EXTENDED REPORT

Attachment to laminin-111 facilitates transforming growth factor β -induced expression of matrix metalloproteinase-3 in synovial fibroblasts

Maik Hoberg, Maximilian Rudert, Thomas Pap, Gerd Klein, Steffen Gay, Wilhelm K Aicher

Ann Rheum Dis 2007;66:446–451. doi: 10.1136/ard.2006.060228

See end of article for authors' affiliations

Correspondence to:
W K Aicher, ZMF, Center for Medical Research,
Eberhard-Karls-University Medical School,
Waldhoernle Strasse 22,
D 72072 Tuebingen,
Germany; aicher@uni-tuebingen.de

Accepted 22 October 2006
Published Online First
23 November 2006

Background: In the synovial membrane of patients with rheumatoid arthritis (RA), a strong expression of laminins and matrix degrading proteases was reported.

Aim: To investigate the regulation of matrix metalloproteinases (MMPs) in synovial fibroblasts (SFs) of patients with osteoarthritis (OA) and RA by attachment to laminin-1 (LM-111) and in the presence or absence of costimulatory signals provided by transforming growth factor β (TGF β).

Methods: SFs were seeded in laminin-coated flasks and activated by addition of TGF β . The expression of genes was investigated by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR), immunocytochemistry and ELISA, and intracellular signalling pathways by immunoblotting, and by poisoning p38MAPK by SB203580, MEK-ERK by PD98059 and SMAD2 by A-83-01.

Results: Attachment of SF to LM-111 did not activate the expression of MMPs, but addition of TGF β induced a fivefold higher expression of MMP-3. Incubation of SF on LM-111 in the presence of TGF β induced a significant 12-fold higher expression of MMP-3 mRNA, and secretion of MMP-3 was elevated 20-fold above controls. Functional blocking of LM-111–integrin interaction reduced the laminin-activated MMP-3 expression significantly. Stimulation of SF by LM-111 and TGF β activated the p38MAPK, ERK and SMAD2 pathways, and inhibition of these pathways by using SB203580, PD98059 or A-83-01 confirmed the involvement of these pathways in the regulation of MMP-3.

Conclusion: Attachment of SF to LM-111 by itself has only minor effects on the expression of MMP-1 or MMP-3, but it facilitates the TGF β -induced expression of MMP-3 significantly. This mode of MMP-3 induction may therefore contribute to inflammatory joint destruction in RA independent of the proinflammatory cytokines interleukin (IL)1 β or tumour necrosis factor (TNF) α .

In the course of rheumatoid arthritis (RA), fibroblast-like cells of the synovial tissue become activated and express elevated levels of matrix-degrading proteases and cytokines.^{1–2} Among the cytokines found to be elevated in RA synovial tissue or fluid, proinflammatory factors such as interleukin (IL)1 β and tumour necrosis factor (TNF) α contribute to synovial pain, swelling and cartilage degradation.^{3–4} Mitogenic factors such as TGF β 1 or bFGF may promote synovial hyperplasia.⁵ However, the synovial fibroblasts (SFs) do not only respond to cytokines. Interactions of these cells with extracellular matrix proteins such as fibronectin, collagens and laminins (LMs) may activate SFs and thus contribute to articular pathologies in RA, synovial inflammation and hyperplasia.^{6–7} It has been shown that fibroblast-like synoviocytes express increased levels of integrins, which correlate with their enhanced binding to matrix proteins.^{8–9} Integrin engagement is known to induce proliferation of SFs.¹⁰ Binding of intact fibronectin to cells via integrins suppressed the induction of matrix metalloproteinases (MMPs), but fibronectin fragments induced elevated MMP expression.¹¹ Further, attachment to fibronectin suppressed p53-mediated apoptosis of fibroblasts.^{7–12} Thus interaction of extracellular matrix compounds with SFs can modulate the joint pathology in RA or other disorders.

LMs are heterotrimeric, extracellular matrix proteins. LMs are composed of α , β and γ chains. In the human genome, five α , three β and three γ chains encoding genes have been identified and characterised. To date, 16 LM isoforms are known, designated LM-111–LM-523.¹³ Elevated expression of different isoforms of LMs was reported in RA specimen when compared with osteoarthritis (OA) samples.⁸ Binding to LM

fragments or LM-derived peptides induced elevated expression of different MMPs in macrophages.^{14–15} In mice, LM-111, previously termed LN-1, is typically expressed early in embryogenesis.¹⁶ In the adult organism, it shows a rather limited expression pattern, but LM-111 was found to be expressed in tumour cells, in normal adult brain, kidney, testis, placenta, adrenal gland, liver and in cultured cells,^{16–17} and it was upregulated in the airway in a murine asthma model.¹⁸ In humans, elevated LM-111 expression was described in endometriotic lesions and anti-LM-111 autoantibodies were found in infertile patients.¹⁹ Enhanced expression of LM-111 was also reported in astrocytes of diseased areas in the frontal cortex of Alzheimer's brain tissues,²⁰ and reduced expression was observed in acinar basement membranes of patients with Sjögren's syndrome,²¹ indicating that LM expression patterns change during pathological processes.

