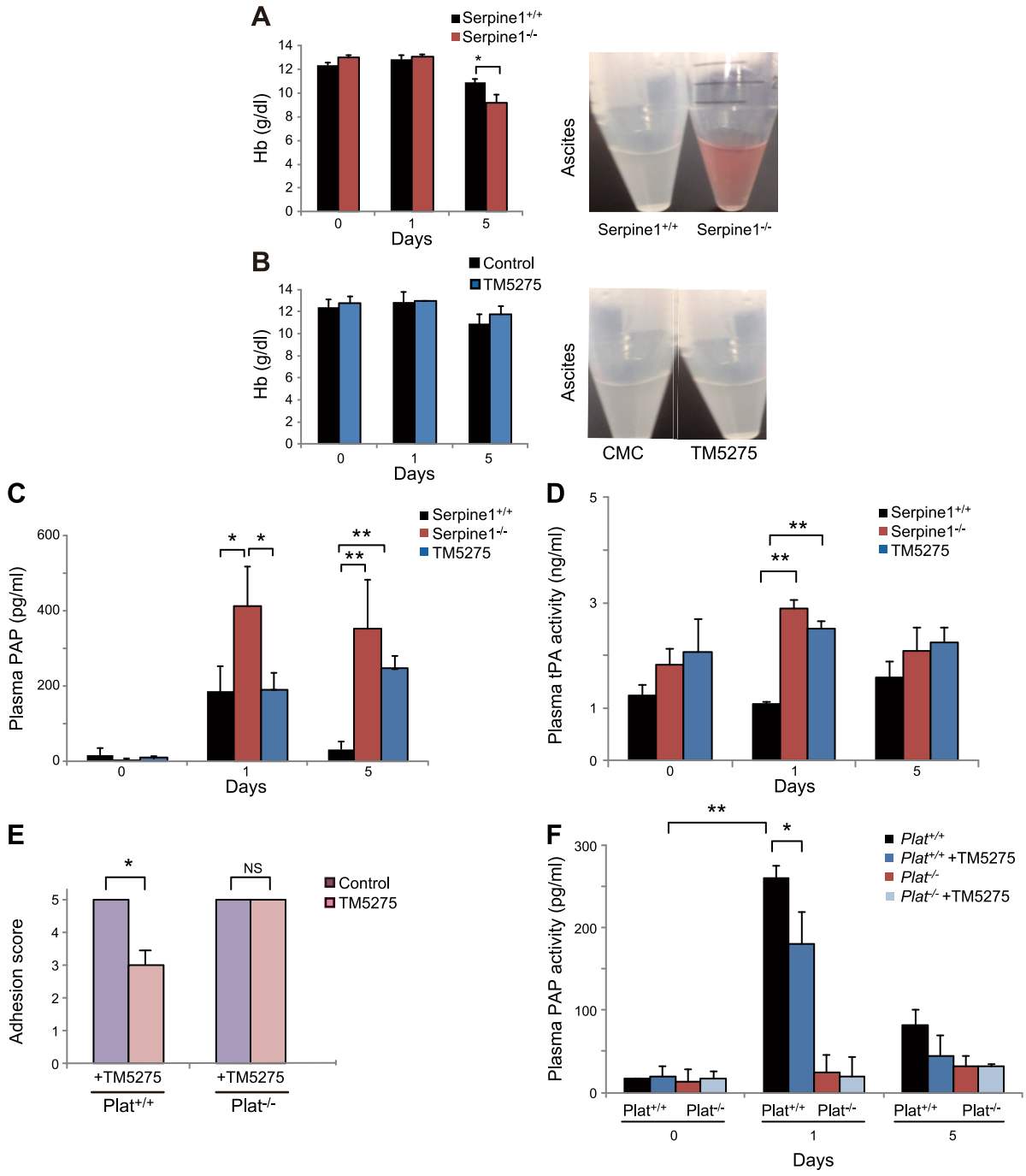

Figure 2. Fibrinolytic factors in pharmacologically and genetic PAI-1–deleted mice during intra-abdominal adhesion. *A*, *B*) Detection of hemoglobin in ascites (bloody ascites) of *Serpine1*$^{+/+}$ *and Serpine1*$^{-/-}$ mice (*A*) and mice treated with or without PAI-1 inhibitor, TM5275 (*B*), was performed 5 d after induction of intra-abdominal adhesion (n = 7–13/group). Macroscopic images of peritoneal lavage fluid (n = 4–10/group; right). *C*, *D*) PAP complex level (*C*) and active tPA (*D*) at d 1 and 5 of treatment of *Serpine1*$^{-/-}$ mice or *Serpine1*$^{+/+}$ mice with PAI-1 inhibitor, TM5275 (n = 3–5/group). *E*) Adhesion score determined 7 d after gauze-induced adhesion formation in *Plat* $^{+/+}$ and *Plat* $^{-/-}$ mice treated with or without TM5275 (n = 5/group). *F*) PAP complex level at d 1 and 5 of treatment of *Plat*$^{-/-}$ mice or *Plat* $^{+/+}$ mice with TM5275 (n = 3/group). CMC, carboxymethylcellulose; NS, not significant. Values represent means ± SEM. *$P < 0.05$, **$P < 0.01$, determined by 2-tailed Student's t test.

