Infectious diseases and fibrotic disorders: Potential novel targets

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Protease activated receptor-1 (PAR-1) deficiency ameliorates skin fibrosis in a mouse model of bleomycin-induced scleroderma.

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ABSTRACT

Objective. Accumulating evidence shows that Protease Activated Receptor-1 (PAR-1) plays an important role in the development of fibrosis, including systemic sclerosis (SSc) induced lung fibrosis. However, whether PAR-1 also plays a role in the development of scleroderma induced by SSc remains elusive. The aim of this study was to determine the role of PAR-1 in the development of skin fibrosis.

Methods. In order to explore possible mechanisms in which PAR-1 could play a role, human dermal fibroblasts and keratinocytes were stimulated with specific PAR-1 agonists or antagonists. To investigate the role of PAR-1 in skin fibrosis, we subjected wildtype and PAR-1 deficient mice to a model of bleomycin-induced skin fibrosis.

Results. PAR-1 activation leads to increased proliferation and extra cellular matrix (ECM) production, but not migration of human dermal fibroblasts (HDF) in vitro. Moreover, transforming growth factor (TGF)-β production was increased in keratinocytes upon PAR-1 activation, but not in HDF. The loss of PAR-1 in vivo significantly attenuated bleomycin-induced scleroderma. The bleomycin-induced increase in dermal thickness and ECM production was significantly reduced in PAR-1 deficient mice compared to wildtype mice. Moreover, TGF-β expression and the number of proliferating fibroblasts were reduced in PAR-1 deficient mice although the difference did not reach statistical significance.

Conclusion. This study demonstrates that PAR-1 contributes to the development of skin fibrosis in a mouse model of SSc. We suggest that PAR-1 potentiates the fibrotic response by inducing fibroblast proliferation and ECM production.

INTRODUCTION

Scleroderma (also referred to as systemic sclerosis (SSc)) is an rheumatic autoimmune disease of unknown etiology characterized by excessive extracellular matrix (ECM) production in (amongst others) skin and lung. The activation, proliferation and migration of resident fibroblasts at the site of trauma induces deposition of ECM proteins like fibronectin and collagen. Inhibiting fibroblast activation may thus provide therapeutic strategies for SSc due to its direct anti-fibrotic effect.

Protease activated receptor (PAR)-1 is a G-coupled receptor belonging to the protease activated receptor family that consists of 4 members (PAR-1-4). PARs show a unique mechanism of activation, being proteolytically cleaved by serine proteases. Removal of the
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N-terminal extracellular domain releases a tethered ligand that binds to the body of the receptor to induce transmembrane signaling. Interestingly, PAR-1 induces proliferation, migration and ECM production of fibroblasts and PAR-1 deficiency (either genetic or pharmacologic) limits experimental fibrosis in lung and liver. In the skin, PAR-1 is expressed on keratinocytes, endothelial cells and fibroblasts and the density of PAR-1 positive fibroblasts is increased in the skin of SSC patients compared to that of healthy controls. Moreover, PAR-1 is expressed in both the epidermis and dermis of normal and hypertrophic scars and in keloid lesions. The functional consequence of PAR-1 expression in scleroderma remains elusive however although accelerated wound healing in mice after topical PAR-1 activation pinpoints PAR-1 as an important receptor in the skin.

Here we show that PAR-1 drives pro-fibrotic responses of human dermal fibroblasts (HDF) and keratinocytes in vitro. Furthermore, we show that PAR-1 plays a pivotal role in the development of skin fibrosis in a murine model of bleomycin-induced skin fibrosis.

METHODS

Animals. Heterozygous PAR-1 KO mice on a C57Bl/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Animals were intercrossed to obtain homozygous PAR-1 KO mice as described before. Wildtype C57BL/6 mice were purchased from Charles River (Maastricht, the Netherlands). All experiments were approved by the Institutional Animal Care and Use Committee of the University of Amsterdam. All mice were maintained according to institutional guidelines. Animal procedures were carried out in compliance with the Institutional Standards for Humane Care and Use of Laboratory Animals. The Animal Care and Use Committee of the Academic Medical Center approved all experiments.

