

Vitronectin-binding PAI-1 protects against the development of cardiac fibrosis through interaction with fibroblasts

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Plasminogen activator inhibitor-1 (PAI-1) promotes or abates fibrotic processes occurring in different organs. Binding of PAI-1 to vitronectin, an extracellular matrix component, may inhibit vitronectin-integrin complex-mediated cellular responses in pathophysiological conditions. To investigate the importance of plasmin suppression vs vitronectin-binding pathways of PAI-1 in cardiac fibrosis, we studied uninephrectomized mice fed a high salt diet and infused with angiotensin II (Ang II) together with different PAI-1 variants, including PAI-1AK (AK) that inhibits plasminogen activators but does not bind vitronectin, PAI-1RR (RR) that binds vitronectin but does not have protease inhibitory effects or control PAI-1 (CPAI), the control mutant that has similar molecular backbone and half-life as AK and RR while retaining all functions of native PAI-1. Compared with RR and CPAI, non-vitronectin-binding AK significantly increased expression of cardiac fibroblast marker, periostin (Ang + AK 8.40 ± 3.55 vs Ang + RR 2.23 ± 0.44 and Ang + CPAI $2.33 \pm 0.12\%$ positive area, both $P < 0.05$) and cardiac fibrosis (Ang + AK $1.79 \pm 0.26\%$ vs Ang + RR $0.91 \pm 0.18\%$ and Ang + CPAI $0.81 \pm 0.12\%$ fibrotic area, both $P < 0.05$), as well as Col1 mRNA (Ang + AK 12.81 ± 1.84 vs Ang + RR 4.04 ± 1.06 and Ang + CPAI 5.23 ± 1.21 fold increase, both $P < 0.05$). To elucidate mechanisms underlying the protective effects of vitronectin-binding PAI-1 against fibrosis, fibroblasts from normal adult human ventricles were stimulated with Ang and different PAI-1 variants. Protease inhibitory AK and CPAI increased supernatant fibronectin, while decreasing plasminogen activator/plasmin activities and matrix metalloproteinase. RR and CPAI variants significantly reduced fibroblast expression of integrin β_3 , vitronectin level in the supernatant and fibroblast adhesion to vitronectin compared with the non-vitronectin-binding AK. Further, RR and CPAI preserved apoptotic, decreased anti-apoptotic and proliferative activities in fibroblasts. Thus, PAI-1 promotes or protects against development of cardiac fibrosis differentially through the protease inhibitory pathway or through its binding to vitronectin.

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Elevated levels of plasminogen activator inhibitor 1 (PAI-1) have been associated with increased cardiovascular risk, progression of glomerulosclerosis and fibrosis in the liver and lung.^{1–4} In cardiac fibrosis, it remains controversial whether PAI-1 is a mediator or inhibitor of cardiac fibrosis.^{5–7} Our previous studies showed that genetic deficiency of PAI-1 or pharmacological inhibition of PAI-1 potentiated angiotensin II (Ang II) or aldosterone-induced fibrosis in the heart.^{8,9} Development of Ang II-associated cardiac fibrosis involves proliferation and infiltration of cardiac fibroblasts,^{10,11} which

are the cells primarily responsible for production and remodeling of the extracellular matrix components.^{12–15}

PAI-1 is the primary *in vivo* inhibitor of plasminogen activators, which convert plasminogen to its active form plasmin. The plasmin/protease system has an important role in matrix degradation, which PAI-1 in turn increases accumulation of extracellular matrix and fibrosis.¹⁶ PAI-1 also binds vitronectin, a matrix protein that interacts with cell-surface integrins. The binding of PAI-1 to vitronectin blocks the vitronectin-integrin $\alpha_V\beta_3$ interaction, and

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regulates variety of cell activities including adhesion, migration, proliferation and survival.^{17–19} Ang II has been shown to upregulate PAI-1 and integrin $\alpha_v\beta_3$ in cardiac fibroblasts.^{20–22} Interestingly, a recombinant PAI-1 protein variant that only has vitronectin-binding activity but does not inhibit protease attenuates glomerular lesions.^{23–25} Thus, we hypothesized that PAI-1 differentially impacts Ang II-induced cardiac fibrosis through its vitronectin-binding and protease inhibitory pathways.

To test this hypothesis, three PAI-1 variants were used: PAI-1AK (AK) that retains all protease inhibitory activity but has no effect on binding vitronectin, PAI-1RR (RR) that binds vitronectin normally but does not inhibit protease and control PAI-1 variant (CPAI) that has all native PAI-1 effects. Different effects between the AK and CPAI variants thus are taken to reflect the lack of vitronectin-binding PAI-1, whereas differences between the RR and CPAI reflect the deficiency of protease inhibition.^{23,26–28} The CPAI, also known as the 14-1B variant, has remarkably increased functional stability compared with the wild-type PAI-1 (wtPAI-1).²⁷ AK, which has further R101A/Q123K mutations on the 14-1B backbone, shows similar functional half-life of ~ 145 h as the CPAI *in vitro*. However, owing to the nature of lacking vitronectin-binding capability, AK is eliminated much faster than CPAI *in vivo*.²⁹ Although RR variant is constructed on the wtPAI-1, its T333R/A335R mutations result in extended half-life both *in vitro* and *in vivo*.^{29,30} Using these PAI-1 protein variants, the present study examined the effects of vitronectin-binding and protease-inhibiting pathways of PAI-1 on cardiac fibrosis in mice with chronic Ang II infusion superimposed on uninephrectomy and high salt intake, a model mimicking human hypertension with reduced nephron number that promotes cardiac fibrosis in both human and rodents. Further studies also explored different effects of PAI-1 variants on cultured cardiac fibroblasts. We revealed that PAI-1 protects against development of cardiac fibrosis through its binding to extracellular vitronectin, which may result in decreased $\alpha_v\beta_3$ integrin effects.

MATERIALS AND METHODS

Animals

Female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME, USA) were housed in AAALAC-certified facilities with access to water *ad lib*. The animal protocols were approved by the Vanderbilt University Institutional Animal Care and Use Committee.