In the RA synovial tissue, elevated levels of integrin receptors and LMs colocalise in the synovial lining layer, with elevated expression of MMPs.⁸ Hence, it was hypothesised that stimuli contributing to enhanced integrin or LM expression might also induce MMPs in synovial cells. However, the metabolic changes in SFs on attachment to LMs have not been studied so far. We reasoned that attachment of RA-SF to LM via integrin could

Abbreviations: JNK, jun-N kinase-1; LM, laminin; mAb, monoclonal antibody; MAP, mitogen-activated protein; MMP, matrix metalloproteinase; OA, osteoarthritis; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; RA, rheumatoid arthritis; SAPK, stress-activated protein kinase; SF, synovial fibroblast; SMAD, transcription factor (homologue to *mothers against DPP* and *SMA* genes); TGF, transforming growth factor; TIMP, tissue inhibitor of metalloproteinase

also modulate the expression of MMPs and, therefore, set out to investigate whether attachment to LMs influences the expression of MMP-1 and MMP-3, two important proteases contributing to cartilage degradation in joints of patients with RA.

We report that TGF β activates SFs to produce, on attachment to LM-111, 20-fold more MMP-3 when compared with mock-treated controls. SFs bind to LM-111 through integrin receptors, and blocking of β 1-integrins reduced this effect of coactivation. TGF β -induced expression of MMP-3 is mediated through the ERK and SMAD2 pathways and enhanced by phosphorylation of p38MAPK and extended or intensified phosphorylation of ERK on LM-111 binding. The SAPK/JNK-1 seems to regulate the expression of MMP-3 as well.

MATERIALS AND METHODS

Cell culture

SFs were isolated from surgical samples of 16 patients with RA diagnosed according to the revised American College of Rheumatology (ACR) criteria for RA and of 12 patients with OA after obtaining written consent²² (table 1 and supplementary files available on <http://ard.bmj.com/supplemental>). The cells were expanded in vitro as described.²³ In brief, fibroblasts were isolated from synovial membranes by cutting the tissue, followed by enzymatic degradation, and expanded for 3 to 4 passages in 10% fetal calf serum-Dulbecco's Modified Eagle's medium. All studies were approved by the local ethics committee.

For induction experiments, cells were stimulated for 24 h with 10 ng/ml recombinant human TGF- β 1, or were incubated on LM-111-coated or LM-511/521-coated flasks. Cells incubated in uncoated cell culture flasks w/o TGF β served as controls.

To modulate the effects of LM, SFs were preincubated with 10 μ g/ml anti-CD29 monoclonal antibodies (anti- β 1 integrin, clone 4B4, Beckman Coulter, Krefeld, Germany) for 60 min on ice to block integrin receptors. Then the cells were incubated for 24 h in the presence or absence of TGF β and LM-111 and changes in the expression of MMPs were enumerated by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR).

Cell signalling

To investigate signal transduction pathways, cells were incubated on LM-111, in the presence of 10 ng/ml recombinant human TGF β , or on LM-111 and TGF β . Untreated cells served as controls. 20 μ M SB203580 was used to block the p38MAPK pathway,²⁴ 20 μ M PD98059 to block the MEK-ERK pathway,²⁵ and ALK-5 blocker A-83-01 to block TGF β dependent SMAD-2 signalling (100 nM, TGF β R-I serine/threonine kinase blocker,²⁶ all from Calbiochem, Schwalbach, Germany) were used. After overnight incubation, cells were harvested to measure changes in the expression of genes by qRT-PCR.

Transcript analysis

The RNA was extracted from cells using a spin column technique (RNeasy, Qiagen, Hilden, FRG) to generate cDNA. Gene-specific cDNA was enumerated by qRT-PCR (qRT-PCR, LightCycler,

Roche, Mannheim, Germany) using commercially available primer pairs (SearchLC, Heidelberg, FRG). The qRT-PCR was performed as touch-down PCR in 35 PCR cycles (denaturing 95°C, 10 s; annealing 5 cycles 68°C, 10 s; then touch-down to 58°C, 10 s; extension 72°C, 16 s). Quantification of GAPDH mRNA and a recombinant standard DNA (Roche) served as controls in each PCR.²⁷ To detect LM-111 mRNA, end point RT-PCRs were performed (30 cycles; denaturing 94°C, 60 s; annealing 56°C, 60 s; extension 72°C, 120 s, respectively), and amplification products were separated and visualised on agarose gels.

ELISA

The secretion of MMPs was measured in SF supernatants by ELISA. The cells were seeded in flasks, activated for 24 h by addition of 10 ng/ml rhTGF β 1, by attachment to LM-111, or with both stimuli. Controls were incubated in uncoated cell culture flasks w/o TGF β or LM-111. Supernatants were harvested after 24 h of incubation, precleared by centrifugation (20 000 $\times$ g, 4°C, 5 min), and MMPs were measured by ELISA (R&D Systems, Minneapolis, Minnesota, USA).