To understand whether endogenous tPA was important for TM5275 effects on adhesion prevention, *Plat*$^{-/-}$ mice were treated with TM5275. Whereas adhesion scores were similar for *Plat*$^{-/-}$ and *Plat*$^{+/+}$ mice, a lower adhesion score was achieved in TM5275-treated *Plat*$^{+/+}$, but not *Plat*$^{-/-}$, mice, which indicated that antiadhesive effects of TM5275 required endogenous tPA (Fig. 2*E*). Given that tPA can generate plasmin, higher PAP levels—indicative of enhanced fibrinolysis—were expected in *Plat*$^{+/+}$ mice, especially when treated with TM5275. Surprisingly, PAP levels were lower in TM5275-treated *vs*. -nontreated *Plat*$^{+/+}$

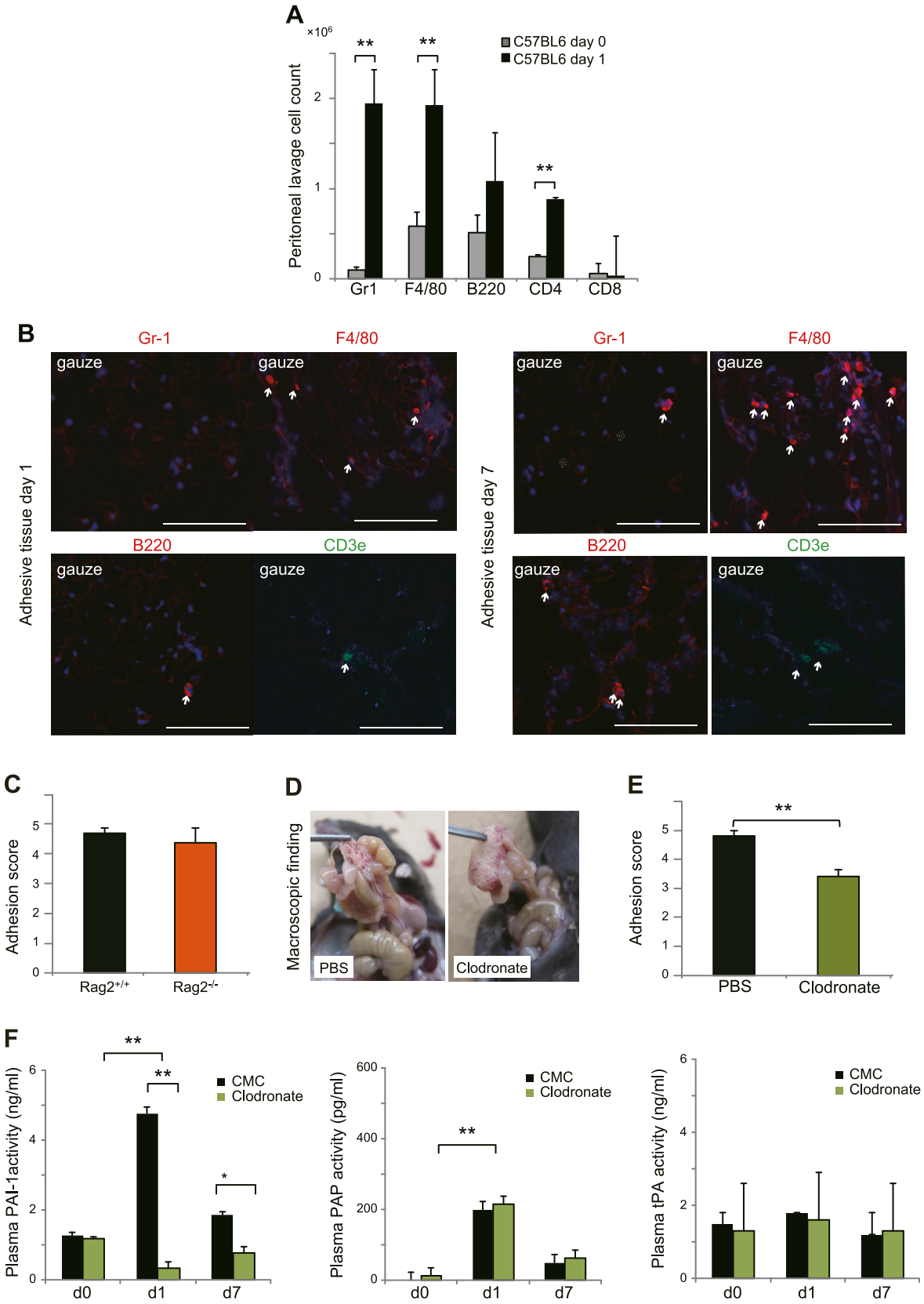

Figure 3. Macrophages are involved in adhesions. *A*) Cell count of Gr1$^+$, F4/80$^+$, B220$^+$, CD4$^+$, and CD8$^+$ cells in spontaneous intra-abdominal fluid/ascites of C57BL/6 mice at d 0 and 1 after surgery as determined by FACS (n = 9/group). *B*) Representative immunofluorescent images of colonic sections retrieved 1 and 7 d after surgery derived from adhesion site after Gr1, F4/80, CD3e, and B220 staining. Arrows indicate positive cells. Scale bars, 50 μm. *C*) Adhesion was induced by gauze in immune-deficient *Rag2* mice. Adhesion score was determined at d 7 (n = 3/group). *D*, *E*) Representative macroscopic pictures of