At 12 weeks of age, the mice were uninephrectomized (UNx) under isoflurane anesthesia, and fed a high sodium diet (3.15% Na, Harlan Teklad, Indianapolis, IN, USA). After 2 weeks, the animals were randomized into five experimental groups to receive chronic Ang II infusion and daily intraperitoneal injection of different PAI-1 variants (1 $\mu\text{g/g}$ body weight, Molecular Innovations, Novi, MI, USA) or phosphate-buffered saline (PBS): UNx/salt control + PBS (Cont, $n = 4$), Ang II/UNx/salt + PBS (Ang + PBS, $n = 7$),

Ang II/UNx/salt + RR (Ang + RR, $n = 9$), Ang II/UNx/salt + AK (Ang + AK, $n = 8$) and Ang II/UNx/salt + CPAI (Ang + CPAI, $n = 8$). The Ang II (1 ng/min/g) was delivered by osmotic mini-pump implanted at week 0 and replaced at 4 weeks (Alzet, Cupertino, CA, USA). Systolic blood pressure (SBP) was measured by tail-cuff plethysmography (BP-2000 Blood Pressure Analysis System, Visitech Systems, Apex, NC, USA) in trained conscious mice. Urine albumin-to-creatinine ratio (UACR) was assessed by ELISA (Exocell, Philadelphia, PA, USA) at weeks 0, 2, 4, 6 and 8. At week 8, plasma and hearts were harvested under isoflurane anesthesia. The ratio of the heart weight-to-body weight was determined as an indicator of cardiac hypertrophy. A transverse section at the base of the left ventricle cut through the cardiac valves was used for histological analyses, and the remaining ventricle tissue was isolated for molecular determinations.

In order to test the dosage of the human PAI-1 variants in mice, another 12 normal mice were injected with CPAI, AK or RR (1 $\mu\text{g/g}$ body weight) intraperitoneally every 24 h for 7 days, with 4 mice for each variant. About 100 μl of blood samples were collected through retro-orbital bleeding at 4 to 24 h after the last injection, and plasma isolated for determination of human PAI-1 by ELISA (Molecular Innovations).

Histologic Assessment for Cardiac Fibrosis

Cardiac fibrosis was assessed by Masson trichrome staining on 3- μm paraffin sections and immunohistochemistry for periostin (1:50, Abcam, Cambridge, MA, USA) on 5- μm cryosections.³¹ Images were acquired by Nikon ECLIPSE E400 system. The percentage of positive area was analyzed by AxioVision software (Carl Zeiss, Gottingen, Germany).

Cardiac Fibroblast Culture

Primary fibroblasts from normal adult human ventricle (NHCF-V, Lonza, Allendale, NJ, USA) were cultured with conditioned fibroblast growth medium (FGM-3, Lonza) containing 10% fetal bovine serum (FBS), hFGF-B, insulin and gentamicin/amphotericin-B, and passaged at 1:3 split. Passage 5–6 cells reached 70% confluence were starved in serum-free medium for 24 h, and then incubated in the experiment medium containing 5% FBS with different PAI-1 variants (1.5×10^{-7} mol/l) with or without Ang II (5×10^{-7} mol/l). The PAI-1 variant was supplemented again at 24 h. Supernatants and cells were collected at 48 h for protein assays by ELISA or western blotting. Each of the eight groups, that is, Cont (without Ang II and any PAI-1 variants), RR, AK, CPAI, Ang + PBS, Ang + RR, Ang + AK and Ang + CPAI, was studied in triplicate.

ELISA

Plasma human PAI-1, active plasmin and total mouse PAI-1 in the heart lysate were determined by ELISA kits (Molecular Innovations). The heart tissue was homogenized in RIPA buffer with EDTA and EGTA (Boston BioProducts,

Boston, MA, USA) containing Complete Proteinase Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany), and 1:5 was diluted before loading onto a microtiter plate. The assay was performed following the manufacturer's instruction. The value was corrected by the heart protein concentration.

Fibronectin in the supernatant of cultured cardiac fibroblasts was measured by QuantiMatrix™ ELISA kit (Millipore, Billerica, MA, USA).

Chromogenic Substrate Assay for Plasmin and Plasminogen Activity

Supernatant plasmin activity was measured using a plasmin-specific chromogenic substrate, Chromozym PL (Roche Molecular Biochemicals).²⁴ Briefly, 100 μ l supernatant and 20 μ l of 3 mM Chromozym PL were added per well. Absorbance was measured at 405 nm at baseline and 24 h. A standard linear curve was generated with serial dilutions of human plasmin (Sigma-Aldrich, St Louis, MO, USA) ranging 0–100 $\times 10^{-4}$ unit/ml against the increase in the absorbance. Plasmin activity of each sample was calculated and corrected by the supernatant protein concentration. Plasminogen activator activity was measured by a similar method with additional supplement of 4 μ M plasminogen (Sigma-Aldrich).

Proliferation and Apoptosis of Fibroblasts

Proliferation of cultured fibroblasts was determined by MTS assay with CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA). Briefly, 5 $\times 10^3$ cells in 100 μ l conditioned fibroblast medium containing Ang II with or without PAI-1 variants were dispensed into the well and pretreated for 48 h. After adding 20 μ l of CellTiter 96 Aqueous One Solution Reagent to each well, the plate was incubated at 37 °C for 4 h in a humidified, 5% CO₂ atmosphere. Absorbance at 490 nm was recorded.

Apoptosis of fibroblasts was detected by TUNEL method (Millipore). Briefly, 2 $\times 10^4$ cells were added to each well of four-well culture slides and treated with Ang II and one of the PAI-1 variants as described above. After fixation in 1% paraformaldehyde in PBS, the slides were incubated with working strength TdT enzyme at 37 °C for 1 h, and then stop buffer was added. For each well, 10 images were acquired at $\times 40$ magnification, and the fraction of positive cells was evaluated.

Migration Assay

The relative migratory ability of fibroblasts was determined by wound-healing assay. Cells were plated onto wells of six-well plates precoated with collagen type I, and allowed to reach full confluence before a scratch was gently created with a pipet tip. The area devoid of cells was photographed at the time the scratch was created and at 9 h. The relative distance of fibroblasts migrated into the denuded area was measured. Migration experiments were performed in triplicate wells.

Cell Adhesion to Vitronectin

To prepare the culture plates for adhesion assay, 5 μ g/ml of human vitronectin (BD Biosciences, Franklin Lakes, NJ, USA) was added to 96-well plate, incubated at 4 °C overnight and the remnant was aspirated. Nonspecific binding to the plate was blocked by incubation with 1% bovine serum albumin at 37 °C for 1 h. After three washes with medium containing 5% FBS, 3 $\times 10^4$ cells were added to each well in the presence of Ang II and PAI-1 variants. Plates were then incubated at 37 °C for 60 min. Non-adherent cells were washed off with PBS. The attached cells were fixated with 4% paraformaldehyde/PBS for 5 min, stained with 0.5% toluidine blue in 4% paraformaldehyde for 5 min, rinsed with water and solubilized with 100 μ l of 1% sodium dodecyl sulfate (SDS). Optical density was read at a wavelength of 595 nm.³²