Immunoblot analysis

Cells were stimulated by LM-111 and TGF β , harvested, lysed and subjected to sodium dodecyl sulphate-polyacrylamide agarose gel electrophoresis and immunoblot analysis as described.²⁸ Aliquots of 100 μ g cellular protein were mixed with Laemmli sample buffer,²⁸ denatured and then separated by electrophoresis in 10% sodium dodecyl sulphate-polyacrylamide agarose gel electrophoresis. Proteins were transferred onto nylon membranes, blocked and probed overnight at 4°C with monoclonal antibody (mAb) specific for phospho-p38MAPK (Thr180/Tyr182), total-p38MAPK, phospho-ERK1/2 (Thr202/Tyr204), phospho-JNK/SAPK (Thr183/Thr185), phospho-SMAD2 (Ser465/467; all from Cell Signalling Technology, Beverly, Massachusetts, USA). After rinsing of the membrane, binding of the primary antibodies was detected by peroxidase-labelled goat anti-rabbit-IgG antiserum (Dianova, Hamburg, Germany). The binding of antisera was visualised by enhanced chemoluminescence (ECL, Amersham Biosciences, Freiburg, FRG) and recorded by a luminescence-sensitive CCD camera system (Diana, Raytest Straubenhardt, FRG).

Statistics

Experimental data were analysed by a two-sided modified Student's t test and p values of datasets ≤ 0.05 , 0.01 or 0.001 were considered to be significant. Each data point given represents the mean value $\pm$ SD from individual experiments using SFs from n different patients ($4 \leq n \leq 14$). A detailed description of all specimens including materials and methods can be found in the supplementary files (available at <http://ard.bmj.com/supplemental>).

RESULTS

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

Elevated expression of LM^s and elevated concentrations of growth factors including TGF β are reported in RA synovial

Table 1 Patients included in the study

Diagnosis	Patients (n)	Sex	Mean age (years)	Mean CRP	Mean BSR	DMARD	Steroids	NSAR
RA	16	M 7/F 9	61.8	0.77	29.1	13/16	10/16	4/16
OA	12	M 5/F 7	66.2	0.41	14.3	0/12	1/12	12/12

BSR, blood sedimentation rate; CRP, C reactive protein; DMARD, disease-modifying anti-rheumatic drug; F, female; M, male; NSAR, non-steroidal anti-rheumatics; OA, osteoarthritis; RA, rheumatoid arthritis.

fluid.²⁹ Expression of LM-111 by SFs in vitro was confirmed (see supplementary fig S1 and files available at <http://ard.bmj.com/supplemental>). Attachment of SFs to LM-111-coated flasks without additional stimulation induced only slight but significant increases of MMP-1 (2.3-fold $\pm$ 1.61 induction, $p \leq 0.015$) or MMP-3 mRNA (1.49-fold $\pm$ 0.66 induction, $p \leq 0.037$, fig 1A). This moderate induction was also observed with other batches of LM-111, and in the presence of anti-TGF β antiserum (not shown). In addition, incubation of SFs on LM-511 was investigated as well, but an effect of LN-511 to MMP expression was not observed (not shown). Activation of SFs by 10 ng/ml TGF β 1 resulted in a significant induction of MMP-3 mRNA (5.3-fold $\pm$ 3.13 induction, $p \leq 0.018$), but with no effects on expression of MMP-1 mRNA (fig 1A). Incubation of SFs in LM-111-coated flasks in the presence of 10 ng/ml TGF β augmented the expression of MMP-1 mRNA to some extent (2.73-fold $\pm$ 1 induction, $p \leq 0.004$), but a significantly stronger induction of MMP-3-encoding message (14.21-fold $\pm$ 7.27 induction, $p < 0.004$, fig 1A) was observed. This expression of MMP-3 was significantly higher when compared with expression of MMP-1 (5.2-fold, $p \leq 0.018$). No difference in the induction patterns of MMP-1 or MMP-3 between RA-SF and OA-SF was observed. The induction of tissue inhibitors of metalloproteinases (TIMPs) and other MMPs was less pronounced (not shown).

The release of MMP-1 and MMP-3 was confirmed by ELISA (fig 1B). SFs produced spontaneously some MMP-1 (mean 49 pg/ml), but activation of MMP-1 production was not induced significantly by either method (mean 70–123 ng/ml, fig 1B). SF spontaneously expressed less MMP-3 (mean 25 ng/ml), but substantially more on activation with TGF β (sevenfold $\pm$ 0.92 induction, $p \leq 0.012$, mean 168 ± 16 ng/ml). The production of MMP-3 was further enhanced by attachment to LM-111 in the presence of TGF β (23.19-fold $\pm$ 1.96 induction, $p < 0.004$, mean (SD) 492 ± 73 ng/ml, fig 1B). This LM-111 and TGF β induced release of MMP-3 was significantly higher when compared with the stimulation of SF by LM-111 only (19-fold, $p \leq 0.004$) or that by TGF β only (3.3-fold, $p \leq 0.009$).

LM-facilitated expression of MMP-3 is mediated by β 1-integrins

Among the different integrins, α 6 β 1 integrin represents a major receptor for LM-111 binding on fibroblasts. The expression of α 6 β 1 integrin was verified on SFs in vitro by immunocytochemistry (fig 2A,B). Preincubation of SF with anti- β 1 integrin mAb before seeding the cell into LM-111-coated flasks reduced the number of cells attached in comparison with mock-treated controls (fig 2C,D). This confirmed that the mAb 4B4 interfered with the LM-integrin binding on the cell surface. In SFs coactivated with TGF β and LM-111, the elevated MMP-3 mRNA expression was reduced significantly ($p < 0.05$) by preincubation of SFs with anti- β 1 integrin mAb (fig 3). The data confirm the expression of β 1 integrin on SF in vitro and suggest a functional role of this integrin chain in the regulation of MMP-3 expression.

