Regulation of matrixmetalloproteinase-3 and matrixmetalloproteinase-13 by SUMO-2/3 through the transcription factor NF- B

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Abstract: OBJECTIVE: Based on previous data that have linked the small ubiquitin-like modifier-1 (SUMO-1) to the pathogenesis of rheumatoid arthritis (RA), we have investigated the expression of the highly homologous SUMO family members SUMO-2/3 in human RA and in the human tumour necrosis factor transgenic (hTNFtg) mouse model of RA and studied their role in regulating disease specific matrixmetalloproteinases (MMPs). METHODS: Synovial tissue was obtained from RA and osteoarthritis (OA) patients and used for histological analyses as well as for the isolation of synovial fibroblasts (SFs). The expression of SUMO-2/3 in RA and OA patients as well as in hTNFtg and wild type mice was studied by PCR, western blot and immunostaining. SUMO-2/3 was knocked down using small interfering RNA in SFs, and TNF- induced MMP production was determined by ELISA. Activation of nuclear factor- B (NF- B) was determined by a luciferase activity assay and a transcription factor assay in the presence of the NF- B inhibitor BAY 11-7082. RESULTS: Expression of SUMO-2 and to a lesser extent of SUMO-3 was higher in RA tissues and RASFs compared with OA controls. Similarly, there was increased expression of SUMO-2 in the synovium and in SFs of hTNFtg mice compared with wild type animals. In vitro, the expression of SUMO-2 but not of SUMO-3 was induced by TNF- . The knockdown of SUMO-2/3 significantly increased the TNF- and interleukin (IL)-1 induced expression of MMP-3 and MMP-13, accompanied by increased NF- B activity. Induction of MMP-3 and MMP-13 was inhibited by blockade of the NF- B pathway. TNF- and IL-1 mediated MMP-1 expression was not regulated by SUMO-2/3. CONCLUSIONS: Collectively, we show that despite their high homology, SUMO-2/3 are differentially regulated by TNF- and selectively control TNF- mediated MMP expression via the NF- B pathway. Therefore, we hypothesise that SUMO-2 contributes to the specific activation of RASF.

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REGULATION OF MATRIX METALLOPROTEINASE -3 AND -13 BY SUMO-2/3 THROUGH THE TRANSCRIPTION FACTOR NF-κB

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ABSTRACT

OBJECTIVE: Based on previous data that have linked the small ubiquitin-like modifier-1 (SUMO-1) to the pathogenesis of rheumatoid arthritis (RA), we have investigated the expression of the highly homologous SUMO family members SUMO-2/3 in human RA and in the hTNFtg mouse model of RA and studied their role in regulating disease specific matrixmetalloproteinases (MMPs).

METHODS: Synovial tissue was obtained from RA and osteoarthritis (OA) patients and used for histological analyses as well as for the isolation of synovial fibroblasts (SFs). The expression of SUMO-2/3 in RA and OA patients as well as in hTNFtg and wt mice was studied by PCR, Western blot and immunostaining. SUMO-2/3 was knocked down using siRNA in SFs, and TNF-alpha induced MMP production was determined by ELISA. Activation of NF-κB was determined by a luciferase activity assay and a transcription factor assay in the presence of the NF-κB inhibitor BAY 11-7082.

RESULTS: Expression of SUMO-2 and to a lesser extent of SUMO-3 was higher in RA tissues and RASFs compared to osteoarthritis controls. Similarly, there was increased expression of SUMO-2 in the synovium and in SFs of hTNFtg mice compared to wt animals. *In vitro*, the expression of SUMO-2 but not of SUMO-3 was induced by TNF-alpha. The knockdown of SUMO-2/3 significantly increased the TNF-alpha and IL-1beta induced expression of MMP-3 and MMP-13, accompanied by increased NF-κB activity. Induction of MMP-3 and MMP-13 was inhibited by blockade of the NF-κB pathway. TNF-alpha and IL-1beta mediated MMP-1 expression was not regulated by SUMO-2/3.