Immunoblot Analysis

Frozen heart tissues and cell samples were homogenized in RIPA buffer with EDTA and EGTA containing proteinase inhibitor. The total protein concentration in each sample was determined by BCA Protein Assay Kit (Thermo Scientific, Pittsburgh, PA, USA). Equal amounts of total protein from each sample (60 μ g for heart tissues, 20 μ g for cell lysate of cultured fibroblasts and 50 μ g for supernatant samples) were separated on 10% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes. Membranes were subsequently blocked with 5% powdered non-fat milk in Tris-buffered saline containing 0.1% TWEEN 20 (TBS-T) and incubated with primary antibody at 4 °C overnight. Primary antibodies used in the present studies included anti-vitronectin (1:400, Santa Cruz), anti-Bcl-xL (1:1000, Cell Signaling Technology, Danvers, MA, USA), anti-Bax (1:1000, Cell Signaling Technology), anti-integrin $\beta 3$ (1:1000, Abcam), anti-integrin α_V (1:400, Santa Cruz) and anti-MMP-9 (1:800, Millipore). After washing in TBS-T, horseradish peroxidase-labeled IgG secondary antibodies (1:2500 in 5% non-fat milk/TBS-T) was added and incubated at room temperature for 1 h. Protein bands on western blots were visualized by ECL Plus (Amersham, Arlington Heights, IL, USA) then developed on film. The amount of loading was detected by using anti-tubulin (1:5000, Abcam) for tissue or cell lysate, or Ponceau S stain for culture supernatant samples. The abundance of each protein expression shown as a specific band was analyzed by ImageJ software, normalized by the loading control and expressed by fold changes relative to the Cont group.

Reverse Transcription and Quantitative Polymerase Chain Reaction

Snap-frozen heart tissue from the ventricles was homogenized in RLT buffer (Qiagen, Germany) containing 2-mercaptoethanol. The total RNA was extracted by RNeasy Mini Kit (Qiagen), then 2 μ g of total RNA with A₂₆₀/A₂₈₀ ranging 1.8–2.3 was reverse transcribed by TaqMan Reverse

Transcription Kit (Applied Biosystems, Branchburg, NJ, USA) according to the manufacturer's protocol. PCR for Col1 was performed on CFX-96 real-time PCR detection system by using SYBR green master mix (Bio-Rad). The 25 μ l PCR reaction mixture contained 0.5 μ l of cDNA and 1.25 μ mol of each PCR primer (forward: 5'-GGCAGGC GGGAGGTCTT-3'; reverse 5'-ACGGCTGCACGAGTCAC AC-3').³³ The initial denaturation was carried out at 95 °C for 3 min, which was followed by amplification with 40 cycles of 95 °C for 20 s, 60 °C for 20 s and 72 °C for 40 s. Cycle threshold (Ct) values were normalized to 18s rRNA measured on the same plate, and fold differences in gene expression were determined using the $2^{-\Delta\Delta C_t}$ method.³⁴

Statistical Analysis

All results are presented as mean \pm s.e.m. Repeated-measures ANOVA and *post hoc* Tukey's test were used to examine the differences among groups and among the different time points. Nonparametric tests were used when data were not normally distributed. A *P*-value <0.05 for two-tailed tests was considered to be significant.

RESULTS

Plasma Level of Human PAI-1 after Injection with PAI-1 Variant

In mice, normal plasma level of mouse PAI-1 is <25–80 pg/ml,³⁰ and rose to >1 ng/ml in some pathophysiologic conditions.³⁵ In our study, CPAI, AK or RR was given intraperitoneally at a daily dose of 1 μ g/g body weight. After 1 week, the mean plasma level of human PAI-1 at 4 h after each injection was 3.2 ng/ml for CPAI, 0.6 ng/ml for AK and 1.2 ng/ml for RR. Those levels were decreased and stabilized after 10 h of injection, ranging 0.15–0.53 ng/ml. There was no significant difference in the plasma human PAI-1 level among CPAI, AK and RR within 10–24 h after injection. The human PAI-1 was not detectable in mice without injection of human PAI-1 variants.

Effects of PAI-1 Variants on SBP, Proteinuria and Cardiac Fibrosis

The body weight of mice did not change significantly throughout the study, and was not significantly different among the groups. Retrospective calculation for the actual amount of Ang II received, based on the body weight and remaining Ang II in the pump, was also similar among the groups, ranging 0.99–1.08 ng/min/g. SBP and heart weight were significantly increased by Ang II infusion in mice with UNx/salt. The various PAI-1 variants did not impact the hypertension or cardiac hypertrophy (Table 1). UACR was significantly increased by Ang II infusion. Compared with the vitronectin-binding PAI-1 variants, RR and CPAI, the non-vitronectin-binding PAI-1 variant, AK, significantly increased UACR (Table 1). Compared with the UNx/salt control mice, Ang II significantly increased cardiac fibrosis, which was further exacerbated by the non-vitronectin-binding PAI-1

variant, AK (Figure 1a). These changes paralleled the results of the periostin staining for fibroblast (Figure 1a) and the mRNA level of collagen type I (Col1) in the ventricles (Figure 1b). Both vitronectin-binding PAI-1 variants, that is, RR without protease inhibition and CPAI with protease inhibition, significantly attenuated the upregulation of periostin and Col1, and reduced cardiac fibrosis compared with the AK.

Changes in Cardiac PAI-1, Plasmin and Vitronectin

ELISA showed that the non-vitronectin-binding PAI-1 variant, AK, significantly increased total mouse PAI-1 protein in the heart compared with its protease inhibitory control variant, CPAI, which also binds vitronectin. As expected, mice injected with protease inhibitory AK and CPAI, numerically lowered active plasmin in the heart compared with the mice treated with non-protease inhibitory RR and the UNx/Salt Cont. Although immunoblotting showed that Ang II significantly increased cardiac vitronectin, this change was numerically alleviated by the vitronectin-binding PAI-1 variants RR and CPAI (Table 2).

Effects of PAI-1 Variants on Plasminogen Activator Activity, Plasmin Activity and Fibronectin in the Supernatant of Cultured Cardiac Fibroblasts

Without Ang II, the RR variant significantly increased plasminogen activator activity in the supernatant, and RR and CPAI increased plasmin activity compared with the Cont. The plasminogen activator activity was significantly lower in the supernatant of AK- and CPAI-treated fibroblasts than that of RR (Figure 2a). With Ang II, AK and CPAI significantly reduced plasmin generation compared with RR. The supernatant level of MMP-9 was not significantly different in non-Ang II-exposed cells, but significantly increased by vitronectin-binding variants, RR and CPAI in Ang II-exposed fibroblasts compared with those of Cont, Ang + PBS and Ang + AK (Figure 2b).

