Co-activation of synovial fibroblasts by laminin-111 and TGF-β induces expression of MMP-3 and MMP-10 independently of NFκB

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Abstract

We showed previously that the attachment of synovial fibroblasts (SFs) to laminin-111 (LM-111) in the presence of TGF-β induces significant expression of the matrix metalloproteinase (MMP)-3. Here we go on to investigate the regulation of additional MMPs and their specific tissue inhibitors of matrix proteases (TIMPs). Changes in steady state mRNA levels encoding TIMPs and MMPs were investigated by quantitative RT-PCR. Production of MMPs was monitored by a multiplexed immunoarray. Signal transduction pathways were studied by immunoblotting. Attachment of SFs to LM-111 in the presence of TGF-β induced significant increases in MMP-3 mRNA (12.35-fold, p<0.001) and protein (mean 62 ng/mL, 6-fold, p<0.008) and in expression of MMP-10 mRNA (11.68-fold, p<0.05) and protein (54 ng/mL, 20-fold, p≥0.02). All other TIMPs and MMPs investigated failed to show this LM-111-facilitated TGF-β response. No phosphorylation of NFκB was observed. We conclude that co-stimulation of SFs by LM-111 together with TGF-β suffices to induce significant expression of MMP-3 and MMP-10 by SFs and that this induction is independent of NFκB phosphorylation.
Introduction

The elevated expression of matrix-degrading proteases in synovial lining cells contributes to the destruction of cartilage and bone. Overexpression of these proteases is activated by a variety of cytokines such as IL-1β or TNF-α [1] and other factors [2]. We showed previously that stimulation of SF by TGF-β and LM-111 significantly raised the expression of MMP-3 [3]. Here we extend our investigation to determine whether other MMPs or TIMPs also respond to this mode of activation.

The activator protein (AP)-1, a heterodimer of jun/fos proteins, was shown to play a critical role in the regulation of MMPs in fibroblasts [4], and co-expression of MMP and c-fos was seen in RA-SF [5]. TIMPs are regulated by other transcription factors, including Egr-1 [6]. In the cytokine-induced kinase pathways, phosphorylation and nuclear translocation of transcription factor NFκB regulate the expression of MMPs in SFs [7, 8]. We therefore hypothesized that the LM-111- and TGF-β-activated expression of MMPs and TIMPs could address transcription factors such as AP-1 or Egr-1 and involve NFκB activity as well.

Co-stimulation of SFs by LM-111 in the presence of TGF-β resulted in significant expression of MMP-3 and MMP-10. No phosphorylation of transcription factor NFκB was observed and no involvement of IL-1β and TNF-α detected. We conclude that the attachment of SFs to LM-111 in the presence of TGF-β suffices to induce overexpression of MMP-3 and -10 by SFs.
Materials and Methods

Cell culture
SFs were isolated from surgical samples of seven patients diagnosed with rheumatoid arthritis (RA) [9] and eight patients with osteoarthritis (OA) and expanded in vitro as described [3]. Briefly, the tissue was minced and degraded in collagenase/PBS. The fibroblasts were expanded in DMEM complete medium. All studies were approved by the local ethics committee.

Transcript analysis
In induction experiments, cells were stimulated for 24 h in the presence of 10 ng/mL rhTGF-β1 and/or in LM-111-coated flasks as described recently [3]. Gene expression was compared to that in non-activated controls. RNA was extracted from 10⁶ SFs to generate cDNA. Transcript quantities in SFs were determined by qRT-PCR and normalized to GAPDH. Recombinant DNA standards served as controls.

Protein analysis by SDS-PAGE and immunoblotting
Cells were incubated overnight on LM-111-coated flasks (10 µg/mL) and stimulated by TGF-β1 (10 ng/mL) and/or LM-111 (10 µg/mL) for 60 min. Cells w/o stimuli or activated by TGF-β1 served as controls. Cells were harvested, lysed and subjected to immunoblot analysis as described recently [3]. The nylon membranes were probed overnight at 4°C with affinity-purified rabbit antisera (all from Santa Cruz or Cell Signalling) specific for total Egr-1 (C-19, 1:1000), total c-Fos (H125, 1:1000), anti-total-NFκB (1:1000), or phospho-NFκB p-65 (1:1000). Blots were stripped (1%SDS/0.5%βME/TBE, 2h, 25°C), washed, blocked, and incubated with affinity purified rabbit antiserum specific for β-actin (13E5, 1:1000) as loading control. Binding of the primary antibodies was detected as described [3].