CONCLUSIONS: Collectively, we show that despite their high homology, SUMO-2/3 are differentially regulated by TNF-alpha and selectively control TNF-alpha mediated MMP expression via the NF-κB pathway. Therefore, we hypothesize that SUMO-2 contributes to the specific activation of RASF.

INTRODUCTION

Hyperplasia of the synovium, infiltration of different inflammatory cells and invasion of synovial fibroblasts into joint structures are characteristic features of human rheumatoid arthritis (RA)(1, 2). Stable activation of RA synovial fibroblasts (RASFs) plays a major role in the rheumatoid destruction of the cartilage matrix(3), mainly through the expression of MMPs which are regulated by inflammatory cytokines such as TNF-alpha and IL-1(4, 5). Small ubiquitin-related modifiers (SUMOs) belong to a subfamily of ubiquitin-like proteins. Posttranslational modification of proteins by SUMO is involved in a variety of cellular processes, including protein localisation(6, 7), transcriptional regulation(8, 9), protein stability, cell survival and death(10-12). There are four mammalian SUMO genes: SUMO-1, SUMO-2, SUMO-3 and SUMO-4. SUMO-2 and SUMO-3 share 96% amino acid sequence identity, while SUMO-1 is approximately 50% similar to SUMO-2 and SUMO-3; thus SUMO-2 and SUMO-3 are referred as SUMO-2/3(13). SUMO-2 and SUMO-3 can be modified *in vivo* by SUMO-1 and SUMO-2/3(14, 15). Interestingly, SUMO can not only be targeted by posttranslational modifications belonging to the ubiquitin family but also by acetylation and phosphorylation. For SUMO-2 it was shown that the lysine residues K11, K32 and K41 can be modified by ubiquitin(16, 17). A recent study, showed that SUMO-3 can be phosphorylated at serine 2, while SUMO-2 cannot be phosphorylated because it has an alanine at this position(18). Thus, these may be functional differences between SUMO-2 and SUMO-3 which, however, have not been investigated in detail.

In line with their important role in cellular functions, the expression and activity of MMPs are tightly regulated at multiple levels of gene transcription, synthesis, and extracellular activity. Previously, it was shown that the MMP-1 promoter is hyperacetylated in RASF compared to SFs from OA patients (OASF). Interestingly, overexpression of SUMO specific protease-1 (SENP-1), which is downregulated in RASF, leads to normalization of the acetylation pattern

in the MMP-1 promoter and decreases the production of MMP-1(19). Cytokines such as TNF-alpha and IL-1beta stimulate the production of MMPs through the activation of cellular signaling pathways involving mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB)(20).

In the current study, we have investigated the expression of SUMO-2/3 in human RASFs and in human TNF-alpha transgenic (hTNFtg) mice(21), where transgenic overexpression of the human tumor necrosis factor alpha (hTNF-alpha) leads to a chronic inflammatory and destructive polyarthritis. Here, we demonstrate for the first time a role of SUMO-2/3 in the activation of TNF-alpha mediated MMP-3 and MMP-13 expression in RASFs. We show that SUMO-2/3 is part of regulatory mechanisms that limit expression of distinct MMP´s by inhibiting the activation of NF-κB.

RESULTS

Elevated expression of SUMO-2/3 in the synovium and in synovial fibroblasts from patients with RA

First, we addressed the question of whether SUMO-2/3 is expressed in synovial fibroblasts from the inflamed synovium of human RA patients. As shown in Fig.1a, immunohistochemical stainings of human RA synovial tissue revealed high expression of SUMO-2/3. The proteins are predominantly localised in the synovial lining layer as well as in the sublining layer, whereas only marginal expression was found in synovial tissue of patients with OA. For further analysis of the subcellular distribution of SUMO-2/3 in RASFs and OASFs, immunocytochemical stainings were performed. SUMO-2/3 was found predominantly within the nucleus (Fig.1b). When compared to OASFs, RA cells exhibited a markedly enhanced nuclear staining for SUMO-2/3. These data were confirmed by Western blot analysis at protein level (Fig.1c) using a SUMO-2 specific antibody (Suppl.Fig.S3) which

additionally recognizes SUMO-3. Interestingly, analysis by quantitative real-time PCR in Fig.1d shows an increased expression of SUMO-2 in fibroblasts isolated from RA synovium compared to the control cells from patients with OA. The mRNA level of SUMO-3 was far less increased in RASFs compared to in OASFs.

