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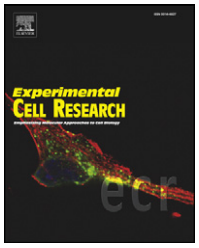

Research Article

Activin A induces a non-fibrotic phenotype in smooth muscle cells in contrast to TGF-β

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ABSTRACT

Aims: Activin A and transforming growth factor-β1 (TGF-β1) belong to the same family of growth and differentiation factors that modulate vascular lesion formation in distinct ways, which we wish to understand mechanistically.

Methods and results: We investigated the expression of cell-surface receptors and activation of Smads in human vascular smooth muscle cells (SMCs) and demonstrated that activin receptor-like kinase-1 (ALK-1), ALK-4, ALK-5 and endoglin are expressed in human SMCs. As expected, TGF-β1 activates Smad1 and Smad2 in these cells. Interestingly, activin A also induces phosphorylation of both Smads, which has not been reported for Smad1 before. Transcriptome analyses of activin A and TGF-β1 treated SMCs with subsequent Gene-Set Enrichment Analyses revealed that many downstream gene networks are induced by both factors. However, the effect of activin A on expression kinetics of individual genes is less pronounced than for TGF-β1, which is explained by a more rapid dephosphorylation of Smads and p38-MAPK in response to activin A. Substantial differences in expression of fibronectin, alpha-V integrin and total extracellular collagen synthesis were observed.

Conclusions: Genome-wide mRNA expression analyses clarify the distinct modulation of vascular lesion formation by activin A and TGF-β1, most significantly because activin A is non-fibrotic.

Introduction

Vascular pathologies such as atherosclerosis, restenosis after angioplasty and vein-graft disease in coronary bypasses eventually result in obstruction of normal blood flow. The lesions that are formed contain inflammatory cells as well as smooth muscle cells (SMCs) and substantial extracellular matrix depositions synthesized by activated SMCs. Transforming growth factor-β1 (TGF-β1) has been linked to excessive extracellular matrix synthesis in atherogenesis and fibrotic disease in the kidney, liver and skin [1,2]. The TGF-β superfamily also comprises activin A [3], which is present in human atherosclerotic tissue [4]. Both TGF-β and activin

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A have been proposed to exhibit atheroprotective functions [1,5], although the role of TGF-β1 is controversial. In mouse models, inhibition of TGF-β1 signaling by means of neutralizing antibodies, recombinant soluble TGF-β type II receptors or dominant-negative TGF-β-receptors in T cells, results in accelerated atherosclerosis [6–8]. This increase has been attributed to increased numbers of inflammatory cells, whereas the number of SMCs remained the same and a decrease in extracellular matrix production was observed. TGF-β1 overexpression has also been demonstrated to provoke increased vascular lesion formation, involving enhanced extracellular matrix production [9–11]. We demonstrated that activin A inhibits SMC-rich lesion formation in human vascular organ cultures and *in vivo* in cuffed femoral arteries of mice [5]. Activin A was shown to retain SMCs in the contractile quiescent state, preventing proliferation and migration of these cells [5].

TGF-β1 and activin A bind distinct type II receptors and TGF-β1 can subsequently interact with the type I receptor activin receptor-like kinase (ALK)-5 and ALK-1, whereas activin A has been described to bind ALK-4. Downstream of their specific receptors both factors activate similar signaling pathways resulting in phosphorylation of Smad2 and -3 [12,13]. TGF-β1 activation of ALK-1 enhances activation of Smad1, -5 and -8 [12,13], a difference with activin A signaling that may explain the observation that TGF-β1 and activin A affect SMC-rich lesion formation divergently. Up to now ALK-1 has been described to be exclusively expressed on endothelial cells, neurons, hepatic stellate cells and fibroblasts of scleroderma patients [14–16], implying that signaling through Smad1, -5 or -8 may not occur in SMCs.

A number of gene expression profiling analyses downstream of either TGF-β or activin A have been performed in distinct cell types [17–20]. However, to understand in detail the underlying mechanism of the function of TGF-β1 and activin A in SMC-rich lesion development, we performed a comprehensive transcriptome analysis in human vascular SMCs treated with either TGF-β1 or activin A within the same experiment. In the present study, we show that ALK-1 is expressed in human SMCs and we demonstrate a novel activin A signaling pathway resulting in Smad1 phosphorylation. Our analyses reveal that activin A and TGF-β1 share many signaling pathways in SMCs, except for SMC extracellular matrix formation where activin A and TGF-β1 function diverge.