In non-Ang II-exposed fibroblasts, PAI-1 variants did not significantly change the level of fibronectin in the supernatant. Although Ang II did not significantly increase fibronectin production in the primary cardiac fibroblasts indicating a phenotype of fibroblasts rather than myofibroblasts, addition of AK or CPAI significantly increased supernatant fibronectin (Figure 2c). These results also indicate that the differential effects of PAI-1 variants on fibroblasts are at least partially dependent of Ang II.

Effects of PAI-1 Variants on Vitronectin, $\alpha_v\beta_3$ Integrin in Ang II-Exposed Cardiac Fibroblasts and Adhesion of Fibroblasts to Vitronectin

Immunoblotting showed that, compared with AK, RR treatment significantly decreased vitronectin level in the supernatant of Ang II-exposed fibroblasts, indicating reduced vitronectin availability for interaction with $\alpha_v\beta_3$ integrin. Although immunoblotting did not show any difference in

Table 1 Effects of different PAI-1 variants on blood pressure, albuminuria and cardiac hypertrophy in mice with Ang II/UNx/salt

	Cont	Ang + PBS	Ang + RR	Ang + AK	Ang II + CPAI
N	4	9	9	10	9
<i>Systolic blood pressure (mm Hg)</i>					
Week 0	112 ± 5	109 ± 4	113 ± 4	111 ± 3	108 ± 2
Week 2	109 ± 5	128 ± 6	150 ± 5 ^a	135 ± 7	141 ± 5 ^a
Week 4	102 ± 4	157 ± 7 ^a	149 ± 8 ^a	166 ± 8 ^a	162 ± 10 ^a
Week 6	117 ± 3	144 ± 11	153 ± 5 ^a	163 ± 8 ^a	138 ± 7
Week 8	112 ± 6	163 ± 12 ^a	164 ± 6 ^a	161 ± 7 ^a	144 ± 4 ^a
<i>Urine albumin-to-creatinine ratio (µg/mg)</i>					
Week 0	22.0 ± 7.5	14.7 ± 4.2	9.2 ± 1.8	13.5 ± 4.7	38.2 ± 30.5
Week 2	6.1 ± 1.2	1060.6 ± 5.9 ^a	522.8 ± 270.4 ^a	1265.6 ± 358.9 ^{a,b,c}	354.3 ± 118.1 ^a
Week 4	33.9 ± 12.0	1117.1 ± 475.9 ^a	961.0 ± 334.8 ^a	1618.3 ± 347.0 ^{a,b}	496.5 ± 186.4 ^a
Week 6	28.1 ± 12.9	1528.8 ± 1156.4 ^a	1050.5 ± 390.9 ^a	2048.0 ± 573.7 ^a	982.4 ± 305.7 ^a
Week 8	29.5 ± 22.6	1595.3 ± 752.5 ^a	1165.8 ± 324.1 ^a	2712.6 ± 573.6 ^{a,c}	1365.4 ± 388.9 ^a
<i>Heart-to-body weight (mg/g)</i>					
Week 8	5.5 ± 0.2	9.8 ± 1.1 ^a	8.8 ± 0.4 ^a	8.4 ± 0.5 ^a	9.2 ± 1.0 ^a
<i>Heart rate (beat/min)</i>					
Week 8	685 ± 15	637 ± 19	656 ± 14	639 ± 14	671 ± 9

Abbreviations: Ang, angiotensin II; AK, PAI-1AK variant; Cont, UNx/salt control; CPAI, control PAI-1 variant; RR, PAI-1RR variant; UNx, uninephrectomy. Data are shown as mean ± s.e.m.

^avs Cont.

^bvs Ang + CPAI.

^cvs Ang + RR, $P < 0.05$ compared at the same time point.

protein expression of the α_V integrin (Cont 0.36 ± 0.09 , Ang + PBS 0.37 ± 0.04 , Ang + RR 0.27 ± 0.12 , Ang + AK 0.26 ± 0.08 and Ang + CPAI 0.25 ± 0.01), the β_3 integrin was significantly higher in the AK-treated than in the RR-treated fibroblasts exposed to Ang II, indicating that binding of PAI-1 to vitronectin downregulates cellular expression of β_3 integrin. The adhesion of Ang II-exposed fibroblasts to vitronectin was significantly inhibited by vitronectin-binding RR and CPAI but not by the AK variant, which only has protease inhibitory effects (Figure 3). These results suggested decreased formation of vitronectin- $\alpha_V\beta_3$ integrin complex by the vitronectin-binding PAI-1.

Effects of PAI-1 Variants on Apoptosis and Proliferation of Ang II-Exposed Cardiac Fibroblasts

The number of TUNEL-positive apoptotic fibroblasts was numerically lower in Ang II-exposed fibroblasts than Cont. Compared with Cont and Ang + PBS, addition of RR and CPAI did not significantly affect the number of apoptotic cells. However, the number of apoptotic fibroblasts was significantly reduced by non-vitronectin-binding AK compared

with that of Ang + PBS and Ang + RR. Expression of pro-apoptotic Bax was not significantly different among the Cont and Ang II groups; however, expression of anti-apoptotic Bcl-xL was significantly decreased by the vitronectin-binding RR and CPAI compared with the Ang + PBS, but not by AK (Figure 4). These results indicate that binding of PAI-1 to vitronectin protects apoptotic activity of fibroblasts in response to Ang II.

Although Ang II did not increase proliferation of cultured fibroblasts, vitronectin-binding RR and CPAI significantly decreased the number of viable fibroblasts that were exposed to Ang II (Figure 5).

Effects of PAI-1 Variants on Migration of Ang II-Exposed Cardiac Fibroblasts

Compared with fibroblasts incubated with Ang II alone, protease inhibitory AK and CPAI significantly increased fibroblast migration determined by wound-healing assay. RR-treated fibroblasts migrated into the scratched region significantly more slowly than fibroblasts incubated with Ang II alone, or treated with Ang II with AK or CPAI (Figure 6).

