Infectious diseases and fibrotic disorders: Potential novel targets

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PAR-2 drives proliferation and extra cellular matrix deposition of dermal fibroblasts and promotes skin fibrosis during experimental scleroderma.

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ABSTRACT

Objective. Protease Activated Receptor-2 (PAR-2) is a G protein-coupled receptor implicated in fibroproliferative disorders. PAR-2 expression increases during disease progression and genetic ablation of PAR-2 limits experimental fibrosis in organs like lung, liver and kidney. In the skin, PAR-2 is expressed in scars but also in dermal fibroblasts of scleroderma patients. The relevance of PAR-2 expression in the skin remains elusive however and here, we aimed to prove or refute the hypothesis that PAR-2 is an essential player in scleroderma.

Methods. The importance of PAR-2 in scleroderma was evaluated by subjecting wildtype and PAR-2 deficient mice to bleomycin-induced scleroderma. The underlying mechanism was evaluated by in vitro stimulations of human dermal fibroblasts (HDF) with PAR-2 (ant) agonists.

Results. Genetic ablation of PAR-2 exerted potent antifibrotic effects and reduced dermal thickening, fibroblast proliferation and extracellular matrix (ECM) deposition during bleomycin-induced scleroderma. Stimulation of bleomycin-pretreated HDF with PAR-2 agonists had no effect on proliferation or ECM deposition. Interestingly, a specific PAR-2 antagonist dramatically reduced HDF proliferation and ECM deposition.

Conclusion. PAR-2 contributes to the progression of scleroderma in a mouse model of SSc. We show that PAR-2 on HDF potentiates the fibrotic response by inducing proliferation and ECM production.

INTRODUCTION

Scleroderma (systemic sclerosis, SSc) is an autoimmune disease of unknown aetiology characterized by progressive fibrosis of the skin and internal organs. The activation, proliferation and migration of resident fibroblasts at the site of trauma induces deposition of extracellular matrix (ECM) proteins like fibronectin and collagen leading to tissue fibrosis. Inhibiting fibroblast activation may thus provide therapeutic strategies for SSc due to its direct anti-fibrotic effect.

Protease activated receptor (PAR)-2 belongs to the family of protease activated receptors that are irreversibly activated by proteolytic cleavage. Interestingly, PAR-2 is implicated in driving fibrotic disease in different organs like lung, liver and kidney by modifying fibroblast proliferation and/or ECM deposition. In the skin, PAR-2 is expressed in myofibroblast...
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rich regions in keloid and hypertrophic scars, whereas PAR-2 is expression is increased in dermal fibroblasts of scleroderma patients. The relevance of PAR-2 expression in the skin for the development/progression of SSc remains elusive however.

Here, we evaluated whether PAR-2 potentiates the progression of skin fibrosis by subjecting wildtype and PAR-2 deficient mice to bleomycin-induced scleroderma. We hypothesized that PAR-2 deficiency would limit fibroblast activity thereby limiting scar formation.

MATERIALS & METHODS

Animals. Ten-week-old wild-type C57Bl/6 mice were purchased from Charles River (Someren, the Netherlands). PAR-2 deficient C57Bl/6 mice were originally provided by Jackson Laboratories (Maine) and bred at the animal care facility of the Academic Medical Center. 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 (Amsterdam, the Netherlands) approved all experiments.

Induction of Scleroderma. Mice received daily intradermal injections of bleomycin (10 μg in 100 μl PBS) or saline into their shaved backs for 10 days. For reducing animal use, the experiments were performed simultaneously with experiments aiming at the role of PAR-1 in SSc and the data of the wildtype mice are consequently similar to the data described in.

Real-time PCR. PAR-2 mRNA levels were quantified by SYBR green real-time PCR using the following primers: 5’-TCCGCTCTTCTGCTATGGAT-3’ and 5’-CAGAGGGCGACAAGGTAGAG-3’. PAR-2 expression levels were normalized to GAPDH expression levels using the following primers: 5’-CTCATGACCACAGTCCATGC-3’ and 5’-CACATTGGGGGTAGGAACAC-3’.

(Immunohistochemistry). Histological examination was performed according routine procedures. In detail, skin sections were fixed in 4% 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 (#HT15-1KT, Sigma) according to routine procedures. In H&E staining, dermal thickness was measured using pictures at 20× magnification.

Proliferative cells were detected using a rabbit anti-Ki67 (#RM-9106; Lab Vision) antibody. 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). Ki67 positive cells of the epidermis, hair follicles and sebaceous glands were excluded in the analysis.

**Cell culture.** Human dermal fibroblasts (HDF) were maintained in DMEM supplemented with 10% FCS. Cells were serum-starved for 4 hours and subsequently stimulated as described.