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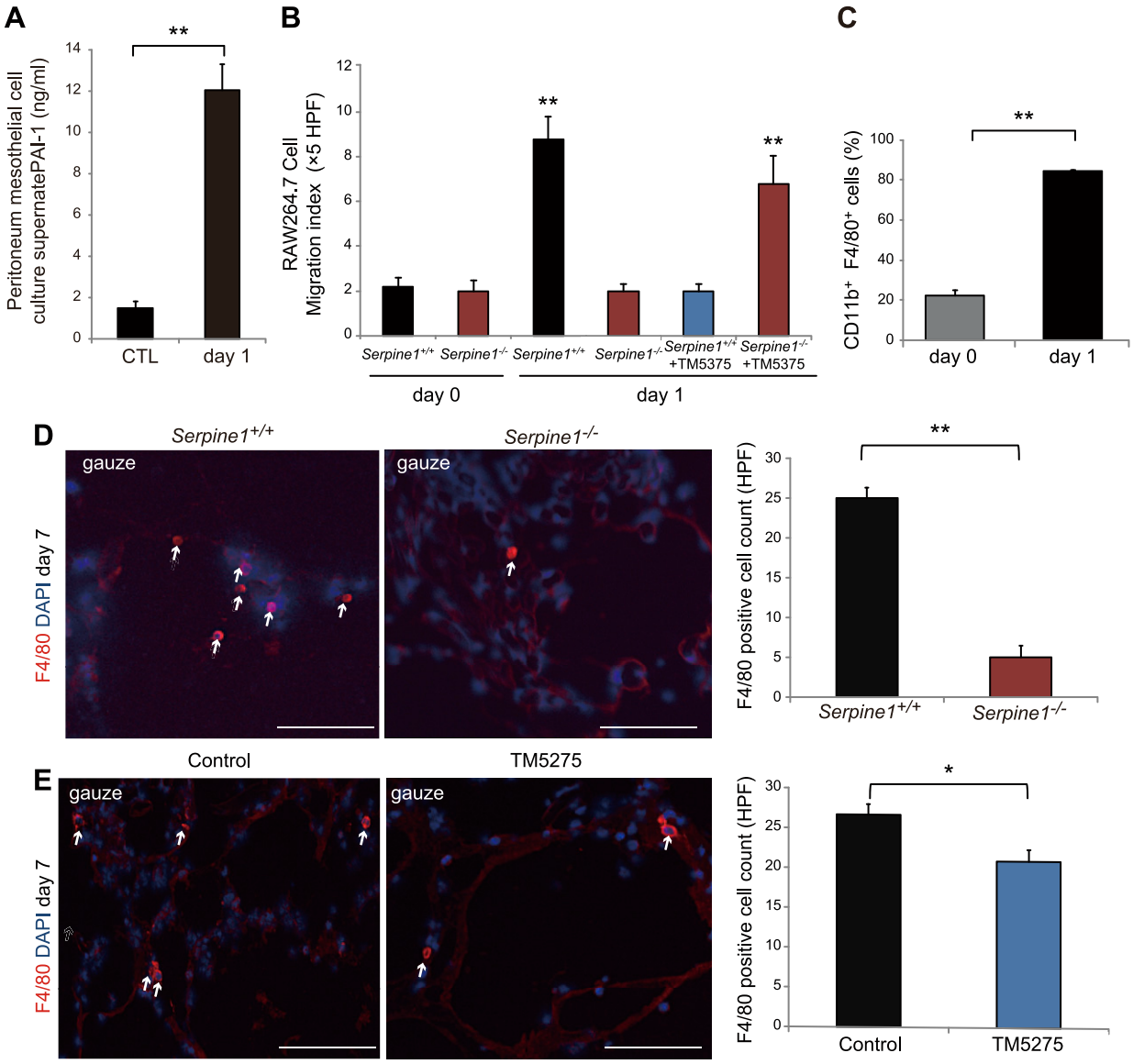

Figure 4. Inhibition of PAI-1 prevented macrophage migration *in vivo*. *A*) Supernatants derived from cultured peritoneal mesothelial cells retrieved from C57BL/6 mice before and 1 d after adhesion induction were analyzed for active PAI-1 by ELISA ($n = 3$/group). *B*) Supernatants of *Serpine1*$^{+/+}$ and *Serpine1*$^{-/-}$ mesothelial cells were used as chemoattractant in a transmigration assay for RAW 264.7 cells. Migrated RAW cells were determined by counting cells in 5 random fields under the microscope. *C*) Percentage of CD11b^{+}F4/80^{+} cells in adhesive lesion at d 0 and 1 after surgery ($n = 9$/group). *D*, *E*) Representative immunohistochemistry images of adhesion tissues showing F4/80 staining 7 d after surgery in *Serpine1*$^{+/+}$ and *Serpine1*$^{-/-}$ mice ($n = 3$/group; *D*) and in C57BL/6 mice treated with or without TM5275 ($n = 6$/group; *E*). Arrows indicate positively stained cells. Scale bars, 50 μm. CTL, control; HPF, high-power field. Values represent means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, determined by 2-tailed Student's *t* test.

mice but were still elevated compared with non-surgical controls (Fig. 2*F*). These data suggest that, although TM5275 enhances fibrinolysis *via* tPA-mediated plasmin generation, antiadhesive effects of TM5275 might involve fibrinolysis-independent functions of tPA.