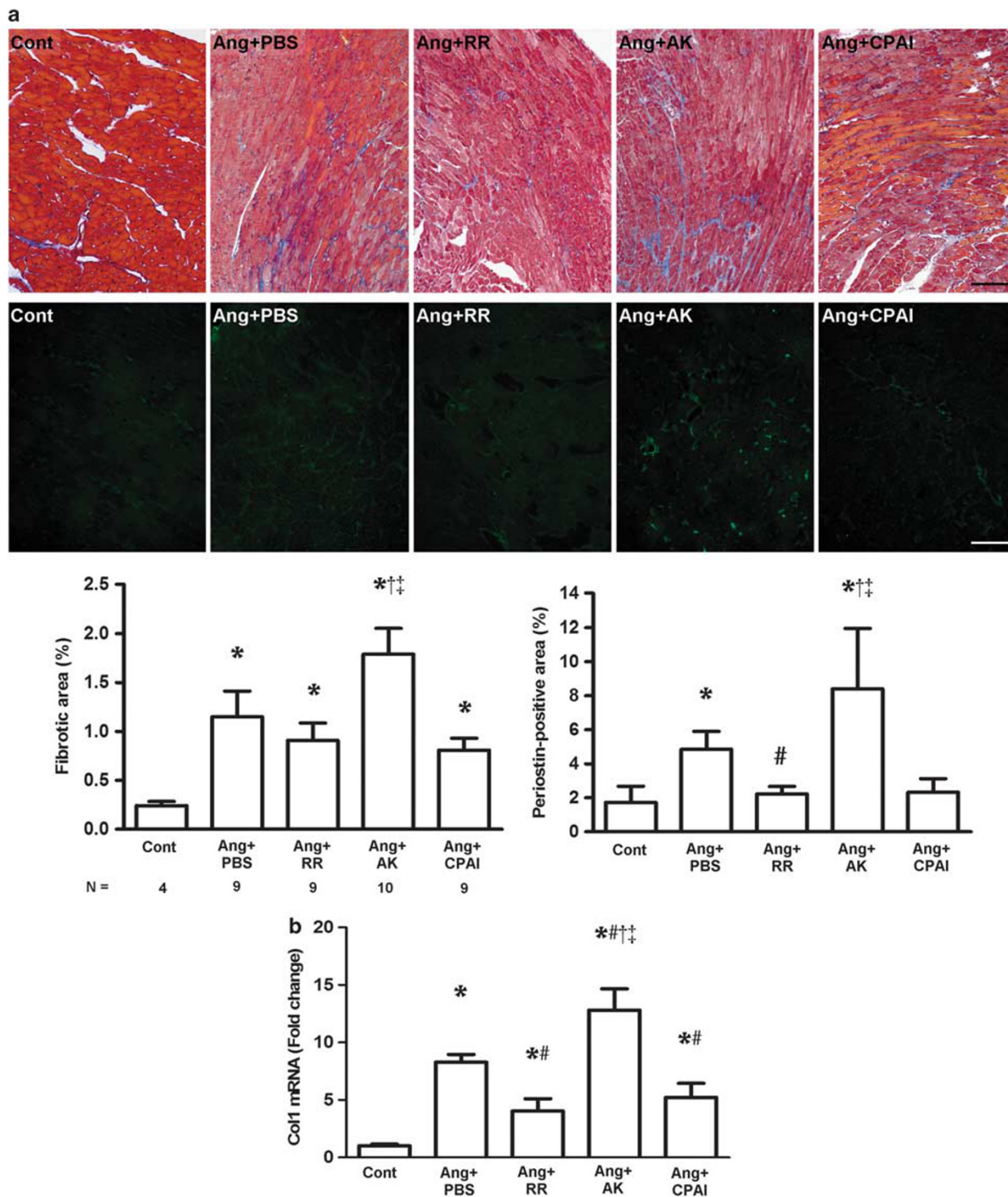


Figure 1 Cardiac fibrosis in Ang II/UNx/salt mice treated with PAI-1 variants. **(a)** Masson trichrome stain (upper panel) showed that, compared with control mice with uninephrectomy and high salt diet (Cont), superimposing chronic infusion of Ang II and PBS (Ang + PBS) increased cardiac fibrosis (shown in blue). The Ang II/UNx/salt mice injected with AK (Ang + AK), the PAI-1 variant without binding capacity to vitronectin, had significantly more cardiac fibrosis than mice injected with RR (Ang + RR), the PAI-1 variant with vitronectin-binding ability but without protease inhibition or CPAI (Ang + CPAI), the control PAI-1 variant with both vitronectin-binding and protease inhibitory capacities. Immunofluorescent staining for periostin, a cardiac fibroblast marker (middle panel, shown in green), showed a significant increase in fibroblasts by Ang II compared with Cont, which was exacerbated by AK and attenuated by RR. **(b)** The histological changes shown in A were also reflected by the mRNA level of collagen type I (Col1) in the ventricles, which was significantly increased in all Ang II-infused mice and further surged in the Ang + AK group. The RR and CPAI variants attenuated this upregulation. *vs Cont, # vs Ang + PBS, † vs Ang + CPAI, ‡ vs Ang + RR, $P < 0.05$. Scale bar = 50 μ m.

Table 2 Cardiac PAI-1, plasmin and vitronectin in mice with Ang II/UNx/salt and PAI-1 variants

	Cont	Ang + PBS	Ang + RR	Ang + AK	Ang + CPAI
N	4	9	9	10	9
PAI-1 (ng/mg)	0.73 ± 0.06	1.10 ± 0.18	1.17 ± 0.19 ^a	1.40 ± 0.23 ^{a,b}	0.93 ± 0.09
Active plasmin (ng/mg)	6.19 ± 3.41	3.60 ± 0.88	4.84 ± 0.70	3.31 ± 0.38	3.68 ± 0.83
Vitronectin (a.u.)	0.21 ± 0.02	0.31 ± 0.01 ^a	0.24 ± 0.03	0.27 ± 0.05	0.22 ± 0.07

Abbreviations: Ang, angiotensin II; AK, PAI-1AK variant; a.u., arbitrary unit by western blotting; Cont, UNx/salt control; CPAI, control PAI-1 variant; RR, PAI-1RR variant; UNx, uninephrectomy.

Data are shown as mean ± s.e.m.

^avs Cont.

^bvs Ang + CPAI, *P* < 0.05.