Activation of ERK phosphorylation by LM-111 attachment and TGF β

Signal transduction pathways contributing to the TGF β and LM-111 induced MMP-3 expression were investigated by immunoblot analysis. A weak phospho-ERK signal was seen in control cells before activation (fig 4A). Addition of 10 ng/ml TGF β to SFs induced a transient phosphorylation of ERK at 10–30 min after induction (fig 4A, top). In contrast, phosphorylation of SMAD2 was not observed in control cells, but only on addition of TGF β (fig 4A, middle). The p38MAPK was detected

in all samples (not shown), but phospho-p38MAPK signals remained below detection levels (fig 4A, bottom).

Coactivation of SFs by TGF β in the presence of LM-111 induced an enhanced and extended phosphorylation of ERK at 10–60 min after induction (fig 4B, top). Again, phospho-SMAD2 was not detected before stimulation, but a long-lasting phosphorylation of SMAD2 was noted (fig 4B, middle). Moreover, phospho-p38MAPK signals were noted at 30 min (fig 4B, bottom) and phosphorylation of SAPK/JNK-1 was induced weakly as well (see supplementary fig S2 and files at <http://ard.bmj.com/supplemental>).

Modulation of signal transduction contributing to elevated expression of MMP-3

We further investigated whether the elevated expression of MMP-3 could be blocked by addition of SB203580, a p38MAPK blocker, or by PD98059, a MEK/ERK blocker, or by A-83-01, interfering with the SMAD2 signalling pathway.²⁶ Attachment of the cells to LM-111 significantly enhanced the TGF β -induced MMP-3 mRNA (3.1-fold, $p < 0.003$; fig 5). Addition of SB203580, PD98059 or A-83-01 before stimulation of the SF reduced the elevated expression of MMP-3 significantly ($p \leq 0.005$), confirming the importance of these pathways in TGF β - and LM-111-dependent induction of MMP-3 (fig 5).

DISCUSSION

Attachment of SFs to LM-111 in the presence of TGF β induced a significant expression of MMP-3, a matrix protease that has been known for its involvement in tissue destruction in RA for a long time.^{30–31} MMP-3 has a rather broad substrate specificity and degrades a variety of extracellular matrix proteins, including proteoglycans, collagens, fibronectin and LMs.³² Hence, MMP-3 plays an important role in the pathomechanisms of RA. The induction of MMP-3 is associated with inflammatory cytokines, especially IL1 β or TNF α .

The additive effects of attachment of SFs to LMs together with TGF β have not been investigated in detail so far, but some hints may have been derived from studies using Matrigel for investigation of the invasive properties of SF.³³ In the present study, we show that attachment of SFs to LM-111 sensitises these cells to TGF β -mediated signalling and significantly increases the expression of MMP-3, a relevant protease in RA.

In recent studies, RA-SF showed a higher invasive score on Matrigel when compared with control cells, and this correlated significantly with a higher expression of MMP-1, MMP-3 and MMP-10.³³ Matrigel contains LM-111 as a major component. It therefore may activate SFs in a way comparable to our experiments. Using a ribozyme approach to limit the expression of MMP-1 in RA-SF confirmed that this increased invasiveness of SF on Matrigel was associated with the expression of MMP-1.³⁴ Our data, presented here, provide a molecular explanation for these findings, as we see an increased expression of MMP-1 in SF on attachment to LM-111. Addition of anti-TGF β antibodies to SF attached to LM-111 did not change the LM-induced effects in our experiments (data not shown). This finding confirms that our LM-111 preparations used did not contain TGF β . For elevated production of MMP-1, cosignalling of LM-111 and TGF β is not sufficient, but LM-111 together with TGF β significantly elevated the expression of both MMP-3 mRNA and protein.

As shown recently, most extracellular proteins derived from articular cartilage or connective tissue enhanced the attachment of SF mediated by integrins on the SF.⁸ Stronger adherence of RA-SF to cartilage oligomeric protein was associated with their expression of α v β 1-integrin.³⁵ In other studies, elevated expression of α 6- and β 1-integrins on RA-SF correlated with their enhanced attachment to matrix proteins.⁹ In mice lacking β 1-integrin expression in chondrocytes, cell

Figure 1 Activation of matrix metalloproteinase (MMP)-1 and MMP-3 expression in synovial fibroblasts (SFs) by laminin (LM)-111 and transforming growth factor (TGF) β . (A) The SFs (10^6 per flask) were incubated on LM-111-coated flasks ($n=14$), in the presence of 10 ng/ml TGF β in normal tissue culture flasks ($n=10$), or in LM-111-coated flasks in the presence of 10 ng/ml TGF β for 24 h ($n=13$). Cells were harvested and mRNA expression patterns of MMP-1 (grey bars) and MMP-3 (black bars) investigated by quantitative reverse transcriptase-polymerase chain in comparison with controls. Attachment of SF to LM-111 in the presence of TGF β induces significant MMP-1 (2.73-fold, $p<0.004$) and MMP-3 (14.21-fold, $p<0.004$) mRNA responses. Induction of MMP-3 was significantly higher (bracket, $p<0.018$) when compared with MMP-1. Each data point represents the mean induction index $\bar{Y} \pm SD$ over not activated controls ($Y_c=1$). (B) Supernatants were harvested after 24 h of incubation of SFs as described above, and MMP-1 (grey bars) and MMP-3 (black bars) concentrations were measured by ELISA. Attachment of SF to LM-111 in the presence of TGF β induces a significant MMP-3 secretion (23.19-fold, $p<0.004$). This coactivation by LM-111 in the presence of TGF β was significantly higher when compared with MMP-3 induction by LM-111 or TGF β alone (brackets, $p \leq 0.004$, and $p \leq 0.009$, respectively). Data show the mean MMP concentrations (pg/ml) $\pm SD$ from quadruplicate experiments using supernatants of SFs from three donors.