Macrophage influx into peritoneal adhering tissues

Inflammatory CD11b^{+} myeloid cells contribute to adhesion. There are circulating, free macrophages and other immune cells present in the peritoneal fluid (20),

adhesive areas are shown (*D*) and adhesion scores were determined on d 7 after surgery on C57BL/6 mice treated with the macrophage depleting agent clodronate ($n = 3$/group; *E*). *F*) PAI-1 and PAP as well as active tPA levels were measured by ELISA in the plasma of clodronate-treated or nontreated C57BL/6 mice ($n = 5$/group). CMC, carboxymethylcellulose. Values represent means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, determined by 2-tailed Student's *t* test.

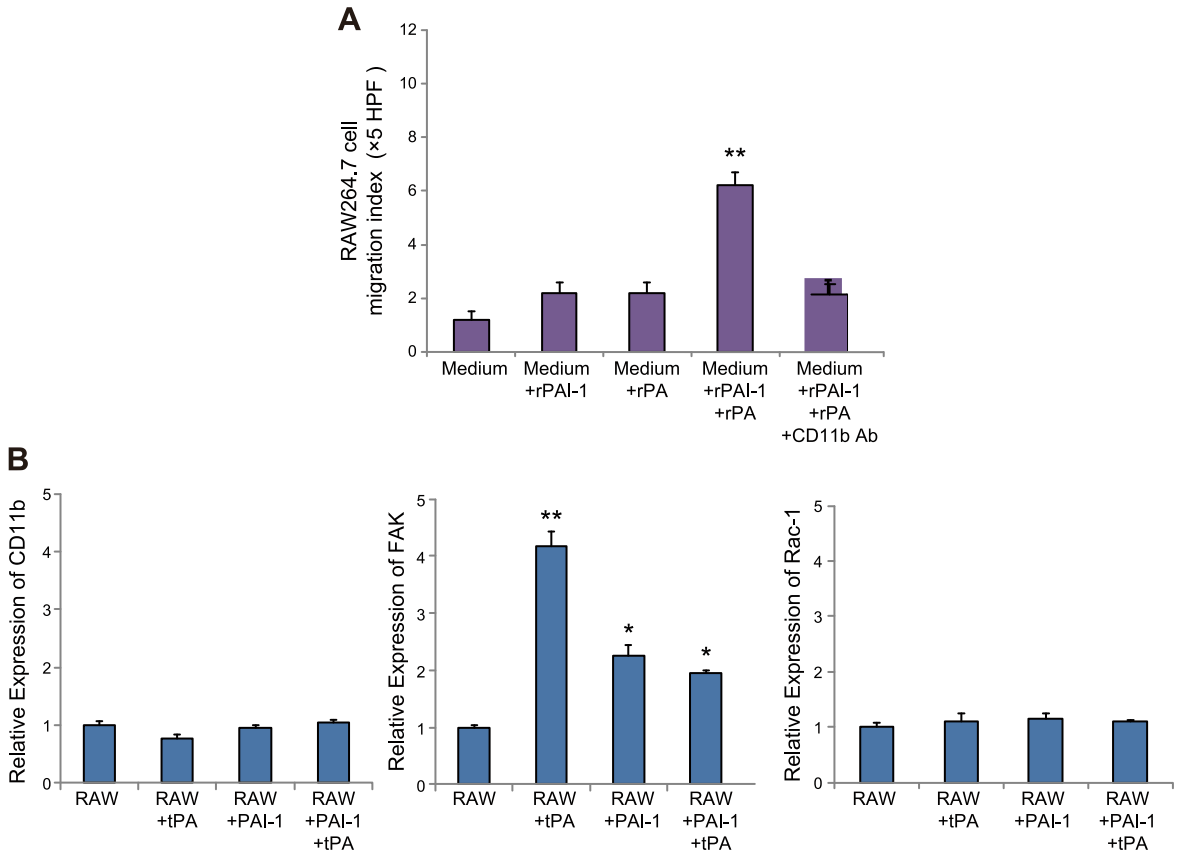

Figure 5. tPA and PAI-1–mediated macrophage migration requires integrin CD11b by enhancing FAK signaling. *A*) RAW 264.7 cell migration toward serum-free medium that was supplemented with or without rPAI-1 and/or rtPA. RAW 264.7 cell migration was also carried out toward serum-free medium that was supplemented with rPAI-1 and tPA in the presence or absence of neutralizing CD11b Abs ($n = 3$/group). Migrated cells were counted in 5 random fields after 6 h under an inverted microscope. *B*) Expression of CD11b (left), FAK (middle), and Rac-1 (right) was determined in RAW 264.7 cells that were treated without or with rtPA and PAI-1 by RT-PCR ($n = 3$/group). HPF, high-power field. Values represent means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, as determined by 2-tailed Student's *t* test.

and we asked whether immune cells are involved in gauze-induced adhesion. Predominantly $Gr1^+$ neutrophils, $F4/80^+$ macrophages, and $CD4^+$ T cells—but not $CD8^+$ T and $B220^+$ B cells—had been recruited into the peritoneal fluid by 24 h after induction of gauze-induced adhesion (**Fig. 3*A***). In accordance with other studies that used different abdominal inducing models, immunohistochemical analysis revealed an increased number of $F4/80^+$ macrophages, $CD3e^+$ T cells, and $B220^+$ B cells, but not $Gr1^+$ neutrophils, in adhesive tissues at d 1 and 7 after surgery (Fig. 3*B*). Our data indicate that among inflammatory cells, although neutrophils were found in similar numbers in the peritoneal lavage of mice after gauze-induced adhesion, macrophages and lymphocytes had predominately been recruited to the adhesive site.