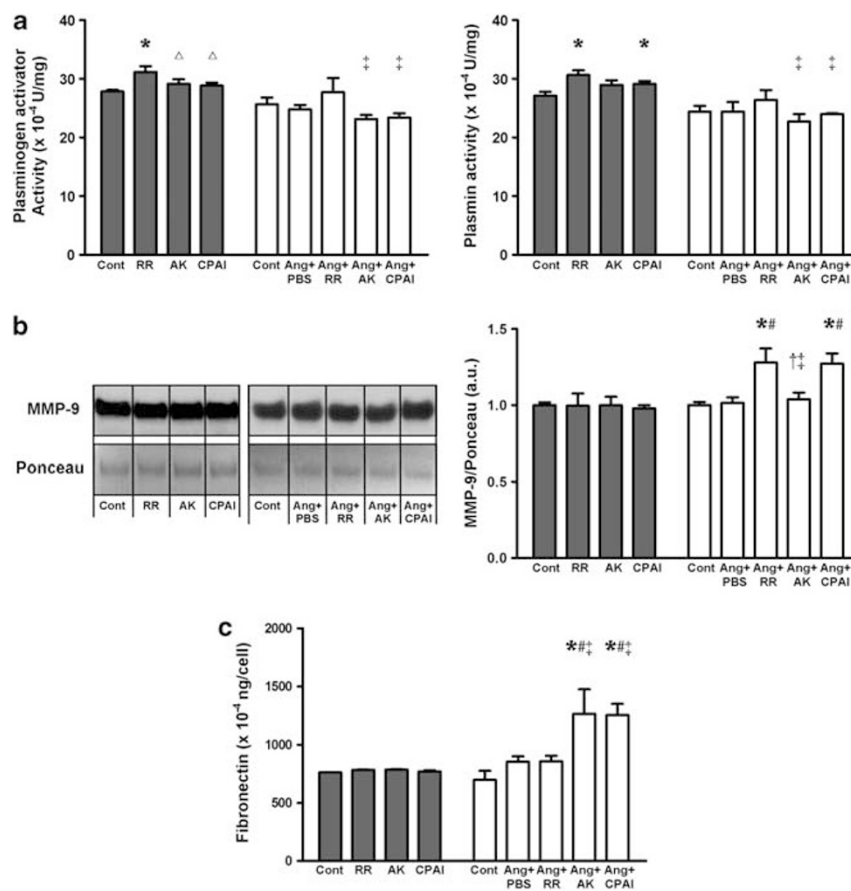


Figure 2 Effects of PAI-1 variants on plasmin and fibronectin in the supernatant of primary cardiac fibroblasts. **(a)** Chromogenic substrate assay: compared with the control fibroblasts (Cont), non-protease inhibitory but vitronectin-binding PAI-1 variant, RR, significantly increased plasminogen activator activity, which was also significantly higher than that of AK or CPAI. RR and CPAI variant also increased plasmin activity in the supernatant. Addition of AK or CPAI to Ang II-exposed cardiac fibroblasts (Ang + AK, or Ang + CPAI) significantly decreased plasminogen activator activity and plasmin activity compared with RR (Ang + RR). **(b)** Western blot: the supernatant level of MMP-9 was not significantly changed by different PAI-1 variant at baseline, but significantly increased by vitronectin-binding RR and CPAI in Ang II-exposed fibroblasts. **(c)** ELISA: different PAI-1 variant did not change supernatant fibronectin level at baseline. Ang II numerically increased supernatant fibronectin, which was significantly increased by AK or CPAI in Ang II-exposed fibroblasts. These results suggest that protease inhibition by PAI-1 has a significant role in extracellular matrix accumulation, which may be protected by vitronectin-bound PAI-1. * vs Cont, ^Δ vs RR, [#] vs Ang + PBS, [†] vs Ang + CPAI, [‡] vs Ang + RR, *P* < 0.05. *N* = 3 for each group.

DISCUSSION

Our *in vivo* results show that cardiac fibrosis caused by chronic infusion of Ang II was exacerbated by a protease

inhibitory but non-vitronectin-binding PAI-1 variant, the AK variant. However, development of cardiac fibrosis induced by Ang II was attenuated by vitronectin-binding PAI variants,

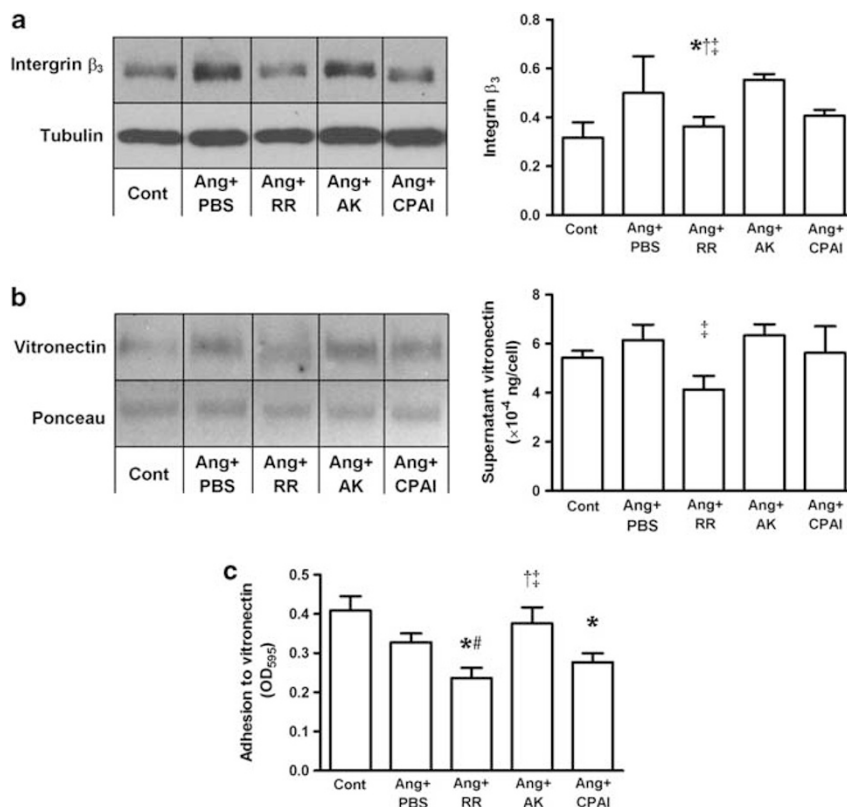


Figure 3 Changes in integrin β_3 expression, supernatant vitronectin and adhesion of cardiac fibroblasts to vitronectin. In cardiac fibroblasts exposed to Ang II, non-vitronectin-binding AK (Ang + AK) significantly upregulated cellular expression of integrin β_3 (western blot, **a**) compared with Ang II alone (Ang + PBS) and the vitronectin-binding PAI-1 variants, RR (Ang + RR) and CPAI (Ang + CPAI). This AK-caused increase in integrin β_3 was accompanied by higher level of supernatant vitronectin than with RR treatment (western blot, **b**) and greater adhesion to vitronectin than those in response to RR and CPAI (**c**). These results suggest a decreased vitronectin- $\alpha_V\beta_3$ interaction by vitronectin-bound PAI-1. * vs Cont, \ddagger vs Ang + PBS, \dagger vs Ang + CPAI, \ddagger vs Ang + RR, $P < 0.05$. $N = 3$ for each group.

RR and CPAI, no matter whether it inhibits activation of plasmin (CPAI) or not (RR). *In vitro* studies on Ang II-exposed cardiac fibroblasts revealed that protease-inhibiting PAI-1 variants, AK and CPAI, increased supernatant fibronectin. On the other hand, fibroblasts treated with vitronectin-binding PAI-1 variants, RR and CPAI, had higher expression level of MMP-9, as well as greater apoptotic and fewer proliferative activities than the cells treated with non-vitronectin-binding AK variant. Further, expression of integrin β_3 and cellular adhesion to vitronectin were significantly lower in RR-treated fibroblasts compared with the AK, indicating that the protective effects of vitronectin-binding PAI-1 involves reduced vitronectin-integrin $\alpha_V\beta_3$ reactions in fibroblasts.