motility and proliferation were impaired,³⁶ and the expression of MMP-3, MMP-9 and MMP-13 was significantly reduced in these cells.³⁷ We corroborated these results as treatment of SF with mAb 4B4 blocked the attachment of SF to LM-111 and significantly reduced the induction of MMP-3. Further, the induction of MMP-3 described here was induced by TGF β and LM-111, without addition of IL1 β or TNF α to the cells.

TGF β is a multifunctional factor regulating cell division and differentiation. Depending on the individual experimental context, TGF β may activate or reduce proliferation and expression of target genes in a dose- and time-dependent manner.³⁸ This factor has also been shown to influence the homeostasis of the extracellular matrix.³⁹ TGF β may repress the PMA-induced expression of MMP-1 by SMAD signalling.⁴⁰ Without prestimulation by PMA, TGF β activates the expression of MMPs including MMP-3 using a p38MAPK signalling pathway.⁴¹ In our experiments, addition of TGF β activated the ERK and SMAD2 but not the p38MAPK pathways. The importance of SMAD2 for regulation of MMPs is supported by the fact that SMAD2 transgenic mice show elevated expression of MMP-1 and MMP-2.⁴²

Activation of ERK1/2 by expression of a recombinant constitutively active MEK1 was sufficient to induce MMP-1

Figure 2 Detection of integrin expression and integrin-mediated attachment by synovial fibroblasts (SFs). Expression of CD49f (integrin $\alpha 6$ -chain) and CD29 (integrin $\beta 1$ -chain) on SFs is shown by staining of the cells with monoclonal antibody (mAb) GoH3 (1:100) followed by Cy3-labelled anti-rat serum (1:1000, A), and with mAb 4B4 (1:100, B) followed by Cy2-labelled anti-mouse serum (magnification $\times 20$, $n=3$). The SFs attach to LM-111-coated surfaces within 60–90 min of inoculation (C, magnification $\times 4$). Preincubation of SF with 10 μ g/ml mAb 4B4 before seeding reduces the number of cells attached to LM-111 (D, magnification $\times 4$, $n=2$).

and MMP-3, but strong expression of MMP-1 and MMP-3 was observed when ERK1/2 were activated in combination with either SAPK/JNK or p38MAPK kinases. Further, ERK was associated with the induction of transcription factors *c-jun*, *junB* and *c-fos*, whereas p38 α regulated MMP mRNA stability.²⁴ In our experiments, SF from one patient failed to induce the expression of MMP-3 when attached to LM-111 in the presence of TGF β . Investigation of these cells showed that phosphorylation of ERK had not occurred (data not shown). However, attachment to LM-111 seems to address the ERK pathway in different mesenchymal cells.⁴³

In our experiments, phosphorylation of p38MAPK was not observed by TGF β alone but on coactivation by LM-111 and TGF β . We also quantified less MMP-3 mRNA after blocking p38MAPK activity in activated SF by the kinase blocker

Figure 3 Modulation of matrix metalloproteinase (MMP)-3 expression by blocking $\beta 1$ -integrin on synovial fibroblasts (SFs). SFs were harvested and 10^6 cells were inoculated on normal plates (= controls), on LM-111-coated plates in the presence of TGF β without ($\emptyset$) or after preincubation with anti- $\beta 1$ integrin monoclonal antibody (mAb) (4B4). Pretreatment with mAb 4B4 reduced MMP-3 mRNA induction significantly (bracket, $p<0.05$). Data show the mean values $\pm SD$ of individual inductions using SF from four donors.

Figure 4 Signal transduction pathways activated by transforming growth factor (TGF) β , and laminin (LM)-111 and TGF β coactivation. The rheumatoid arthritis-synovial fibroblasts (RA-SFs) were incubated for different periods of time ranging from 10 min to 22 h with 10 ng/ml recombinant human TGF β 1 (A) or by LM-111 and TGF β coactivation (B). Phosphorylation of ERK1/2, SMAD2 and p38MAP kinases was investigated by immunoblot. A recombinant protein mix ($\emptyset$) and cells w/o TGF β activation (0) served as negative controls (left), and a phosphorylated molecular size protein marker (M) was used to confirm the transfer of proteins (right). Addition of TGF β induced a transient phosphorylation of ERK (A, top) and SMAD2 (A, middle), but with no p38MAPK signal (A, bottom). By coactivation using TGF β in the presence of LM-111, an extended ERK-phosphorylation (B, top), transient SMAD2 (B, middle) and p38MAPK (B, bottom) signals were seen. The molecular weight of proteins (MW) is shown in kDa. For detection of phosphorylated p38MAPK, extracts of UV-treated NIH/3T3 cells were used as positive controls (P).