Detection of MMPs in cell culture supernatants
The production of cytokines and MMPs was measured in SF supernatants with a multiplexed immunoassay (Luminex®, Austin, TX). Cells were activated for 24 h by the addition of 10 ng/mL rhTGF-β1, by attachment to LM-111 or by attachment to LM-111 in the presence of 10 ng/mL TGF-β1. Controls were incubated in normal tissue culture flasks without stimuli. Supernatants were harvested and pre-cleared by centrifugation, and aliquots mixed with fluorochrome-labeled microbeads coated with antibodies reactive with IL-1β, TNF-α, or MMP-1, -2, -3, -7, -8, -9, and -13 (R&D Systems, Abingdon, UK). To detect MMP-10, fluorochrome-labeled microbeads were coated with affinity-purified monoclonal antibodies. Beads were washed, incubated with PE-labeled detection antibodies and analyzed in a Luminex-HTS® device in flow cytometry mode as described by the supplier. Serial dilutions of recombinant proteins served as controls.

Statistics
A two-sided modified Student’s t-test was performed. Data sets with p-values equal to or lower than 0.05, 0.01 or 0.001 were considered statistically significant and marked accordingly (.*, **, *** ) in the artwork. Each data point represents the mean ± standard deviation from individual experiments using SFs from n different patients (6 ≤ n ≤ 14).
Results

Regulation of MMP expression by TGF-β and by attachment to LM-111

Activation of SFs by TGF-β1 caused significant induction of transcripts encoding TIMP-3 (4.9±2.6, p≤0.02), MMP-3 (4.8-fold±2.7, p≤0.001) and MMP-10 (3.25-fold±3.09, p<0.04, Fig. 1A). Attachment of SFs to LM-111-coated flasks did not induce significant responses (not shown). Activation of SFs by TGF-β and LM-111 failed to further augment expression of TIMP-3 (4.1±1.6, p≤0.001) compared to SFs activated by TGF-β alone (Fig. 1B), but it significantly enhanced expression of MMP-3 (12.34-fold±8, p<0.0001) and MMP-10 (11.68±20, p<0.05) (Fig. 1B). MMP-3 expression increased 2.55-fold (p<0.01) and MMP-10 expression increased 3.6-fold compared to cells activated by TGF-β alone. Transcripts encoding MMP-1, -9 and -13 were significantly enhanced, but without enhanced co-activation. No statistically significant difference in induction of any of the MMPs or TIMPs investigated was observed between SFs from RA and those from OA patients. The expression of other TIMPs and MMPs remained low or did not differ significantly from that of cells induced by either LM-111 or TGF-β (Fig. 1B).

Induction of MMP production by attachment to LM-111 in the presence of TGF-β

The SFs spontaneously secreted low amounts of MMP-3 (mean 9.89±10 ng/mL), but more MMP-3 was secreted upon activation with TGF-β (mean 39±28 ng/mL). This was significantly enhanced by the attachment to LM-111 in presence of TGF-β (mean 62.67±42.5 ng/mL, p<0.05; Fig. 1C). Furthermore, SFs spontaneously produced low levels of MMP-10 (mean 2.6±1.4 ng/mL), which was upregulated significantly by TGF-β (mean 31.36±27.4 ng/mL p<0.02) and even more by attachment to LM-111 in the presence of TGF-β (mean 54.27±44.36 ng/mL, p<0.02; Fig. 1D). Some MMP-9 was detected in supernatants of SFs w/o stimulation (mean 35±30 ng/mL); it was enhanced by attachment to LM-111 in the presence of TGF-β (61.6±14.8 ng/mL, p<0.02; not shown). MMP-13 protein expression was not detected in any of the investigated samples (detection limit 0.3 ng/mL, not shown), and we saw no release of TNF-α (detection limit 0.5 pg/mL) or involvement of IL-1β (mean 13 pg/mL, not shown).

Expression of transcription factors Egr-1 and c-Fos without activation of NFκB

Transcription factors involved in this regulation of MMPs were investigated one hour after the stimulation of cells. Phosphorylation of NFκB was observed in SFs after activation with FCS, but not after activation by either TGF-β, LM-111 or the two stimuli together. Total NFκB was detected in all extracts (Fig. 2). The expression of Egr-1 and c-Fos was used as a positive control and activation of SFs by TGF-β, LM-111 or both induced enhanced expression of these transcription factors. Reprobing the immunoblot with an antibody to β-actin served as loading control (Fig. 2).