We examined how a lack of lymphocytes would affect adhesion formation by using RAG-$2^{-/-}$ mice, a mouse strain known to lack mature lymphocytes as a result of an inability to initiate V(D)J rearrangement (21). Adhesion formation was unaltered in *Rag2*$^{-/-}$ mice compared with controls, which indicated that, in our model, it is not lymphocytes, but rather $F4/80^+$ macrophages, that exacerbate intra-abdominal adhesion (Fig. 3*C*).

Macrophage depletion prevents intra-abdominal adhesion formation

Studies have shown that fibrinolytic factors like tPA and PAI-1 are involved in $CD11b^+$ cell migration (22). To further study the role of macrophages *in vivo*, C57BL/6 mice treated with clodronate, a drug known to deplete macrophages showed a lower adhesion score than control mice (Fig. 3*D*, *E*) suggesting the importance of macrophages for adhesion in our model. Active PAI-1, but not PAP or tPA plasma levels, was lower in C57BL/6 mice treated with clodronate 1 and 7 d after surgery, as determined by ELISA, indicating that macrophages directly or indirectly contribute to the upregulation of PAI-1 expression *in vivo* (Fig. 3*F*).

PAI-1 enhances macrophage migration during intestinal adhesion

Human omental tissue mesothelial cells produce large amounts of tPA *in vitro*, together with PAI-1 and PAI-2 (23). More PAI-1 was released from mesothelial cells that were isolated from mice with intra-abdominal

adhesion compared with control mice (**Fig. 4*A***). Supernatants from *Serpine1*$^{+/+}$, but not *Serpine1*$^{-/-}$, mesothelial cells that were isolated from mice with intra-abdominal adhesions showed macrophage chemoattractive activity (Fig. 4*B*). This activity was absent in supernatants from steady-state nonadhesive mesothelial cells. Addition of PAI-1 inhibitor, TM5275, blocked the macrophage migration-stimulating potential of *Serpine1*$^{+/+}$-derived mesothelial cell supernatants. Similarly, addition of rPAI-1 enhanced the chemoattractive potential of supernatants, which indicated that mesothelial-derived PAI-1 altered the potential of mesothelial supernatants to stimulate migration.

We next examined whether PAI-1 could enhance macrophage migration. PAI-1 can modulate macrophage migration (16), and plasmin enhances the influx of inflammatory cells (24). By using FACS analysis, CD11b$^+$ macrophages were markedly increased at the adhesive site in C57BL/6 mice 1 d after surgery (Fig. 4*C*). Increased numbers of F4/80$^+$ cells were found at the adhesion site in *Serpine1*$^{+/+}$, but not in *Serpine1*$^{-/-}$, mice which suggested that PAI-1 has a role in recruitment of monocytes and macrophages during adhesion (Fig. 4*D*). Similarly, reduced numbers of F4/80$^+$ cells were found at the adhesion site of TM5275-treated mice (Fig. 4*E*).

CD11b integrin signaling is required for tPA- and PAI-1–mediated macrophage migration

It has been reported that tPA promotes CD11b/Mac-dependent macrophage motility, a process that is fine-tuned by PAI-1 interaction with lipoprotein receptor–related protein, which facilitates macrophage transition to cell retraction (25). Although macrophages did not migrate toward rPAI-1 or rtPA when either protein was added alone to the lower chamber of a transmigration assay, macrophages migrated faster toward wells that contained both rPAI-1 and rtPA in tandem (**Fig. 5*A***). rtPA/rPAI-1–mediated macrophage migration was blocked by using CD11b neutralizing Ab, which demonstrated the importance of integrin CD11b/Mac1 for rtPA/rPAI-1–mediated macrophage migration (Fig. 5*A*).

Integrin signaling *via* FAK is known to modulate cell motility (26). Recent studies have demonstrated that FAK and Rac-1 are indispensable for tPA-induced CD11b$^+$ macrophage migration (26). To identify the signaling cascade of macrophage migration, we examined the expression of CD11b, FAK, and Rac-1. Whereas CD11b and Rac-1 expression did not change in tPA- and/or PAI-1–treated RAW 264.7 cells (Fig. 5*B*), FAK expression was up-regulated in the presence of rtPA or PAI-1, alone or in combination. These data demonstrate that tPA and PAI-1 augment macrophage mobility *via* FAK activation in a CD11b-dependent manner.