SB203580, corroborating that p38MAPK is involved in LM-111 and TGF β induced expression of MMP-3 expression. A very weak signal of phospho-SAPK/JNK was noted in LM-111 and TGF β activated SF, but not in SF activated by TGF β alone (see supplementary fig S2 and files at <http://ard.bmj.com/supplemental>), indicating that additional MAP kinases are addressed by these stimuli.⁴⁴ However, a detailed investigation of the contribution of other MAP kinases to the TGF β and LM-111 induced expression of MMPs in SF must await additional experiments.⁴⁵

In summary, we show that attachment of RA-SF and OA-SF to LM-111 in the presence of TGF β is sufficient to yield a significant MMP-3 expression. Our data corroborate the importance of ERK, p38MAPK and SMAD2 signalling for efficient MMP-3 induction by TGF β and attachment to LM-111 in SF. Other cytokines such as IL1 β or TNF α were not necessary for the expression of MMP-3 under these conditions. We conclude that pathological expression of LMs in the synovium contributes to the elevated expression of MMP-3 in RA. This pathway may also explain in part the progression of cartilage destruction when patients with RA are treated with anti-TNF (or anti-IL1) therapies, as levels of TGF β did not change during etanercept treatment.⁴⁶

Figure 5 Modulation of matrix metalloproteinase (MMP)-3 activation by blocking the signal transduction pathway. The rheumatoid arthritis-synovial fibroblasts (RA-SFs) were activated by incubation on laminin (LM)-111-coated flasks by addition of transforming growth factor (TGF) β , or after coactivation in the presence of SB203580, PD98059 or A-83-01. The MMP-3 encoding mRNA amounts were enumerated by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) in activated cells in comparison with untreated controls (n=8, left). Coactivation induced MMP-3 transcripts significantly (3.1-fold $\pm$ 1.5 induction, $p < 0.005$, n=8). Transcript amounts encoding MMP-3 were reduced significantly by addition of SB203580 (4.2-fold, $p \leq 0.005$, n=5), PD98059 (3.9-fold, $p \leq 0.001$, n=8) or A-83-01 (2.3-fold, $p < 0.004$, n=7).

ACKNOWLEDGEMENTS

We thank Mrs T Abruzzese, A Hack and M Weis-Klemm for excellent experimental support, Dr D Alexander for technical advice, and Prof Klaus von der Mark (University of Erlangen, FRG) for providing purified murine EHS LM-111 for coating of culture flasks.

Supplementary files are available on <http://ard.bmj.com/supplemental>

Authors' affiliations

Maik Hoberg, Maximilian Rudert, Department of Orthopedic Surgery, CRONA University Hospital, Tübingen, Germany

Thomas Pap, Division of Molecular Medicine of Musculoskeletal Tissue, Department of Orthopedics, Münster University Hospital, Münster, Germany

Gerd Klein, Center for Medical Research (ZMF), Section for Transplantation Immunology, University of Tübingen, Tübingen, Germany
Steffen Gay, WHO Center for Exp. Rheumatology, University Hospital Zürich, and Zürich Center for Integrative Human Physiology (ZIHP), Zürich, Switzerland

Wilhelm K Aicher, Center for Medical Research (ZMF), Department of Orthopaedic Surgery, Eberhard-Karls-University Medical School, Tübingen, Germany

Funding: This project was supported by DFG-grants Ai16/10-2 and Ai16/14-1 to WKA and in part by institutional funding. SG was supported by the European Community's FP6 funding. This publication reflects only the authors' views. The European Community is not liable for any use that may be made of the information herein. There are no disclosures for this study by any of the authors.

Competing interests: None declared.

REFERENCES

- 1 Pap T, Müller-Ladner U, Gay RE, Gay S. Fibroblast biology: role of synovial fibroblasts in the pathogenesis of rheumatoid arthritis. *Arthritis Res Ther* 2000;2:361-7.
- 2 Firestein GS. Evolving concepts of rheumatoid arthritis. *Nature* 2003;423:356-61.
- 3 Mengshol JA, Vincenti MP, Coon CI, Barchowsky A, Brinckerhoff CE. Interleukin-1 induction of collagenase 3 (matrix metalloproteinase 13) gene expression in chondrocytes requires p38, c-Jun N-terminal kinase, and nuclear factor kappaB.