Methods

SMC and HUVEC isolation and culture

Primary cultures of human SMCs were obtained from saphenous vein explant cultures. The vessels were stripped of the adventitia and endothelial cells were scraped off. The media was cut into small fragments (1–3 mm^2), which were cultured in 1 ml of Dulbecco's modified Eagle's medium (DMEM) with L-glutamine, supplemented with 10% (v/v) fetal calf serum (FCS), 100 U/mL penicillin and 100 μg/mL streptomycin (GIBCO-Invitrogen Life Technology, Breda, The Netherlands). The cells were characterized by immunostaining with an antibody directed against SM α-actin (1A4, 1:500, Dako, Glostrup, Denmark), which was detected with a goat anti-mouse-HRP conjugated antibody (PowerVision, ImmunoLogic, Duiven, The Netherlands). The cells were used at passages 5–7. For experiments, the cells were serum starved and treated with recombinant human activin A (50 ng/ml, Sigma-Aldrich, Saint Louis, MO, USA) or recombinant human TGF-β1 (10 ng/ml, R&D systems Europe, Abingdon, UK) for the indicated time. Human umbilical cord endothelial cells (HUVECs) were isolated and cultured as described [21,22]. The culture medium was composed of Medium-199 (M199, Invitrogen, Carlsbad, CA), supplemented with 20% (vol/vol) fetal bovine serum (FBS), 50 μg/mL heparin (Sigma-Aldrich), 12.5 μg/mL endothelial cell growth supplement (ECGS; Sigma-Aldrich), and 100 U/mL penicillin/streptomycin (Invitrogen). All culture surfaces were fibronectin coated. HUVECs were used at passages 1–2. For further characterization of the primary cell cultures, see supplemental data (Fig. 1). The investigation conforms to the principles outlined in the Declaration of Helsinki. Approval to use human tissue was approved by a local ethics review board.

Real-time RT-PCR

RNA was isolated from untreated SMCs or HUVECs, or from activin A- or TGF-β1-treated SMCs using the Absolutely RNA miniprep kit (Stratagene, La Jolla, CA, USA). cDNA was generated by reverse transcription of 500 ng of RNA with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Veenendaal, The Netherlands). Real-time RT-PCR was performed using an iCycler thermal cycler system (Bio-Rad Laboratories) with a 10× dilution of the cDNA samples in 15 μl reactions with specific forward and reverse primers and SYBR Green Supermix (Bio-Rad Laboratories). The primer sequences for the PCR are listed in the online data supplement (online Table 1). The settings for the thermal cycler were 95 °C for 3 min; 40 cycles of 95 °C for 15 s, and 60 °C for 15 s, 72 °C for 20 s; followed by 95 °C for 1 min and termination at 65 °C. After amplification, mRNA levels were normalized with the housekeeping large ribosomal phosphoprotein P0.

Immunohistochemistry

Normal human aortic specimens were fixed in 4% (w/v) paraformaldehyde and embedded in paraffin. Five μm sections were mounted on glass slides and routine immunohistochemical staining was performed using a 1 h incubation with the primary antibody against ALK-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:100 in PBS with 1% BSA. After rinsing in PBS, the sections were incubated with biotin-labeled rabbit-anti-goat antibody (1:500, Zymed, South San Francisco, CA, USA) for 1 h, again washed in PBS, and incubated for 30 min with SABC-mix (DAKO, The Netherlands). Following thorough rinsing with PBS, the sections were treated with diaminobenzidine tetrahydrochloride (DAB) for approximately 8 min and rinsed in tap water. The sections were counter-stained with Hematoxylin, dehydrated, and mounted in pertex. The investigation conforms to the principles outlined in the Declaration of Helsinki. Approval to use human tissue was approved by a local ethics review board.

Western blotting

Western blot analysis was performed using standard techniques. SMC and HUVEC were treated for the indicated time period with either activin A or TGF-β1. In short, cell lysates were prepared in RIPA buffer (150 mM NaCl, 50 mM Tris–HCl (pH 7.6), 1% (v/v) Triton X-100, 0.5% (w/v) sodium-deoxycholate, 0.1% (w/v) SDS, 1 mM EDTA), supplemented with mammalian protease inhibitor cocktail (Sigma-Aldrich). To establish equal loading, protein