Divergent roles of PAI-1 in development of fibrosis are associated with the complexity of PAI-1 effects on inflammation, cellular adhesion, migration, proliferation and survival, which differ in varying pathological conditions.^{1,8,9,24,30,36–38} The protective or injurious effects of PAI-1 are mediated not only by its inhibition of plasmin/protease system, but also by PAI-1: vitronectin binding that affects interactions with cell-surface integrins.^{17–19} Indeed, recent studies suggest that the pro-fibrotic effects of PAI-1

may be independent of plasmin activity.^{39,40} Vitronectin is an abundant adhesive extracellular glycoprotein that has binding sites for PAI-1, urokinase-type plasminogen activator receptor (uPAR), $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins. Dynamic binding with these factors regulates many pathophysiological processes, such as hemostasis, wound healing, angiogenesis, tissue remodeling and tumor metastasis.^{41–46} The vitronectin-bound PAI-1 competes for uPAR and integrins binding to vitronectin, thereby inhibiting uPAR- or integrin-mediated downstream cellular effects such as proliferation, adhesion and migration.^{46–48} Results of our *in vivo* studies showed that supplement of vitronectin-binding PAI-1 variants attenuated fibrosis markers, whereas the variant without vitronectin-binding capacity but with protease inhibitory activity significantly exacerbated cardiac fibrosis. These results are the first to differentiate the vitronectin-binding effects of PAI-1 from its protease-inhibitory effects on cardiac fibrosis. Binding to vitronectin also stabilizes PAI-1 from cleavage in locations of injury areas.^{49,50} In our study, the vitronectin level in the heart was not significantly different among the groups with different PAI-1 variants. However, AK significantly increased endogenous mouse PAI-1 level in the heart of Ang II-infused mice, possibly because of more binding to

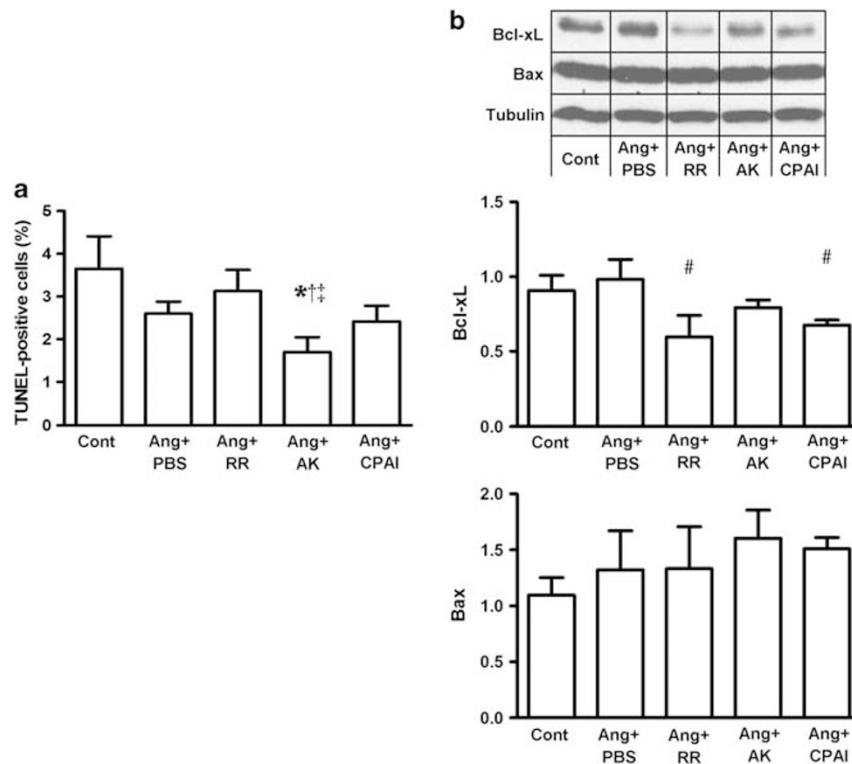


Figure 4 Apoptosis of Ang II-exposed cardiac fibroblast. Treatment with non-vitronectin-binding AK significantly reduced apoptosis, suggesting proapoptotic effects of PAI-1 by binding to vitronectin on Ang II-exposed cardiac fibroblasts (TUNEL, **a**). This effect was further shown by significantly downregulated Bcl-xL, an anti-apoptotic marker, but not pro-apoptotic Bax, in vitronectin-binding RR and CPAI-treated fibroblasts (western blot, **b**). These results indicate that the pro-apoptotic effect of vitronectin-binding PAI-1 involves decreased activity of anti-apoptotic factors. * vs Cont, † vs Ang + PBS, ‡ vs Ang + RR, $P < 0.05$. $N = 3$ for each group.

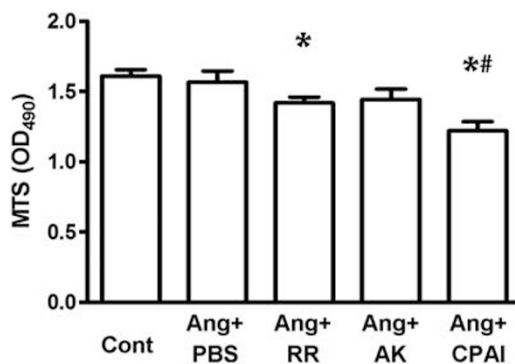


Figure 5 Proliferation of cardiac fibroblasts (MTS assay). Proliferation of Ang II-exposed cardiac fibroblasts was significantly decreased by vitronectin-binding PAI-1 variants, RR and CPAI, but not by AK, which only inhibits protease activity. * vs Cont, † vs Ang + PBS, $P < 0.05$. $N = 3$ for each group.

vitronectin and less cleavage of native mouse PAI-1 than those in RR- or CPAI-treated mice.