- differential regulation of collagenase 1 and collagenase 3. *Arthr Rheum* 2000;**43**:801–11.
- 4 **Görtz B**, Hayer S, Tuerck B, Zwerina J, Smolen J, Schett G. Tumour necrosis factor activates the mitogen-activated protein kinases p38 α and ERK in the synovial membrane in vivo. *Arthritis Res Ther* 2005;**7**:R1140–7.
 - 5 **Bucala R**, Ritchlin C, Winchester C, Cerami A. Constitutive production of inflammatory and mitogenic cytokines by rheumatoid synovial fibroblasts. *J Exp Med* 1991;**173**:569–74.
 - 6 **Shiozawa S**, Yoshihara R, Kuraki Y, Fujita T, Shiozawa K, Imura S. Pathogenic importance of fibronectin in the superficial region of articular cartilage as a local factor for the induction of pannus extension on rheumatoid articular cartilage. *Ann Rheum Dis* 1992;**51**:869–73.
 - 7 **Kitagawa A**, Miura Y, Saura R, Mitani M, Ishikawa H, Hashimoto A, et al. Anchorage on fibronectin via VLA-5 (alpha5beta1 integrin) protects rheumatoid synovial cells from Fas-induced apoptosis. *Ann Rheum Dis* 2006;**65**:721–7.
 - 8 **Kontinen YT**, Li TF, Xu JW, Tagaki M, Pirila L, Silvennoinen T, et al. Expression of laminins and their integrin receptors in different conditions of synovial membrane and synovial membrane-like interface tissue. *Ann Rheum Dis* 1999;**58**:683–90.
 - 9 **Rinaldi N**, Schwarz-Eywill M, Weis D, Leppelmann-Jansen P, Lukoschek M, Keilholz U, et al. Increased expression of integrins on fibroblast-like synoviocytes from rheumatoid arthritis in vitro correlates with enhanced binding to extracellular matrix proteins. *Ann Rheum Dis* 1997;**56**:45–51.
 - 10 **Sarkissian M**, Lafyatis R. Integrin engagement regulates proliferation and collagenase expression of rheumatoid synovial fibroblasts. *J Immunol* 1999;**162**:1772–9.
 - 11 **Huhtala P**, Humphries MJ, McCarthy JB, Tremble PM, Werb Z, Damsky CH. Cooperative signalling by alpha5beta1 and alpha4beta1 integrins regulates metalloproteinase gene expression in fibroblasts adhering to fibronectin. *J Cell Biol* 1995;**129**:867–79.
 - 12 **Ilic D**, Almeida EAC, Schlaepfer DD, Dazin P, Aizawa S, Damsky CH. Extracellular matrix survival signals transduced by focal adhesion kinase suppress p53-mediated apoptosis. *J Cell Biol* 1998;**143**:547–60.
 - 13 **Aumailley M**, Bruckner-Tuderman L, Carter WG, Deutzmann R, Edgar D, Ekblom P, et al. A simplified laminin nomenclature. *Matrix Biol* 2005;**24**:326–32.
 - 14 **Adair-Kirk T**, Atkinson J, Broeckelmann T, Dor M, Tryggvason K, Miner J, et al. A site on laminin $\alpha 5$, AQARSAASKVKVSMKV, induces inflammatory cell production of MMP-9 and chemotaxis. *J Immunol* 2003;**171**:398–406.
 - 15 **Faisal Khan KM**, Laurie GW, McCaffrey TA, Falcone DJ. Exposure of cryptic domains in the alpha 1-chain of laminin-1 by elastase stimulates macrophages urokinase and matrix metalloproteinase-9 expression. *J Biol Chem* 2002;**277**:13778–86.
 - 16 **Ekblom P**, Lonai P, Talts JF. Expression and biological role of laminin-1. *Matrix Biol* 2003;**22**:35–47.
 - 17 **Sasaki T**, Giltay R, Talts U, Timpl R, Talts JF. Expression and distribution of laminin $\alpha 1$ and $\alpha 2$ chains in embryonic and adult mouse tissue: an immunochemical approach. *Exp Cell Res* 2002;**275**:185–99.
 - 18 **Christie PE**, Jonas M, Tsai CH, Chi EY, Henderson WR Jr. Increase in laminin expression in allergic airway remodelling and decrease by dexamethasone. *Eur Resp J* 2004;**24**:107–15.
 - 19 **Inagaki J**, Kondo A, Lopez LR, Shoenfeld Y, Matsuura E. Anti-laminin-1 autoantibodies, pregnancy loss and endometriosis. *Clin Dev Immunol* 2004;**11**:261–6.
 - 20 **Palu E**, Liesi P. Differential distribution of laminins in Alzheimer disease and normal human brain tissue. *J Neurosci Res* 2002;**69**:243–56.
 - 21 **Laine M**, Virtanen I, Salo T, Kontinen YT. Segment-specific but pathologic laminin isoform profiles in human labial salivary glands of patients with Sjögren's syndrome. *Arthr Rheum* 2004;**50**:3968–73.
 - 22 **Arnett FC**, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthr Rheum* 1988;**31**:315–24.
 - 23 **Aicher WK**, Heer AH, Trabandt A, Bridges SL Jr, Schroeder HW Jr, Stransky G, et al. Overexpression of zinc-finger transcription factor Z-225/Egr-1 in synoviocytes from rheumatoid arthritis patients. *J Immunol* 1994;**152**:5940–8.
 - 24 **Reunanen N**, Li SP, Ahonen M, Foschi M, Han J, Kahari VM. Activation of p38 alpha MAPK enhances collagenase-1 (matrix metalloproteinase (MMP)-1) and stromelysin-1 (MMP-3) expression by mRNA stabilization. *J Biol Chem* 2002;**277**:32360–8.