**Cell viability assays.** HDF (5000 cells/well in 96-well plates), were stimulated with PAR-2 agonist peptide (AP) after which cell viability was determined using MTT assays as described before. If indicated, cells were pre-incubated with P2pal18S for 30 minutes.

**Western Blot.** Samples were separated by electrophoresis on a 10% SDS-PAGE gel, after which proteins were transferred onto Immobilon-P membranes (Millipore). Blots were blocked for 1 hour at room temperature in 5% BSA in TBS + 0.1% Tween-20 (TBS-T). Goat anti-fibronectin (sc-6953; Santa Cruz Biotechnology) and mouse anti-α-tubulin (sc-23948, Santa Cruz Biotechnology) were diluted 1:1000 in TBS-T, goat anti-collagen type I (#1310-01, Southern Biotech) and mouse anti-smooth-muscle-α-actin (αSMA; sc-32251, Santa Cruz Biotechnology) were diluted 1:800 in TBS-T. Membranes were incubated overnight, washed in TBS-T, and incubated with a HRP-linked secondary antibody (1:1,000 in TBS-T). After 1 hour incubation at room temperature, blots were washed in TBS-T and imaged using Lumi-Light (12015200001; Roche) and an ImageQuant™ LAS 4000 biomolecular imager for chemiluminescence (GE Healthcare).

**TGF-β detection.** Active-TGF-β levels were measured by ELISA (R&D Systems) according to the manufacturer’s recommendations.

**Statistical Analysis.** Differences between the groups were tested by non-parametric Mann–Whitney $U$ test. MTT data were analysed using nonparametric Kruksal-Wallis tests followed by Dunns multiple comparison tests.
RESULTS

**PAR-2 expression in the skin aggravates bleomycin-induced scleroderma.** In order to determine whether PAR-2 plays a role in the development of scleroderma, wildtype and PAR-2 deficient mice were exposed to daily intradermal bleomycin injections. Interestingly, PAR-2 mRNA expression levels increased upon bleomycin treatment in the skin of wildtype mice (figure 1A). Next, we determined dermal thickening in both wildtype and PAR-2 deficient mice upon bleomycin treatment. As shown in figure 1B, PAR-2 deficiency significantly limited dermal thickening, which was already obvious from macroscopically visible lesions at the injection site of wildtype mice that were absent in PAR-2 deficient mice (figure 1C-D).

The production of ECM is a hallmark of the fibrotic process and therefore we performed Masson’s trichrome stainings on skin sections of wildtype and PAR-2 deficient mice. As shown in figure 1E-F, ECM deposition, stained in blue, was significantly reduced in skin sections of PAR-2 deficient mice as compared to wildtype controls. Taken together, these data show that PAR-2 potentiates bleomycin-induced scleroderma.

**PAR-2 activation increases fibroblast proliferation, but does not affect TGF-β production.** In order to get insight into the mechanisms by which PAR-2 contributes to bleomycin-induced scleroderma, we determined active TGF-β levels in skin homogenates of wildtype and PAR-2 deficient mice. As shown in figure 2A, TGF-β production was however not different between wildtype and PAR-2 deficient mice.

Fibroblast proliferation, which has been shown to be induced upon PAR-2 activation in fibroblasts of different origin, contributes to a great extent to the fibrotic process. Consequently, we hypothesized that PAR-2 would affect fibroblast proliferation in the setting of scleroderma. Indeed, the number of proliferating Ki67 positive cells in the dermis was significantly lower in PAR-2 deficient mice as compared to wildtype mice (figure 2B-D). Overall, PAR-2 seems to contribute to the pro-fibrotic process at the level of proliferation but not TGF-β production.

**PAR-2 activation leads to fibroblast proliferation and ECM production.** One of the most significant observations in the scleroderma model is that the number of proliferating cells in the dermis is reduced in PAR-2 deficient mice. Consequently, we set out *in vitro* experiments in which we assessed the role of PAR-2 on proliferation of HDF. As shown in figure 3A, the inhibition of PAR-2 signalling using a specific PAR-2 antagonist (p2pal18S) did not significantly inhibit fibroblast proliferation, whereas PAR-2 activation by PAR-2-AP or trypsin (data not shown) also did not induce proliferation of HDF. This may be explained...
by the fact that PAR-2 levels are low in HDF during control conditions. As PAR-2 expression can be induced by bleomycin treatment, we subsequently pre-incubated HDF with bleomycin for 24 hours after which the cells were incubated with PAR-2 (ant)agonists. Intriguingly, PAR-2 inhibition by p2pal18S treatment reduced proliferation by around 65%. (Figure 3B). PAR-2-AP treatment did not further increase proliferation of bleomycin-treated HDF as compared to control.