Induction of Scleroderma. Eight to ten-weeks old mice (n=8 per group) received daily intradermal injections of bleomycin (100 μl containing 10 μg bleomycin sulphate in PBS) or saline into their shaved backs for 10 consecutive days.

Histological Analysis of skin. After sacrifice, skin sections were fixed in formalin, embedded in paraffin and 4-μm-thick sections were subsequently deparaffinized, rehydrated and washed in deionized water. Slides were stained with H&E and Masson’s trichrome according to routine procedures. Dermal thickness was measured on H&E stained slides using pictures taken at 10× magnification. The average of three measurements per section was used for each skin section.
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**In vivo proliferation.** Proliferative cells were detected using a rabbit anti-Ki67 (#RM-9106; Lab Vision) antibody essentially as described before. In short, after deparaffinization and endogenous peroxidase inhibition, sections were boiled in citrate buffer (pH 6.0) for 10 minutes, blocked with Normal Goat serum for 30 minutes and incubated over-night with the primary antibody (1:500) at 4°C. Subsequently, slides were incubated with Brightvision PolyHRP-anti-rabbit IgG (DPVR-110HRP; Immunologic) for 30 minutes at room temperature and stained using DAB (BS04-999; Immunologic). Proliferating cells of the epidermis, hair follicles and sebaceous glands were excluded in the analysis.

**Detection of fibronectin.** Fibronectin stainings were performed using a fibronectin antibody (1:50; sc-6953, Santa Cruz Biotechnology). In short, sections were boiled in citrate buffer (pH 6.0) for 20 minutes, blocked with 5% normal rabbit serum in PBS for 30 minutes and incubated over-night with the primary antibody at 4°C. Subsequently, slides were incubated with a rabbit-anti-goat-HRP antibody (1:100; P0160, Dako) for 30 minutes at room temperature and stained using DAB (BS04-999; Immunologic).

**Cell culture.** Human keratinocytes (HaCaT cells; a gift from Dr Versteeg; passages 50-55) and human dermal fibroblasts (HDF; ATCC; passages 2-6) were maintained in DMEM supplemented with 10% FCS. Unless stated otherwise, cells were serum-starved for 4 hours and subsequently stimulated as described. Cells were lysed in Laemmli lysis buffer, incubated for 5 minutes at 95°C and stored at -20°C for further analysis. Medium of the cells was stored at -20°C for TGF-β detection.

**Lenti-viral knockdown of PAR-1.** For lenti-viral PAR-1 silencing, PAR-1 and control shRNA in the pLKO.1-puro backbone were purchased from Sigma (MISSION® shRNA library). We selected clones TRCN0000003689 (CCGGCCCAGTCATTTCCTCAGGACTCGAGTCTGAGAAGATGACCGGGTTTTTT), TRCN0000003691 (CCGGCCTACTACTTCTCAGCCTTCTCTGAGAAGGCTGAGAAGTAGTAGGTTT), and SHC004 (CCGGCGTGATCTTCACCGACATCTCGAGATCTTGTCGGTGAAGATCACGTTTT; control; shTGFP). Lenti-viral production and cell transduction was performed using standard protocols and shRNA transduced HDF were selected in the presence of 2 µg/ml puromycin for 72 hours.

**Cell viability assays.** HDFs, seeded in 96-well plates at a concentration of 5000 cells/well, were stimulated with thrombin (1 U/mL), PAR-1 agonist peptide (H-SFLLRN-NH₂; PAR-1-AP;
100 μM) or human recombinant TGF-β (5 ng/ml) after which cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay according to routine procedures. When indicated, cells were pre-incubated for 30 minutes with P1pal-12 (palmitate-RCLSSAVANRS-NH2; 5 μM).