Macrophage-derived EGF induces PAI-1 secretion at the adhesion site

To identify macrophage-derived factors that exacerbate the adhesion or fibrosis process and that might exacerbate the PAI-1 increase, macrophages were isolated from postoperative adhesion-generated ascites of C57BL/6 mice and examined for expression of TGF-β, FGF2, and EGF. It has been reported that EGF is produced in the peritoneal cavity by peritoneal macrophages and mesothelial cells (12). TGF-β levels are increased in patients after abdominal surgery (27), but serum TGF-β levels were similar in *Serpine1*$^{+/+}$ or *Serpine1*$^{-/-}$ animals after adhesion induction (data not shown). We observed that immunoreactive EGF, but not FGF2, was found in F4/80$^+$ macrophages in control (**Fig. 6*A***). No immunoreactive EGF or F4/80 was found in mice that were treated with the macrophage-depleting agent, clodronate (Fig. 6*B*), which indicated that macrophages are a source of EGF during adhesion. Immunoreactive EGF$^+$F4/80$^+$ cells were increased at the adhesion site in *Serpine1*$^{+/+}$, but not *Serpine1*$^{-/-}$, mice (Fig. 6*C*).

EGF and its receptors, HER-1 and HER-4, are expressed by peritoneal mesothelial cells and peritoneal macrophages (12). What, then, could be the role of macrophage-derived EGF for PAI-1 induction at the adhesive site? Supernatant of peritoneal mesothelial cells that were cultured in the presence of recombinant EGF showed increased active PAI-1 levels (Fig. 6*D*). Cultured mesothelial cells expressed EGF receptor family HER1–4 at baseline levels as determined by RT-PCR. To understand whether EGF could alter HER expression, we treated mesothelial cells *in vitro* with EGF. EGF treatment significantly up-regulated HER1, but not HER2, 3, or 4, expression as determined by quantitative PCR and immunohistochemistry (Fig. 6*E*). These data indicate that EGF-EGF receptor signaling enhances PAI-1 release from mesothelial cells.

We next determined whether EGF-treated supernatants showed increased macrophage chemotactic activity (Fig. 6*F*). EGF-stimulated mesothelial cells demonstrated macrophage chemotactic activity when rtPA was added and that this activity could be blocked when PAI-1 inhibitor, TM5275, was added. This suggests that the chemotactic activity after EGF stimulation was a result of PAI-1 released into supernatants. These data confirm our initial findings on the requirement of both tPA and PAI-1 for macrophage migration.

It has been reported that inhibition of the HER1-mediated signaling cascade in rat kidney fibroblast suppressed PAI-1 expression (12, 28). To understand the consequences of EGF-HER1 signaling for PAI-1–mediated effects on intra-abdominal adhesion, mice were treated with the HER1 inhibitor, cetuximab. Gauze-induced adhesion was reduced after cetuximab treatment in *Serpine1*$^{+/+}$, but not in *Serpine1*$^{-/-}$, animals (Fig. 6*G*). Our data indicate that HER1 stimulation by EGF *via* up-regulation of PAI-1 mitigates adhesion formation by local shift of balance to an antifibrinolytic condition. Thus, macrophage-derived EGF enhances up-regulation of its receptor, HER1, on peritoneal mesothelial cells, which leads to PAI-1 secretion. In turn, PAI-1, in combination with tPA, increases macrophage recruitment to the adhesive site, with macrophages further fueling the vicious cycle of impaired fibrinolysis at the adhesive site.