TNF-alpha regulates SUMO-2, but not SUMO-3

The expression data led us to investigate whether SUMO-2/3 is regulated by inflammatory cytokines such as TNF-alpha. To this end, we stimulated synovial fibroblasts from RA patients (n=5) with recombinant human TNF-alpha and analysed whether there is a dose-response effect of TNF-alpha (0,1; 1; 10 and 100 ng/ml) on SUMO-2 and SUMO-3 at transcriptional level and subsequently analysed the expression of SUMO-2/3 at protein level. As shown in Fig.1e, TNF-alpha (100ng/ml) stimulation of synovial fibroblasts from RA patients for 24h resulted in an upregulation of SUMO-2/3 at protein level. In addition, the conjugation of target proteins by SUMO-2/3 was increased. Interestingly, TNF-alpha was only able to induce a dose-dependent increase in SUMO-2 mRNA expression, whilst SUMO-3 remained unaffected (Fig.1f). These data suggest that only SUMO-2 is regulated by TNF-alpha and that although SUMO-2 and SUMO-3 proteins are closely similar in their amino acid sequence, they perform different functions during inflammatory conditions. Next, we investigated the expression of SUMO-2/3 in human TNF-transgenic (hTNFtg) mice(21), as a model for inflammatory polyarthritis. For this, we first performed immunohistochemical stainings of hTNFtg mice in tissue sections. As shown in Fig.2a, we confirmed the increased expression of SUMO-2/3 in tissue sections from hTNFtg mice as well as in Western blot analysis of primary synovial fibroblasts in comparison to wild type samples (Fig.2c). SUMO-2 mRNA level was strongly increased in synovial fibroblasts from hTNFtg mice compared to wt mice, but the SUMO-3 RNA level was not increased to the same extent (Fig.2b).

Knockdown of SUMO-2/3 affects MMP-3 and MMP-13 expression in synovial fibroblasts from RA and OA patients

To investigate the role of elevated SUMO-2/3 expression in RA, we analysed the production of MMP-1, MMP-3 and MMP-13 in synovial fibroblasts obtained from RA and OA patients. As expected, the levels of MMP-1, MMP-3 and MMP-13 expression were strongly upregulated in RASF compared to in OASF. Interestingly, we found that knockdown of both SUMO-2 and SUMO-3 using specific siRNA, leads to a significant upregulation of MMP-3 and MMP-13 after stimulation with TNF-alpha in comparison to the cells transfected with mock siRNA (Fig.3a) without changing TNF-Receptor I expression (Suppl.Fig.1a). In contrast, the production of MMP-1 was not affected by silencing SUMO-2/3 (Fig.3a). These observations could be confirmed at the transcriptional level by semiquantitative PCR (Fig.3b). We found a significant upregulation of TNF-alpha induced MMP-3 expression in RASFs, but not of MMP-1 after knockdown of SUMO-2/3. In addition to TNF-alpha stimulation we used the cytokine IL-1beta, where a similar effect on MMP-3 and MMP-13 production after SUMO-2/3 knockdown was observed (Suppl. Fig.2a).