**Western Blot.** Samples were subjected to SDS-PAGE (10% gel), after which proteins were transferred onto Immobilon-P membranes (Millipore) as described before. Next, membranes were blocked for 1h at room temperature in either Odyssey Blocking buffer (OBB; LI-COR Biosciences)/PBS (for p44/42) or 5% BSA in TBS + 0.1% Tween-20 (TBS-T; for fibronectin, collagen type I and α-tubulin). The membranes were incubated overnight at 4 °C with antibodies against fibronectin (1:1000 in TBS-T; sc-6953, Santa Cruz Biotechnology), α-tubulin (1:1000 in TBS-T; sc-23948, Santa Cruz Biotechnology), collagen type I (1:800 in TBS-T; #1310-01, Southern Biotech) or phospho-p44/42 (1:1000 in OBB/PBS-T; #9106, Cell Signaling). After washing, the blots were incubated with secondary antibodies (HRP-conjugated for fibronectin, collagen type I and α-tubulin (1:1000 in TBS-T) or IRDye 700-GAR for phospho-p44/42 (1:5000 in OBB/PBS-T with 0.04% SDS). Finally, membranes were imaged using Lumi-Light (12015200001; Roche) on an ImageQuant™ LAS 4000 biomolecular imager (GE Healthcare) or on a LI-COR Odyssey IR Imager.

**TGF-β detection.** Active-TGF-β levels were measured by ELISA (R&D Systems) according to the manufacturer’s recommendations. For in vitro experiments, total TGF-β levels were determined. Before start of the assay, samples were incubated with 1 N HCl in order to cleave all pro-TGF-β and subsequently with 1.2N NaOH/0.5M HEPES to neutralize the reaction.

**Statistical Analysis.** For the in vivo experiment, differences between groups were analyzed by t-test or Mann-Whitney U-test for nonparametric values. For the in vitro experiments, 1-way-ANOVA analysis or Kruksal-Wallis test (for nonparametric values) was performed, followed by Bonferroni’s or Dunns multiple comparison tests respectively. Analyses were performed using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA).

**RESULTS**

PAR-1 induces fibrotic responses in keratinocytes and dermal fibroblasts. To assess whether PAR-1 drives fibrotic responses in vitro, HDF were cultured with or without thrombin and/or TGF-β (positive control). As shown in figure 1A, thrombin-dependent PAR-1 activation and TGF-β stimulation both increased proliferation of HDF, whereas the com-
combination of thrombin and TGF-β further increased proliferation. Thrombin did not induce proliferation in the presence of a specific PAR-1 antagonist (i.e. P1pal-12) showing that thrombin indeed acts via PAR-1 (figure 1B). Interestingly, PAR-1 inhibition not only prevented thrombin-induced proliferation but actually significantly decreased HDF proliferation compared to control conditions. Overall, these data suggest that HDF proliferation is PAR-1

**Figure 1:** PAR-1 activation increases proliferation and ECM production in HDF and TGF-β production in HaCaT cells. (A) MTT assay of HDF stimulated with thrombin (1 U/ml), TGF-β (5 ng/ml) or the combination of both. (B) MTT assay of HDF pre-treated for 30 minutes with p1pal-12 before stimulation with thrombin (1 U/ml). (C) Western blot analysis of phospho-p44/42 in cell lysates of PAR-1 (or control) shRNA transduced HDF stimulated with PAR-1-AP (100 μM) for 5 or 10 minutes. (D) MTT assay of PAR-1 shRNA transduced HDF cultured for 24 hours under normal culture conditions. (E) Western blot analysis of fibronectin, collagen type I and α-tubulin in HDF stimulated with thrombin (1 U/ml), p1pal-12 (5 μM) and TGF-β (5 ng/ml) or combinations thereof. (F) Total-TGF-β production of HaCaT cells upon PAR-1 activation with thrombin or PAR-1-AP. Figures are representative of three independent experiments. Data are means ± SEM. * p<0.05, ** p<0.01, *** p<0.001.
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dependent.