Discussion

Attachment of SFs to LM-111 facilitated the TGF-β-induced expression of MMP-3 but failed to induce a high MMP-1 response [3]. Here we show that MMP-10, but no other MMP or TIMP investigated, is significantly expressed in activated SFs as well.
IL-1β and TNF-α induce the expression of MMPs in synoviocytes [2]. In some RA patients however, radiological progression is observed when biologically active TNF-α is neutralized [10]. As TGF-β levels were not reduced under this treatment [11] we hypothesized that it could be involved in regulation of MMPs. TGF-β utilizes pathways to induce expression of TIMP-1 and MMP-1 via AP-1 and SMAD [12] and a variety of MMPs [13]. We recently showed that TGF-β and LM-111 utilize SMAD2, p38 and p42/44 MAP-kinases to activate expression of MMP-3 independently of IL-1β or TNF-α [3]. Both IL-1β and TNF-α cause phosphorylation of NFκB and its translocation into the nuclei of activated cells [14]. The data presented here confirm that neither IL-1β nor TNF-α are involved in this mode of co-activation of MMP-3 and MMP-10. We did not detect phosphorylation of NFκB in extracts of TGF-β or LM-111 activated cells, either.

Stimulation of SFs by LM-111 and TGF-β is most likely not an RA-specific mode of cellular activation as both RA-SF and OA-SF responded equally well to both stimuli. Pathological integrin signaling contributes to proliferation of cells, elevated MMP-3 expression, and even cancer due to genomic instability [15]. Our experimental set-up might reflect such pathological matrix signaling due to elevated expression of laminins in RA synovial tissue [16]. It may also mimic changes in the extracellular matrix stiffness, as fibrosis of synovial tissues accompanies the RA pathology [17].

In summary we show here that activation of SFs by attachment to LM-111 in the presence of TGF-β activates MMP-3 and MMP-10, and to some extent MMP-9. The IL-1β- and TNF-α-associated NFκB pathway is not involved in this mode of activation.

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Abbreviations:
βME  mercaptoethanol  
LM  laminin  
MAP  mitogen-activated protein  
MMP  matrix metalloproteinase  
OA  osteoarthritis  
qRT-PCR  quantitative reverse transcriptase-polymerase chain reaction  
RA  rheumatoid arthritis  
SF  synovial fibroblasts  
SMAD  homologue to mothers against DPP and SMA genes  
TIMP  tissue inhibitor of metalloproteinase

References:


Figure Legends

Figure 1: 
*Induction of expression of matrix-metalloproteinases by activated synovial fibroblasts*

**A/B:** The SFs were incubated in the presence of 10 ng/mL TGF-β in normal tissue culture flasks (n = 10 individuals, Fig. 1A) or in LM-111 coated flasks in the presence of 10 ng/mL TGF-β for 24 hours (n = 14 individuals, Fig. 1B). Cells were harvested and gene expression patterns investigated by qRT-PCR, comparing them to mock-treated controls. Each data point represents the mean induction index ± standard deviation over non-activated controls (=1).

**C/D:** The SFs were incubated in tissue culture flasks (= control), in LM-111-coated flasks in the absence of TGF-β (= LM-111), in tissue culture flasks in the presence of 10 ng/mL TGF-β (= TGF-β), or in LM-111 coated flasks in the presence of 10 ng/ml TGF-β (= LM+TGF-β) at a starting density of 10^6 cells per flask. After incubation for 24 h supernatants were harvested and concentrations of MMP-3 (Fig. 1C) and MMP-10 (Fig. 1D) were measured by multiplexed analysis (Luminex®). Data show the mean values ± standard deviations from quadruplicate experiments using supernatants of seven SF populations. Statistically significant differences between MMP expression in activated cells and that in controls are marked by asterisks.

Fig. 2 
*Activation of transcription factors Egr-1, c-Fos, and NFκB by TGF-β and LM-111 signaling*

The SFs were incubated in LM-111-coated flasks and activated by the addition of TGF-β (10 ng/mL) and LM-111 (10 μg/mL, **T+L**, lane 1), or of LM-111 only (10 μg/mL, **L**, lane 2), or they were incubated in normal cell culture flasks in the presence of TGF-β only (10 ng/mL, **T**, lane 3) or in normal cell culture flasks w/o stimulation as controls (**c**, lane 4). After 60 min. of stimulation cell extracts were then investigated for expression of Egr-1 (top) or c-Fos (below), for phosphorylation of NFκB (middle), or for expression of total NFκB (below). Detection of β-actin served as loading control (bottom). The molecular weight (MW) of the proteins (kDa) is marked on the left.

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