SUMO-2/3 regulates the activity of MMP-3 and MMP-13 regulation via the NF-κB pathway

Based on our findings, we analysed underlying mechanisms of how SUMO-2/3 regulates MMP-3 and MMP-13. As MMP-3 and MMP-13 are regulated predominantly through the NF-κB pathway, we analysed the functional consequences of SUMO-2/3 knockdown on the activity of NF-κB. Firstly, in light of data showing an effect of SUMO-1 on NF-κB activity via the modification of IκB-alpha(22), we measured the expression of SUMO-1 after SUMO-2/3 knockdown using real time pcr and found that the expression of SUMO-1 was not affected (Suppl. Fig.1b). The direct role of SUMO-2/3 in NF-κB activation was assessed following SUMO-2/3 knockdown using siRNA. As shown in Fig.4a and Fig.4b, the loss of SUMO-2/3 enhanced the activity of NF-κB after stimulation with 100 ng/ml TNF-alpha for 16h. Transfection of HeLa cells with siRNA against SUMO-2/3 resulted in a knockdown that was detectable at the protein level (Fig.4a). These results indicate that downregulation of

SUMO-2/3 positively regulates NF-κB transcriptional activity and hence may increase the expression of MMP-3 and MMP-13. Based on this observation, we used the NF-κB inhibitor BAY 11-7082 to determine whether upregulation of MMP-3 and MMP-13 via SUMO-2/3 was indeed dependent on the NF-κB pathway. Figure 5a and 5b show that inhibition of TNF-alpha induced phosphorylation of IκB-alpha and NF-κB activation using the BAY 11-7082 inhibitor totally blocked SUMO-2/3 induced MMP-3 and MMP-13 production. The MMP-13 levels after treatment with BAY 11-7082 were undetectable.

MATERIALS AND METHODS

Isolation of synovial fibroblasts and cell culture

All studies were approved by the ethics committees of the Medical University of Vienna and the University Hospital Muenster. Samples of synovial tissues from patients with RA or OA were obtained from joint replacement surgery and provided by the Department of Orthopaedic Surgery, St. Joseph Hospital, Sendenhorst, Germany, the Department of Orthopaedic Surgery of the University of Magdeburg School of Medicine, Magdeburg, Germany, and the Department of Orthopaedic Surgery, KMG-Kliniken Kyritz, Germany. Murine synovial fibroblasts were isolated from tarsus of hind paws of wild type and hTNFtg mice. Synovial fibroblasts were isolated by enzymatic digestion and were cultured in DMEM (Invitrogen) + 10% FCS (PAA) + antibiotics/antimycotics (PAA). All cells were cultured in DMEM with 10% FCS at 37°C and 5% CO_2 from passage 3 to 5 for all experiments.

Immunohistochemical analysis of human and mouse synovial tissue

For immunohistochemical analysis, deparaffinized, ethanol-dehydrated tissue sections of human synovial tissue were pretreated with trypsin, blocked with 10% horse serum, stained with antibodies against SUMO-2/3 (Zymed Laboratories), and counterstained with methyl green (Sigma-Aldrich).

siRNAs (small interfering RNAs) and Transfection

A single siRNA was used to suppress both SUMO-2 and SUMO-3 expression (SUMO-2+3 siRNA). Negative control siRNA (target sequence AATTCTCCGAACGTGTCACGT) were synthesized by QIAGEN. Transfection of siRNAs was performed by using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. Synovial fibroblasts were transfected 48h before further analysis.

Semi-quantitative and quantitative RT-PCR

Total RNAs from RASF/OASF were prepared by RNeasy miniprep kit (QIAGEN) according to the manufacturers protocol. cDNAs were synthesized by reverse-transcriptase. Quantitative real-time PCR was performed using the Bio-Rad iQ2 system.

The following primers were used: MMP-1 sense 5`-CTGAAGGTGATGAAGCAGCC-3´, antisense 5´-AGTCCAAGAGAATGGCCGAG-3´; MMP-3 sense 5´-CTCACAGACCTGACTCGGTT-3´, antisense 5´-CACGCCTGAAGGAAGAGATG-3´; SUMO-1 sense 5´-GACCAGGAGGCAAAACCTTCAACTG-3,´ antisense 5-TCTCACTGCTATCCTGTCCAATGACT 3`; SUMO-2 sense 5´-CACACCTGCACAGTTGGAAATGG-3´, antisense 5´-ACCTCCCGTCTGCTGTTGGAA -3´; SUMO-3 sense 5´-GACACTCCAGCACAGCTGGAGATG-´3, antisense 5´-AAACTGTGCCCTGCCAGGCT-3´; GAPDH sense 5´-GGTGAAGGTCGGAGTCAACGGATT-3´,
antisense 5´-TGGTGACCAGGCGCCCAATACGA -3´; β-Actin sense 5´-CCACACCCGCCACCAGTTCG -3´, antisense 5´-TGCTCTGGGCCTCGTCACCC -3´.