Cardiac fibroblasts are the predominant sources of the extracellular matrix protein in the heart.^{12–15,51} Marked phenotypic alterations in fibroblast adhesion and migration have been correlated with myocardial disease.^{52–55} Cardiac fibroblasts can also secrete vitronectin and express vitronectin receptor $\alpha_V\beta_3$ integrin.^{20,56} Ang II increases adhesion

of cardiac fibroblasts to the extracellular matrix, which is associated with upregulated integrins α_V and β_3 .²¹ Integrin $\beta_3^{-/-}$ cardiac fibroblasts exhibited a significant reduction in cell–matrix adhesion, cell spreading, proliferation and migration.⁵⁷ In our study with primary cardiac fibroblasts that were exposed to Ang II, the levels of supernatant vitronectin and $\alpha_V\beta_3$ integrin expression were significantly reduced by the non-protease-inhibiting but vitronectin-binding RR variant, indicating decreased vitronectin–integrin $\alpha_V\beta_3$ interaction by vitronectin-binding PAI-1.⁵⁸ We also found that vitronectin-binding PAI-1 variants prevented the adhesion of fibroblasts, whereas the non-vitronectin-binding AK variant did not. This result is consistent with other studies on different cell types.⁵⁹

Ang II promotes fibroblast proliferation and infiltration, as well as increased PAI-1 level in the heart. Vitronectin is a key determinant of the anti-proliferative effects of PAI-1.⁶⁰ PAI-1 binding to vitronectin on vascular cells also correlates with apoptosis,⁶¹ and vitronectin–integrin complex is linked to prevention of cultured cells from undergoing apoptosis.^{62,63} Our *in vitro* study shows that vitronectin-binding PAI-1 variants caused more apoptosis but less proliferation in Ang II-exposed cardiac fibroblasts. These findings complement the *in vivo* findings of fewer fibroblasts in the heart with the vitronectin-binding variants.

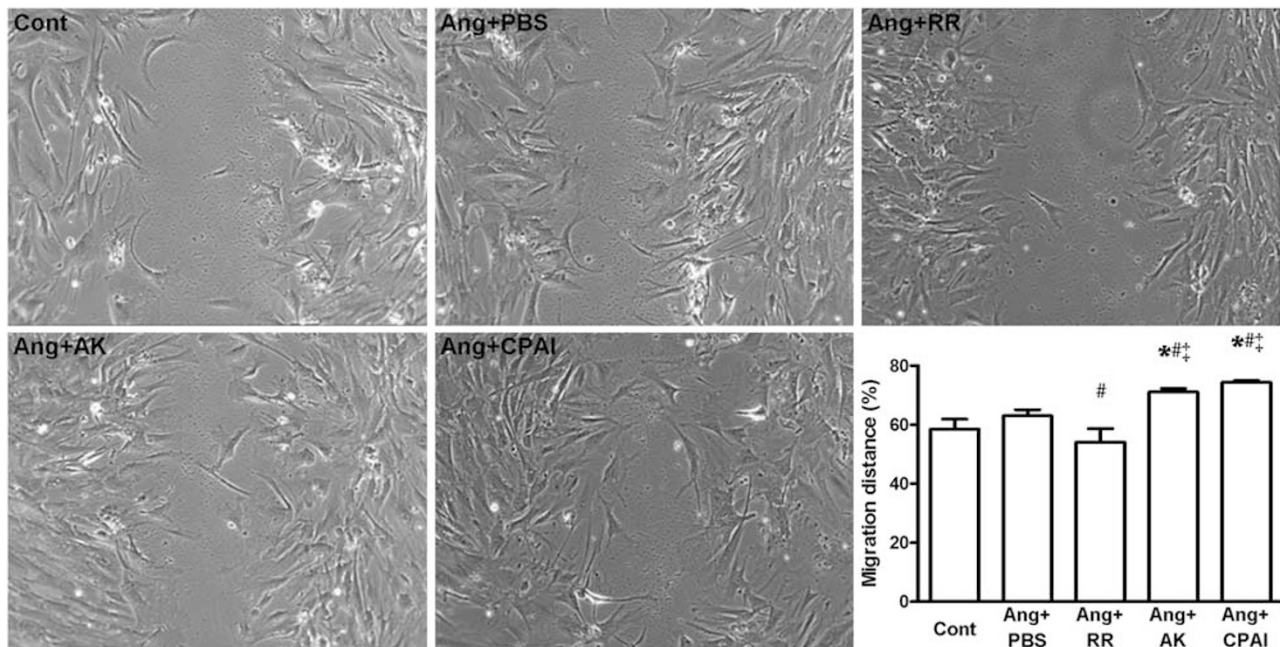


Figure 6 Migration of Ang II-treated cardiac fibroblasts (wound-healing assay). Compared with Ang II treatment alone, protease inhibitory AK and CPAI significantly increased cardiac fibroblast migration, whereas vitronectin-binding RR without protease inhibition significantly decreased migration. These results indicate that the migration of cardiac fibroblasts is impacted by both protease inhibition and vitronectin-binding of PAI-1. * vs Cont, † vs Ang + PBS, ‡ vs Ang + RR, $P < 0.05$. $N = 3$ for each group.

PAI-1 inhibits cell migration through its vitronectin-binding^{19,64–66} and plasmin-inhibiting effects.^{67–69} Our *in vitro* study supported that the inhibitory effects of PAI-1 on cell migration through the vitronectin-binding mechanisms, as the vitronectin-binding only variant, RR, significantly reduced the migration of cardiac fibroblasts. However, the protease inhibitory PAI-1 variants, AK and CPAI, increased cardiac fibroblast migration. This may also be influenced by the decrease in apoptosis, and/or the increase in proliferation, thus promoting increased cellularity in the cultured fibroblasts.

Although the protective effects of RR may be partially through preservation of plasmin activity that reflects competition with endogenous native PAI-1 to inhibit proteases,²⁵ similar effects observed with the CPAI variant, which has both protease-inhibiting and vitronectin-binding capacities indicate a predominant role of vitronectin binding-mediated protection. In align with this, a most recent study with cultured primary human corneal fibroblasts revealed that vitronectin-bound PAI-1 protected against uPA-induced fibroblast-to-myofibroblast transdifferentiation was independent of plasmin activity.⁷⁰

Ang II can induce PAI-1 expression in various cell types, including not only fibroblasts, but also endothelial cells, myocytes, vascular smooth muscle cells, etc. Compared with the wild-type, genetic vitronectin-deficient mice have delayed wound healing,⁷¹ or similar renal interstitial fibrosis induced by ureteral obstruction.^{71,72} In addition, cardiac fibroblasts are not unique sources of extracellular matrix and PAI-1

production. These notions prompt future studies using inducible tissue- or cell-specific PAI-1 expression models.

In summary, we used different PAI-1 variants to investigate the relative contributions of protease-inhibiting and vitronectin-binding pathways of PAI-1 in cardiac fibrosis. Our *in vivo* and *in vitro* data showed protective effects of vitronectin-binding PAI-1 against development of Ang II-induced cardiac fibrosis. By binding with vitronectin, PAI-1 may block integrin $\alpha_v\beta_3$ action, induce apoptosis, and suppress proliferation, adhesion and migration of cardiac fibroblasts. Our study results suggest therapeutic implications of vitronectin-binding PAI-1 for cardiac fibrosis.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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