Although P1pal-12 is frequently used as a specific PAR-1 inhibitor\(^5\) and no toxicity has been described, we next aimed to refute this latter suggestion. To this end, we lenti-virally transduced HDF with two different PAR-1 shRNA constructs (designated 3689 and 3691) and a control construct (004). As shown in figure 1C, PAR-1 activation by PAR-1-AP rapidly induces phosphorylation of the well-known PAR-1 target p44/42. Importantly, PAR-1 signaling was effectively reduced in HDF transduced with shRNA 3691 as evident from the absence of p44/42 phosphorylation upon stimulation with PAR-1-AP. shRNA 3689 was less effective in reducing PAR-1 signaling as evident from residual p44/42 phosphorylation after PAR-1 activation.

As expected, effective inhibition of PAR-1 signaling (i.e. shRNA 3691) led to similar effects on proliferation as compared to P1pal-12 treatment, whereas non-effective PAR-1 inhibition (i.e. shRNA 3689) only showed a 15% reduction in proliferation as compared to control HDF (Figure 1D). Taken together, these data show PAR-1 dependent proliferation of HDF. As opposed to proliferation, PAR-1 did not influence HDF migration (data not shown).

Next we assessed whether PAR-1 would modify ECM production by HDF. Indeed, thrombin stimulation of HDF slightly increased production of collagen type I, but it did not modify fibronectin levels (Figure 1E). PAR-1 inhibition by P1pal-12 pre-treatment abolished thrombin-induced collagen production but also diminished baseline collagen levels showing that PAR-1 induces ECM synthesis. As expected, TGF-\(\beta\) stimulation also induced collagen and fibronectin production. Interestingly, the combination of thrombin and TGF-\(\beta\) further induced fibronectin levels without significantly affecting collagen levels.

PAR-1 deficiency reduces TGF-\(\beta\) levels during pulmonary fibrosis\(^6\) and next to direct pro-fibrotic effects of PAR-1 on HDF, PAR-1 may also drive skin fibrosis by inducing TGF-\(\beta\) production. As shown in Figure 1F, both thrombin and PAR-1-AP stimulation of HaCaT cells indeed induced TGF-\(\beta\) production. TGF-\(\beta\) levels produced by HDF were low and did not change upon PAR-1 activation (data not shown). Overall these data suggest an important role for PAR-1 during dermal fibrosis by modifying HDF proliferation and ECM production and potentially by modifying TGF-\(\beta\) production by keratinocytes.

Amelioration of bleomycin-induced scleroderma by loss of PAR-1. To determine whether PAR-1 is critical for skin fibrosis, we subjected PAR-1 deficient and wildtype mice to a bleomycin-induced scleroderma model. As shown in Figure 2A-C, dermal thickness was significantly reduced in PAR-1 deficient mice as compared to wildtype mice. In line, the accumulation of ECM was also reduced in PAR-1 deficient mice as compared to wildtype
mice as evident from reduced collagen content in Masson’s trichrome stained skin sections (Figure 2D). Together these data show that PAR-1 deficient mice are protected against bleomycin-induced skin fibrosis.

**Modification of profibrotic processes in PAR-1 deficient mice.** In our *in vitro* experiments we showed that PAR-1 drives fibroblast proliferation and consequently we next assessed the number of proliferating cells (excluding cells of the epidermis, hair follicles and sebaceous glands) in the dermis of wildtype and PAR-1 deficient mice during bleomycin-induced skin fibrosis. As shown in figure 3A-B, the number of proliferating cells as analyzed...
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Figure 3: Modification of profibrotic processes by PAR-1 in vivo. (A) Immunohistochemical staining of Ki67 positive cells in skin sections of wildtype and PAR-1 deficient mice upon bleomycin treatment (10x magnification). (B) Quantification of Ki67 positive cells in the dermis of wildtype and PAR-1 deficient mice upon bleomycin treatment. (C) TGF-β levels in skin homogenates of wildtype and PAR-1 deficient mice upon bleomycin treatment. (D) Representative pictures of fibronectin expression in skin sections from wildtype and PAR-1 deficient mice upon bleomycin treatment (10x magnification).