Protein extraction and Western blot analysis

Total protein from human and murine synovial fibroblasts were extracted in RIPA lysis buffer (50mM Tris/HCl, 150mM NaCl, 1mM EDTA, 0,1% SDS, 0,5% deoxycholate, and 1% Triton

X-100) containing complete protease inhibitor cocktail (Roche Applied Science) and 10mM N-ethylmaleimide (NEM). For Western blotting, 30 µg of total cellular protein was separated by gradient SDS-PAGE (4-15%), transferred electrophoretically onto a PVDF membrane (Millipore) and blocked for 1h with PBS containing 5% non-fat milk, then incubated with primary antibody overnight at 4°C, followed by three PBS washes. Endogenous SUMO proteins were visualized using a SUMO-2/3 antibody recognising both SUMO-2 and SUMO-3 isoforms (Zymed Laboratories). Detection was performed using horseradish peroxidase-conjugated secondary antibodies and chemiluminescent substrates (ECL Western Blotting Detection Reagents; Amersham).

MMP expression in human RASF/OASF

MMP-1, MMP-3 and MMP-13 production by synovial fibroblasts from RA and OA patients after stimulation with recombinant human TNF-alpha or IL-1beta for 24h were measured by ELISA (R&D systems) measurement of the supernatants. Before stimulation with TNF-alpha the cells were treated with the NF-κB inhibitor BAY 11-7082, 5µM (Adipogen) or with DMSO for 30 min.

NF-κB p65 transcription factor assay

HeLa cells were transfected with specific siRNA against SUMO-2/3 for 48h.
Activation of NF-κB p65 transcription factor was achieved by the stimulation of HeLa cells with 100ng/ml TNF-alpha for 16h. The nuclear extracts were prepared and NF-κB p65 transcription factor assay was performed according to the manufacturers protocol (Active Motive).

Luciferase reporter assay

Hek 293T cells were co-transfected with siRNA SUMO-2/3 or siRNA mock, in addition to the reporter plasmid, pNF-κB-Luc (Stratagene) and pRL-TK control reporter vector (Promega) using Lipofectamine2000 (Invitrogen). The pNF-κB-Luc plasmid contains a firefly luciferase reporter gene that was derived from a basic promoter element joined to five tandem repeats of an NF-κB consensus-binding element. The pRL-TK plasmid (Promega), which expresses *Renilla* luciferase, was used for the normalization of transfection efficiency. After 12 h of stimulation with 100ng/ml TNF-alpha, the cells were harvested and their luciferase activities were measured using the dual luciferase reporter assay system (Promega). The data represent the means±S.E.M., and each transfection was performed in triplicate.

Statistical Analysis

Data are shown as arithmetic means ± SEM. Statistical analysis were performed using GraphPad Prism Software, 5.0c (Graph Pad Software Inc., San Diego, CA). According to data distribution and number of groups, a parametric (t test) or non-parametric (Mann-Whitney) test was performed. Values from ELISA data were compared by paired Student´s t test. *P ≤ 0,05; **P ≤ 0,01; ***P ≤ 0,001 was considered as statistically significant.

DISCUSSION

In this study, we have shown for the first time that SUMO-2/3 is involved in the activation of synovial fibroblasts in RA and, thus, the disregulation of sumoylation is likely to contribute to the pathogenesis of the disease. SUMO-2 is not only upregulated in RASF but also regulates TNF-alpha and IL-1beta induced expression of MMP-3 and MMP-13.