 - 25 **Alessi DR**, Cuenda A, Cohen P, Dudley DT, Saltiel AR. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. *J Biol Chem* 1995;**270**:27489–94.
 - 26 **Tojo M**, Hamashima Y, Hanyu A, Kajimoto T, Saitoh M, Miyazono K, et al. The ALK-5 inhibitor A-83-01 inhibits Smad signaling and epithelial-to-mesenchymal transition by transforming growth factor-beta. *Cancer Sci* 2005;**96**:791–800.
 - 27 **Alexander D**, Judex M, Meyringer R, Weis Klemm M, Gay S, Müller-Ladner U, et al. Transcription factor Egr-1 activates collagen expression in immortalized fibroblasts or fibrosarcoma cells. *Biol Chem* 2002;**383**:1845–53.
 - 28 **Aicher WK**, Alexander D, Haas C, Kuchen S, Pagenstecher A, Gay S, et al. Transcription factor early growth response 1 activity up-regulates expression of tissue inhibitor of metalloproteinase 1 in human synovial fibroblasts. *Arthr Rheum* 1989;**169**:291–6.
 - 29 **Fava R**, Olsen N, Kseki-Oja J, Moses H, McCabe SM. Active and latent forms of transforming growth factor β activity in synovial effusions. *J Exp Med* 1989;**169**:291–6.
 - 30 **Gravallese EM**, Darling JM, Ladd AL, Katz JN, Glimcher LH. In situ hybridization studies of stromelysin and collagenase messenger RNA expression in rheumatoid synovium. *Arthr Rheum* 1991;**34**:1076–84.
 - 31 **Brinckerhoff CE**. Joint destruction in arthritis: metalloproteinases in the spotlight. *Arthr Rheum* 1991;**34**:1073–5.
 - 32 **Okada Y**, Nagase H, Harris ED Jr. A metalloproteinase from human rheumatoid synovial fibroblasts that digests connective tissue matrix components. Purification and characterization. *J Biol Chem* 1986;**261**:14245–55.
 - 33 **Tolboom TCA**, Pieterman E, van der Laan WH, Toes REM, Huidekoper AL, Nelissen RGHH, et al. Invasive properties of fibroblast-like synoviocytes: correlation with growth characteristics and expression of MMP-1, MMP-3 and MMP-10. *Ann Rheum Dis* 2002;**61**:975–80.
 - 34 **Rutkauskaite E**, Zacharias W, Schedel J, Müller-Ladner U, Mawrin C, Seemayer CA, et al. Ribozymes that inhibit the production of matrix metalloproteinase 1 reduce the invasiveness of rheumatoid arthritis synovial fibroblasts. *Arthr Rheum* 2004;**50**:1448–56.
 - 35 **Neidhart M**, Zaucke F, von Knoch R, Jungel A, Michel BA, Gay RE, et al. Galectin-3 is induced in rheumatoid arthritis synovial fibroblasts after adhesion to cartilage oligomeric matrix protein. *Ann Rheum Dis* 2005;**64**:419–24.
 - 36 **Aszodi A**, Hunziker EB, Brakebusch C, Fässler R. Beta1 integrins regulate chondrocyte rotation, G1 progression, and cytokinesis. *Genes Dev* 2003;**17**:2465–79.
 - 37 **Aszodi A**. Poster # 90 at the Annual Meeting of the German Connective Tissue Society, Cologne 2005.
 - 38 **Wahl SM**. Transforming growth factor beta: the good, the bad, and the ugly. *J Exp Med* 1994;**180**:1587–90.
 - 39 **Massague J**, Blain SW, Lo RS. TGF-beta signaling in growth control, cancer, and heritable disorders. *Cell* 2000;**103**:295–309.
 - 40 **Hall MC**, Young DA, Waters JG, Rowan AD, Chantry A, Edwards DR, et al. The comparative role of activator protein 1 and Smad factors in the regulation of TIMP-1 and MMP-1 gene expression by transforming growth factor-beta 1. *J Biol Chem* 2003;**278**:10304–13.
 - 41 **Kim HS**, Shang T, Chen Z, Pflugfelder SC, Li DQ. TGF-beta1 stimulates production of gelatinase (MMP-9), collagenases (MMP-1, -13) and stromelysins (MMP-3, -10, -11) by human corneal epithelial cells. *Exp Eye Res* 2004;**79**:263–74.
 - 42 **Dixon IM**, Hao J, Reid NL, Roth JC. Effect of chronic AT(1) receptor blockade on cardiac Smad overexpression in hereditary cardiomyopathic hamsters. *Cardiovasc Res* 2000;**46**:286–97.
 - 43 **Klees RF**, Salaszyk RM, Kingsley K, Williams WA, Boskey A, Plopper GE. Laminin-5 induces osteogenic gene expression in human mesenchymal stem cells through an ERK-dependent pathway. *Mol Biol Cell* 2005;**16**:881–90.
 - 44 **Cha H-S**, Boyle DL, Inoue T, Shoot R, Tak PP, Pine P, et al. A novel tyrosine kinase inhibitor blocks c-Jun N-terminal kinase-mediated gene expression in synoviocytes. *J Pharmacol Exp Ther* 2006;**317**:571–8.
 - 45 **Inoue T**, Boyle DL, Corr M, Hammaker D, Davis RJ, Flavell RA, et al. Mitogen-activated protein kinase 3 is a pivotal pathway regulating p38 activation in inflammatory arthritis. *Proc Natl Acad Sci USA* 2006;**103**:5484–9.
 - 46 **Drynda S**, Kuhne C, Kekow J. Soluble tumour necrosis factor receptor treatment does not affect raised transforming growth factor beta levels in rheumatoid arthritis. *Ann Rheum Dis* 2002;**61**:254–6.