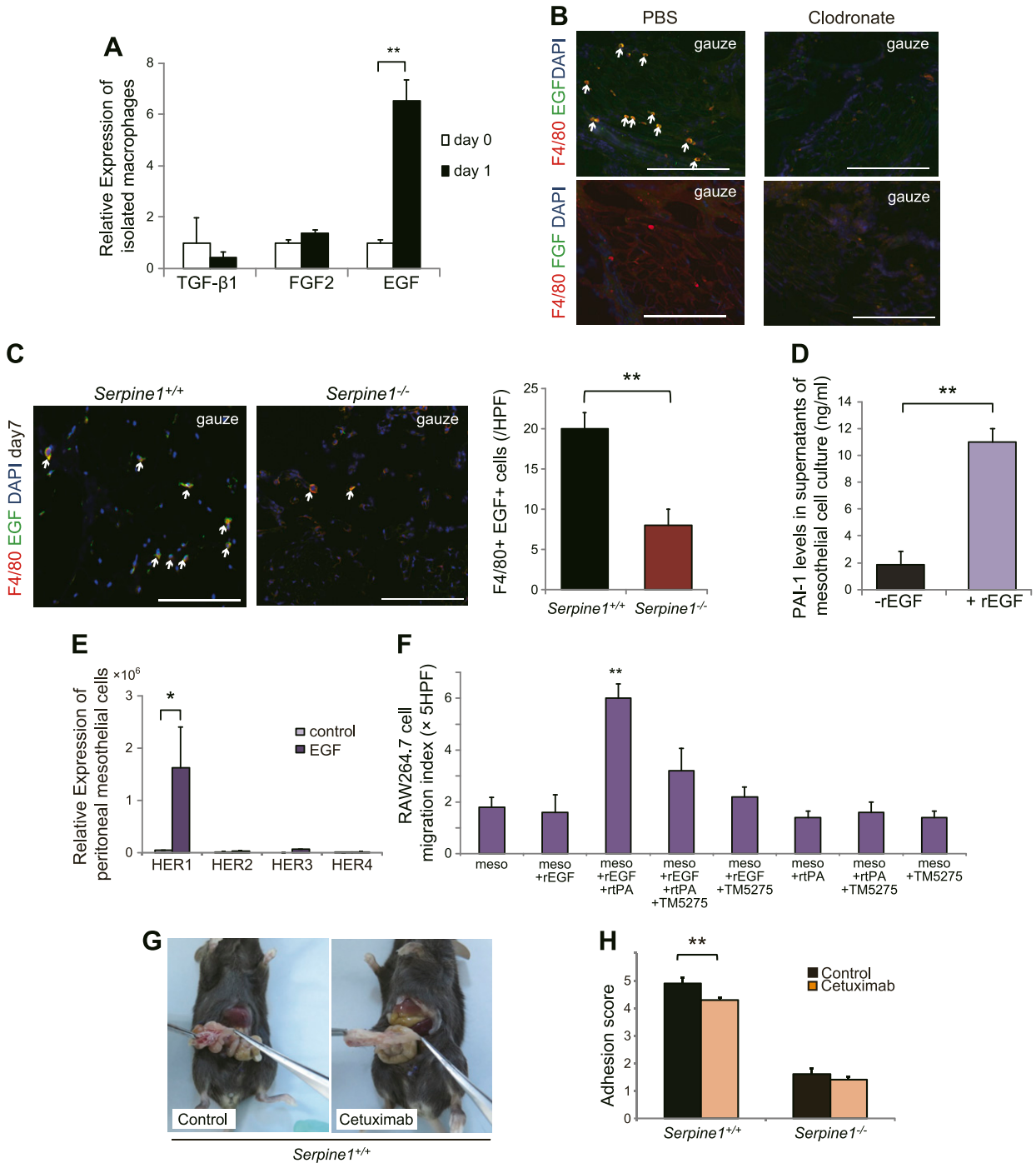

Figure 6. Role for EGF-EGF receptor signaling in the regulation of PAI-1 secretion from mesothelial cells during peritoneal adhesion. *A*) Gene expression of TGF-β1, FGF2, and EGF were measured by quantitative PCR on F4/80$^+$CD11b$^+$ peritoneal cells that were isolated from mice at d 0 and 1 after adhesion induction (normalized to the expression of *Gapdh*; $n = 4$–6/group). *B*) Representative immunohistochemical images of F4/80/EGF- and F4/80/FGF2-stained adhesion areas taken from tissue sections retrieved 7 d after surgery treated with or without clodronate. Arrows indicate double-positive cells. Scale bars, 50 μm. *C*) Representative immunohistochemistry images of adhesion tissues from *Serpine1*$^{+/+}$ and *Serpine1*$^{-/-}$ mice showing F4/80 and EGF staining 7 d after surgery. Arrows indicate positively stained cells. Scale bars, 50 μm (left). Quantification of F4/80$^+$EGF$^+$ cells per high-power field (HPF; right). *D*) Supernatants derived from cultured peritoneal mesothelial cells treated with or without recombinant EGF (rEGF) retrieved from C57BL/6 mice before and 1 d after adhesion induction were analyzed for active PAI-1 by ELISA ($n = 3$/group). *E*) Gene expression of HER1–4 on peritoneal mesothelial cells treated with or without EGF as determined by quantitative PCR (left; $n = 4$–6/group). *F*) Macrophage migration toward EGF-stimulated supernatants that have been supplemented with or without rtPA with or without TM5275 ($n = 3$/group). *G*, *H*) Representative macroscopic images (*G*) and adhesion scores (*H*) were determined in EGF receptor inhibitor (cetuximab) *Serpine1*$^{+/+}$ and *Serpine1*$^{-/-}$ mice at d 7 ($n = 5$/group). Values represent means ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, determined by 2-tailed Student's *t* test.

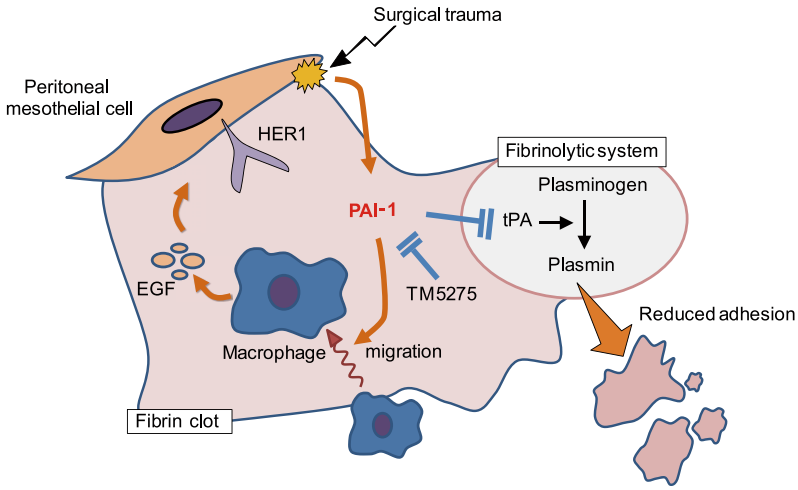

Figure 7. Schematic diagram showing various molecules that are involved in the adhesive effect of PAI-1. After surgery, PAI-1 secreted from, for example, mesothelial cells after surgery suppresses the fibrinolytic activity, thereby stabilizing the fibrin clot. PAI-1 in the presence of tPA promotes migration of EGF-secreting peritoneal macrophage to the adhesion site. EGF, in turn, can induce HER1 expression in peritoneal mesothelial cells, which leads to further PAI-1 release and macrophage recruitment.