Following previous data that have demonstrated the increased expression of SUMO-1 in synovial fibroblasts from patients with RA(23), we investigated the expression of SUMO-2/3 in RASF as well as in the hTNFtg mouse model. In our study, we have demonstrated an increased expression of SUMO-2/3 in synovial tissue sections from RA patients compared to

OA patients as well as in synovial fibroblasts from these two patient groups. Based on this data, we wanted to investigate the effect of the proinflammatory cytokine TNF-alpha, which is one of the major players contributing to RA. Interestingly, we found a dose-dependent increase in SUMO-2 expression at mRNA level, while, expression of SUMO-3 was unchanged. Additionally, protein levels of both free SUMO-2/3 and of the conjugated proteins was increased. Therefore, we conclude that TNF-alpha selectively regulates the expression of SUMO-2, but not SUMO-3. SUMO-3, but not SUMO-2, can be phosphorylated at serine 2(18). This published functional difference between SUMO-2 and SUMO-3 may be the reason for the different contributions of TNF-alpha in the expression of SUMO-2 and SUMO-3. Furthermore, we have also confirmed increased expression of SUMO-2/3 in synovial tissue sections from hTNFtg mice and in synovial fibroblasts obtained from these mice compared to wt mice, suggesting that chronic exposure to TNF-alpha increases the expression of SUMO-2/3.

To find out which functional role SUMO-2/3 has in contributing to the pathogenesis of RA, we knocked down SUMO-2/3 and measured TNF-alpha and IL-1beta induced MMP expression. Surprisingly, knockdown of SUMO-2/3 enhances TNF-alpha and IL-1beta induced expression of MMP-3 and MMP-13, but not of MMP-1. TNF-alpha is one of the key regulators in the pathogenesis of rheumatoid arthritis and is overexpressed in synovial fibroblasts from RA patients. These data suggest that the increased expression of SUMO-2 may be part of a protective rather than a disease-promoting mechanism in synovial fibroblasts from RA patients to counteract and limit the increased and unbalanced expression of MMP-3 and MMP-13. These data raise the question of which transcription factors are involved in increasing MMP-3 and MMP-13 expression following TNF-alpha stimulation of SUMO-2/3 silenced cells. NF-κB plays an important role in RA pathogenesis(24) and cytokine activity e.g. TNF-alpha induces the activation of NF-κB in RA(25). Inactive NF-κB is composed of a heterodimer of p50 and p65 subunits in complex with an inhibitory IκB subunit. Activation of

this factor requires phosphorylation, ubiquitination and proteasomal degradation of IκB. Using site-directed mutagenesis, it was shown that lysine K21 in IκB-alpha is the primary site on the target protein for sumoylation(22). SUMO-1 was shown to regulate NF-κB activity by stabilizing the IκBalpha-NF-κB complex(22). Induction of MMPs by cytokines is both cell type and MMP-specific. In articular chondrocytes and in SW1353 cells, inhibition of the transcription factor NF-κB suppresses TNF-alpha induced MMP-13 expression(25). NF-κB activity is also essential for upregulation of MMP-1 and MMP-3 in rabbit and human vascular smooth muscle cells(26). Our results show that silencing of SUMO-2/3 by specific siRNA upregulates the transcriptional activity of NF-κB in response to TNF-alpha. Our observed data is in line with a recent study, which has shown that mouse SUMO-2 modifies IκB-alpha and inhibits the translocation of NF-κB into the nucleus in dendritic cells(27).

Our observations suggest that after knockdown of SUMO-2/3, NF-κB translocates into the nucleus and promotes increased transcription and secretion of MMP-3 and MMP-13.