by the number of Ki67 positive cells per skin section was reduced in PAR-1 deficient mice as compared to wildtype mice, although the difference did not reach statistical significance. As already indicated, TGF-β production by keratinocytes was induced by PAR-1 activation in vitro, and TGF-β is important in the pathogenesis of scleroderma. As shown in Figure 3C however, active TGF-β levels were not significantly different in skin homogenates of wildtype and PAR-1 deficient mice upon bleomycin treatment. Finally, we determined fibronectin expression in the skin of wildtype and PAR-1 deficient animals and, in line with our in vitro experiments, fibronectin levels per skin section were reduced in PAR-1 deficient mice as compared to wildtype mice (Figure 3D).
DISCUSSION

PAR-1 may play an important role during the initiation and progression of both lung and liver fibrosis. Here, we determined whether PAR-1 would also drive skin fibrosis in the setting of Ssc. Indeed, PAR-1 activation leads to increased proliferation and ECM production in HDF, whereas it also potentiates TGF-β production of HaCaT. In line with these in vitro data, we also show reductions in dermal thickening and collagen/fibronectin deposition in PAR-1 deficient mice during experimental skin fibrosis. Overall, our data thus pinpoint PAR-1 as a novel mediator of skin fibrosis.

Our in vitro data suggest that PAR-1 modifies scleroderma by acting as a pleiotropic mediator affecting multiple profibrotic responses (i.e. proliferation, TGF-β production and ECM deposition). However, during experimental scleroderma, PAR-1 deficiency only showed significant effects on ECM deposition whereas the differences in fibroblast proliferation did not reach significance. It may thus be that PAR-1 mainly affects ECM deposition during bleomycin-induced scleroderma although the observed variability in fibroblast proliferation in vivo may explain the lack of significance. TGF-β production was also not significantly reduced in PAR-1 deficient mice which may be due to the fact that PAR-1 only contributes to TGF-β production in keratinocytes and not in dermal fibroblasts. As PAR-1 modifies inflammation during pulmonary fibrosis, it may be tempting to speculate that PAR-1-dependent inflammation may potentiate skin fibrosis. Importantly however, inflammatory cell influx (neutrophils and macrophages) and cytokine production were similar in wildtype and PAR-1 deficient mice (data not shown).

An interesting finding of our manuscript is that PAR-1 inhibition (both pharmacologically and genetically) reduces both proliferation and ECM production of HDF. These data strongly suggest that HDF secrete an endogenous PAR-1 agonist that drives proliferation and ECM production under normal culture conditions in an autocrine manner. Expression levels of the endogenous PAR-1 ligand seem to be rate limiting for these pro-fibrotic processes as exogenous PAR-1 activation still further increases both proliferation and ECM production. Although several PAR-1 agonist (like MMP13 and kallikreins have been described to be produced in skin fibroblasts, the exact nature of the endogenous PAR-1 agonist remains elusive and is currently under investigation.

We used a bleomycin-induced model of scleroderma that largely mimics human scleroderma. Indeed, dermal thickening is accompanied by deposition of dense packed collagen fibrils that are extensively cross-linked in both murine and human scleroderma. The fact that we show that PAR-1 deficiency limits skin fibrosis in this model, together with the fact that PAR-1 is upregulated in the skin during human Ssc and that PAR-1 activation on...
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human cell types potentiates key profibrotic processes, suggests that PAR-1 may have clinical relevance for human scleroderma. One should realize however that PAR-1 also mediates platelet activation and targeting PAR-1 could lead to bleeding complications. It may consequently be better to target the, still elusive, endogenous PAR-1 agonist.

In conclusion, we identify PAR-1 as a novel potential mediator of skin fibrosis which may open novel therapeutic treatment strategies for limiting scleroderma (or other cutaneous fibrotic disorders) for which no effective treatment strategy is available at this time.

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REFERENCES
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