Interestingly, we also observed reduced ECM deposition in PAR-2 deficient mice (figure 1).
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1E-F). This may be a direct consequence of the reduced number of fibroblasts, but PAR-2 may also stimulate ECM production by activated fibroblasts directly. Consequently, we first assessed whether PAR-2 would modify myofibroblast differentiation. In line with the proliferation data, p2pal18S pre-treatment reduced αSMA levels in bleomycin-treated HDF, whereas the stimulation with PAR-2-AP did not increase αSMA expression (Figure 3C). TGF-β (positive control) did increase αSMA expression in HDF and this TGF-β-induced response was not enhanced by PAR-2-AP. Next, we assessed whether PAR-2 activation would modify ECM deposition by HDF. Again, as for αSMA production and proliferation, p2pal18S pre-treatment reduced collagen type I and fibronectin deposition levels below

Figure 2: PAR-2 contributes to fibroblast proliferation during scleroderma. (A) TGF-β levels in skin homogenates of wildtype and PAR-2 deficient mice upon bleomycin treatment (n=8). (B) Quantification of Ki67 positive cells in the dermis of wildtype and PAR-2 deficient mice upon bleomycin treatment (n=6-7). (C-D) Immunohistochemical staining of Ki67 positive cells in skin sections of wildtype (C) and PAR-2 (D) deficient mice upon bleomycin treatment (10x magnification) as quantified in (B). Data are means ± SEM. * p<0.05.
baseline (most evident after 48 hour incubations). Again, we did not observe any effect of PAR-2 activation by PAR-2-AP on collagen type I and fibronectin deposition, whereas TGF-β stimulation did increase these ECM proteins. Taken together, these data suggest autocrine PAR-2 dependent proliferation and ECM deposition of bleomycin-stimulated HDF.

DISCUSSION

PAR-2 recently emerged as a key factor in fibroproliferative disease and PAR-2 deficiency indeed limits bleomycin-induced pulmonary fibrosis, CCl₄-induced hepatic fibrosis and UUO-induced renal fibrosis.⁴⁻⁶ Interestingly, PAR-2 is expressed in human scars (normal, hypertrophic and keloid) and in the skin of SSc patients but the importance hereof has not yet been established. Here, we show that dermal PAR-2 is not an epiphenomenon but actually contributes to disease progression. Indeed, PAR-2 drives bleomycin-induced scleroderma as evident from reduced dermal thickening, fibroblast proliferation and ECM deposition in PAR-2 deficient mice.

Several papers suggest that PAR-2 is expressed at very low levels in HDF cultures, and in line with this notion we did not observe any effect of PAR-2 on in vitro proliferation or ECM deposition of HDF. Interestingly however, bleomycin treatment increases PAR-2 levels in HDF cultures⁸ and we indeed show that PAR-2 inhibition effectively reduces proliferation and ECM deposition in bleomycin pre-treated HDF. These data strongly suggest that HDF secrete an endogenous PAR-2 agonist that drives fibrotic responses in an autocrine manner. At a first glance this may not be that surprising considering the fact that several PAR-2 agonists (e.g. tryptase, trypsine and kallikrein-related peptidases ((KLK)-5, 6 and 14) are abundantly expressed in the skin.¹¹⁻¹³ However, these agonists are expressed in keratinocytes/epithelial cells (e.g. KLK-5, KLK-6, KLK-14 and trypsin) or mast cells (e.g. tryptase) and are therefore not the most likely PAR-2 activating proteases in HDF. The endogenous PAR-2 agonist in HDF that drives PAR-2 dependent fibrotic effects needs thus to be characterised. As opposed to PAR-2 inhibition, PAR-2 activation did not increase fibrotic responses of HDF in vitro. This may be rather unexpected, also in light of previous reports showing that PAR-2 activation leads to increased proliferation and/or ECM deposition in fibroblasts of different origin (i.e. liver, lung, pancreas⁴⁻⁵,¹⁴), but it may reflect low PAR-2 levels even on bleomycin-treated HDF or high endogenous PAR-2 agonist expression by HDF.

We used a bleomycin-induced model of scleroderma that largely mimics human scleroderma.¹⁵ 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-2 reduces scleroderma in this model, together with the notion
that PAR-2 expression is increased in SSc skin\(^6\) and that PAR-2 activation on fibroblasts potentiates key profibrotic processes suggest that PAR-2 may be an attractive target for the treatment of human scleroderma. However, further studies with specific drugs targeting PAR-2 (or its endogenous agonist) are needed to assess the potential clinical applicability.

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