With respect to the differential regulation of individual members of the MMP family, it has been well established that in addition to NF-κB, MMPs are regulated by other pathways and transcrition factors, such as AP-1(2). Therefore, our results on the one hand may refelect the different levels to which MMPs are regulated specifically by NF-κB. On the other hand, sumoylation pathways have been demonstrated to regulate MMPs also through complex epigenetic pathways, which also may explain the differences(19). Additionally we have confirmed these results by pretreatment of RA synovial fibroblasts with the highly specific inhibitor of IκB-alpha phosphorylation BAY 11-7082. Addition of BAY 11-7082 inhibitor totally blocked SUMO-2/3 induced MMP-3 and MMP-13 production, indicating that SUMO-2/3 regulates MMP-3 and MMP-13 expression through NF-κB pathway. Furthermore, there is a growing body of evidence showing that hypoxia and SUMO activity are closely linked through regulation of HIF1-alpha. Different studies demonstrate this controversial hypothesis

concerning the regulation of HIF1-alpha expression and subsequent regulation of MMP-1 and MMP-3(28-30). In our study, although we demonstrate a role of SUMO-2/3 in regulation of TNF-alpha mediated MMP expression via the NF-κB signalling pathway, we cannot exclude the effect of other integrating signalling pathways upon the overall regulation of MMPs in RA. In conclusion, our results have demonstrated that downregulation of SUMO-2/3 enhances TNF-alpha and IL-1beta induced expression of MMP-3 and MMP-13 through upregulated transcriptional activity of NF-κB. Overall, our results suggest that the fine tuning and balance of sumoylation pathways is important in the pathogenesis of RA. Furthermore, because many proteins which are regulated by SUMO modification are also targets of drugs and therapies against rheumatoid arthritis, it is important to invest in a deeper understanding of this mechanism in order to provide us with novel targets for drug design against RA.

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FIGURE LEGENDS

Figure 1. Expression of SUMO-2/3 in synovial fibroblasts from patients with RA. a) SUMO-2/3 expression was assessed by immunohistochemical staining using SUMO-2/3 antibody in synovial tissue from patients with RA compared to OA. The nuclei were counterstained with methyl green. b) When investigated at the protein level by fluorescence microscopy, SUMO-2/3 (green staining) was detected in the nucleus. c) Westen blot analysis demonstrates SUMO-2/3 expression as well as conjugated proteins in RASF versus OASF. d) mRNA levels of SUMO-2 and SUMO-3 were determined in primary synovial fibroblasts isolated from patients with RA (n=9) and OA (n=8) by quantitative real time PCR (normalized to gapdh). e) Western blotting for SUMO-2/3 in RASFs treated for 24 h with 100ng/ml TNF-alpha. f) Synovial fibroblasts from RA patients (n=5) were treated for 6 h with a dose response of TNF-alpha (0,1; 1; 10 and 100 ng/ml) on SUMO-2 and SUMO-3, mRNA levels were measured by real time PCR. Values were normalized for the housekeeping gene gapdh. The data are mean ± S.E.M. of three independent experiments. *$P \leq 0,05$; **$P \leq 0,01$; ***$P \leq 0,001$ was considered as statistically significant.

Figure 2. Expression of SUMO-2/3 in synovial tissue and synovial fibroblasts from hTNFtg mice. a) Immunohistochemical staining of SUMO-2/3 expression in synovial tissue from hTNFtg mice (n=4) compared to wildtype (wt) (n=4) using SUMO-2/3 antibody or isotype control. Arrows point to areas of synovial fibroblasts. b) mRNA levels of SUMO-2 and SUMO-3 (normalized for the housekeeping gene beta-actin) as well as protein levels (c) were determined in synovial fibroblasts isolated from hTNFtg (n=3) and wt (n=3) mice. The data are mean ± S.E.M. of three independent experiments. *$P \leq 0,05$; **$P \leq 0,01$; ***$P \leq 0,001$ was considered as statistically significant.

Figure 3. SUMO-2/3 regulates TNF-alpha induced MMP-3 and MMP-13 expression, but not MMP-1 in synovial fibroblasts. a) Expression levels of MMP-3, MMP-13 and MMP-1 in supernatants from RASF (n=3) and OASF (n=3) were measured by ELISA. b) Semiquantitative PCR demonstrates TNF-alpha induced production of MMP-1 and MMP-3 in

synovial fibroblasts. The data are mean ± S.E.M. of three independent experiments. *P ≤ 0,05; **P ≤ 0,01; ***P ≤ 0,001 was considered as statistically significant.