DISCUSSION

In this study, we provide genetic and functional evidence that PAI-1 inhibition prevents postoperative adhesion, in part by reducing influx of EGF-secreting macrophages and by interfering with EGF-EGF receptor signaling. Our findings are summarized in **Fig. 7**.

Macrophages are involved in adhesion formation (29), but their role in this process is not well defined. We demonstrate that macrophages make up the dominant cell type in peritoneal exudates at 24 h after surgery and show that macrophage depletion prevented gauze-induced intra-abdominal adhesion. Our data contrast with a study that showed that macrophage depletion in Mafia (Macrophage Fas-induced Apoptosis) mice caused peritoneal adhesion formation when the peritoneal cavity was exposed to an irritant (29). The reason for these divergent results is not clear but might be a result of differences in the way macrophage depletion was achieved as well as the adhesion formation stimulus. Macrophage depletion in Mafia transgenic mice that express a Fas-FKBP construct under the control of the murine *c-fms* promoter was achieved by dimerization of Fas with a synthetic dimerizer, with caused Fas-induced macrophage apoptosis. Clodronate administration caused macrophage depletion after phagocytosis of the drug. In addition, whereas mechanical stress in our model caused adhesion, no surgical mechanical stress was necessary to induce intra-abdominal adhesion in Mafia mice.

Activation of the fibrinolytic system can enhance myeloid $CD11b^+$ cell migration (24, 30). Here, we show that rtPA in synergy with rPAI-1 enhanced transmigration of macrophages, but not when either was added alone. Our data are in accordance with a study by Cao *et al.* (25) that showed that endocytic low density lipoprotein receptor-related protein (LRP), together with tPA and PAI-1, coordinates Mac-1/LRP complex-dependent macrophage migration on fibrin. Previous studies have demonstrated that adhesion is suppressed in fibrin(ogen) knockout mice, which suggests that fibrin formation stimulates macrophage adhesion *in vivo* (31). Similar to our data that show impaired infiltration of macrophages into adhesive sites after genetic and pharmacologic PAI-1 inhibition, *Serpine1*$^{-/-}$ mice with unilateral ureteral obstruction had significantly fewer interstitial macrophages (5).

EGF can bind to EGF receptors, such as HER1–4, and is involved in fibrosis (12). Compared with macrophages that were isolated from nonadhesive lesions, those isolated from adhesive lesions expressed higher levels of the profibrinogenic factor, EGF, but not of FGF2 or TGF-β1. We showed that EGF treatment up-regulated HER1 expression in mesothelial cells and led to release of PAI-1 from mesothelial cells. In addition, drug-induced EGF receptor inhibition prevented i.p. adhesion in *Serpine1*$^{+/+}$, but not in *Serpine1*$^{-/-}$, mice. These data indicate that PAI-1 may be upstream of EGF and EGF receptor signaling. EGF-treated breast adenocarcinoma and glioma cells rapidly up-regulate PAI-1 expression (32, 33). TGF-β–induced PAI-1 expression requires EGF receptor–mediated signaling (34). It has been shown that TGF-β1 failed to induce PAI-1 synthesis in *EGFR*$^{-/-}$ fibroblasts, and that overexpression of EGFR1 in $EGFR^{-/-}$ cells rescued PAI-1 response to TGF-β1 (35).

We show that supernatants from *Serpine1*$^{+/+}$, but not from *Serpine1*$^{-/-}$, mesothelial cells that were isolated from adhesive tissues promoted macrophage migration. Pharmacologic blockade of PAI-1 abolished the macrophage-attracting potential of mesothelial cell supernatants. These data leave open the question as to whether PAI-1–mediated promigratory effects on macrophages are direct or indirect—for example, those resulting from enzymatic alterations caused by changing the thrombotic/fibrinolytic local tissue balance that would result in the activation/deactivation of growth factors/chemokines released from mesothelial cells. Further studies will be necessary to address this question.

Postsurgical adhesion prevention strategies include the use of barriers and improvements in surgical technique. These methods have reduced the incidence of

postsurgical adhesions but have not eliminated them. rtPA diminishes adhesions in animal models without a detrimental effect on wound or anastomotic healing (36) but is not widely used, as bleeding is a major adverse effect. We have shown that pharmacologic inhibition of PAI-1 can prevent adhesion without the fear of bleeding, anastomotic disruption, and wound dehiscence. In conclusion, our data introduce PAI-1 inhibition using TM5275 as a novel treatment option to prevent surgically induced adhesion. FJ

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AUTHOR CONTRIBUTIONS

K. Honjo, S. Munakata, K. Hattori, and B. Heissig designed the study and developed the study concept; K. Honjo and S. Munakata acquired data; K. Honjo and S. Munakata analyzed and interpreted data; S. Munakata, K. Sakamoto, K. Hattori, and B. Heissig drafted the manuscript; S. Munakata critically revised the manuscript; Y. Tashiro, K. Hattori, and B. Heissig obtained funding; A. Ichimura, T. Dan, T. Miyata, and K. Takeda provided material support; and Y. Salama H. Shimzu, S. Eiamboonsert, D. Dhahri, and K. Takeda provided technical support.

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Plasminogen activator inhibitor-1 regulates macrophage-dependent postoperative adhesion by enhancing EGF-HER1 signaling in mice

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Supplementary fig 1.