Figure 4. SUMO-2/3 regulates NF-κB p65 activation. a) Downregulation of SUMO-2/3 leads to enhanced activation of NF-κB p65 after stimulation for 16 h with TNF-alpha in nuclear extracts from HeLa cells (n=3). Transfection efficiency was assessed by Western blot for SUMO-2/3. b) Hek 293T cells were co-transfected with siRNA for SUMO-2/3 or control siRNA, in addition to reporter plasmid, pNF-κB-Luc and pRL-TK control reporter vector. The pRL-TK plasmid, which expresses *Renilla* luciferase, was used for the normalization of transfection efficiency. After 12 h of stimulation with 100ng/ml TNF-alpha, the cells were harvested and their luciferase activities were measured using the dual luciferase reporter assay system. The data represent the means±S.E.M. and each transfection was performed in triplicate. *P ≤ 0,05; **P ≤ 0,01; ***P ≤ 0,001 was considered as statistically significant.

Figure 5. Inhibition of NF-κB activation reduces the expression of MMP-3 and MMP-13. RA synovial fibroblasts were pretreated with IκB phosphorylation inhibitor BAY 11-7082 for 30 min and then stimulated with TNF-alpha (100 ng/ml) for 24 h. Expression levels of MMP-3 and MMP-13 in supernatans from RASF (n=3) were measured by ELISA. The results for MMP-3 (a) and MMP-13 (b) are shown. The data are mean ± S.E.M. of three independent experiments. *P ≤ 0,05; **P ≤ 0,01; ***P ≤ 0,001 was considered as statistically significant.

Supplementary Material

Materials and Methods

RASFs were transfected with siRNA against SUMO-2/3. Whole cell extracts were isolated from this cells and subjected to Western blot. TNF-Receptor I was visualized using a TNF-Receptor I antibody (cell signaling). RNA was isolated, then cDNAs were synthesized and the expression of SUMO-1, -2 and -3 were determined by using real time PCR.

Figure S1. Expression of TNF-Receptor I and SUMO-1 after knockdown of SUMO-2/3. RASFs were transfected with siRNA against SUMO-2/3 and protein extracts were subjected to western blotting. As shown in Fig. S1a knockdown of SUMO-2/3 does not affect the expression of the TNF-Receptor I. Expression of SUMO-1 was not changed after knockdown of SUMO-2/3, as shown in Fig.S1b. All experiments were performed in three independent trials. *P ≤ 0,05; **P ≤ 0,01; ***P ≤ 0,001 was considered as statistically significant.

Figure S2. SUMO-2/3 regulates IL-1beta induced MMP-3 and MMP-13 expression, but not MMP-1 in synovial fibroblasts. a) Synovial fibroblasts form RA and OA patients were transfected with siRNA against SUMO-2/3 and stimulated with IL-1beta for 24h, supernatants were collected. Expression levels of MMP-3, MMP-13 and MMP-1 in supernatants from RASF (n=3) and OASF (n=3) were measured by ELISA. The data are mean ± S.E.M. of three independent experiments. *P ≤ 0,05; **P ≤ 0,01; ***P ≤ 0,001 was considered as statistically significant.

Figure S3. Specifity of the SUMO-2/3 antibody. Flag-SUMO-2, Flag-SUMO-3 constructs or Flag-tag empty control vector were transfected into HeLa cells and lysed 24 hours later. The lysates were separated by SDS-PAGE and the expressed proteins were visualized by western blotting using the SUMO-2/3 antibody. Flag-SUMO-2 and Flag-SUMO-3 transfected HeLa cells show a higher level of sumoylation in comparison to the basal levels seen in both untransfected and empty plasmid control transfected cells.

Contributorship statement

Svetlana Frank: performed research, analyzed data, wrote the manuscript
Marvin A. Peters: designed research, analyzed data
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Christine Seyfert: provided tissue samples

Steffen Gay: designed research

Thomas Pap: designed research, wrote the manuscript

There are no competing interests

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