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Interleukin-1α induces ADAMTS-mediated aggrecanolysis in the bovine meniscus: Evidence for a primary role of ADAMTS-4 in meniscus destruction

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Abstract

Objective: Pro-inflammatory cytokines are known to induce meniscal matrix-degradation and inhibition of endogenous repair mechanisms. However, the pathogenic mechanisms behind this are mostly unknown. Therefore, we investigated interleukin-1 α (IL-1α)-induced proteoglycan degradation in an in vitro-model. Design: Meniscal, and for comparison articular cartilage explant disks (3 mm diameter x 1 mm thickness) were isolated from 2-year-old cattle. After 3 or 6 days of IL-1α-treatment, GAG loss (DMMB assay), biosynthetic activity ($[^{35}SO_4]$-sulfate and $[^3H]$-proline incorporation), gene expression and activity of matrix-degrading enzymes (quantitative RT-PCR, and gelatin/casein zymography from culture supernatants), and aggrecan fragmentation (western blot, immunohistochemistry) was determined. Results: Meniscal tissue had a 4-fold lower GAG content per wet weight than articular cartilage, and cells within constitutively expressed MMP-1, -2, -3, as well as ADAMTS-4 mRNA, whereas ADAMTS-5 expression was low or hardly detectable. IL-1α significantly decreased the overall biosynthetic activity and increased the GAG release, MMP-2, MMP-3, and ADAMTS-4 expression (MMPs as protein pro-forms). Aggrecan fragments G1-NITEGE, G1-VDIPEN, and the m-calpain-generated C-terminal GVA708 were constantly detectable in the tissue. However, IL-1 induced additional formation and release of the aggrecanase-specific NITEGE, GELE and KEEE epitopes. Moreover, TIMP-3 but not TIMP-1 and -2 inhibited IL-1-dependent GAG loss and NITEGE production. Conclusion: Our study shows that aggrecanase activity, most likely ADAMTS-4, is involved in the early IL-1-mediated aggrecanolysis, and might therefore be responsible for meniscal degeneration or a reduced healing capacity of meniscal defects. MMP-2 and -3 expression, which is also IL-1-dependently induced, may be relevant for subsequent meniscal degradation.

Key words: Meniscus, Interleukin-1, MMP, aggrecanase, aggrecan

Introduction

Pathological changes of the knee meniscus, due to direct injury or acute joint inflammation have been shown to contribute to the initiation and progression of knee joint diseases like osteoarthritis (OA) (DiCarlo 1992; Rupp et al. 2002; Roos et al. 1998; Carlson et al. 2002). Therefore it is important to understand the biological mechanisms leading to meniscal degradation and changes which might prevent intrinsic or therapeutic meniscal repair.

Increased levels of proinflammatory cytokines like interleukin-1 (IL-1) have been found in the synovial fluid of inflamed joints, and it has recently been shown that IL-1 and TNF-α are able to inhibit the intrinsic meniscal repair response after injury in vitro (Schlaak et al. 1996; Hennerbichler et al. 2007; McNulty et al. 2007; Wilusz et al. 2008). While this inhibition is accompanied by increased total matrix-metalloproteinase (MMP) activity in a 2-week in vitro-model (McNulty et al. 2009), the potential pathogenic role for specific MMPs and the possible involvement of other matrix degrading activities like aggrecanase activity is indicated but not fully understood (Bluteau et al. 2001: Pennock et al. 2007; Uppton et al. 2006). Increased levels of matrix-degrading enzymes have been found in the synovial fluid of patients with OA and RA, and it is hypothesised that the meniscus is a potential source for these enzymes (Lohmander et al. 1993; Lark et al. 1997; Yoshihara et al. 2000). Since aggrecan is present in significant amounts in the meniscus, and depletion of aggrecan due to increased proteolytic cleavage is one of the earliest changes in degeneration of articular cartilage, it seems reasonable to assume that aggrecanases also play a major role in the IL-1-dependent meniscal aggrecan degradation (McNicol and Roughley 1980; Roughley 1981; Adams et al. 1983; Wildley and McDevitt 1998; Valiyaveettil et al. 2005).

Aggrecan consists of a central core protein with three globular domains (G1, G2, G3) (Hardingham and Fosang 1992; Roughley and Lee 1994; Roughley 2006). Members of the MMP family cleave aggrecan between Asn^{341}-Phe^{342}, forming the VDIPEN fragment (Fosang et al. 1991; Flannery et al. 1992; Fosang et al. 1993; Fosang et al. 1994; Fosang et al. 1996; Imai et al. 1997; Fosang et al. 1998). Members of the ADAMTS-(a disintegrin and metalloproteinase with thrombospondin motifs) family are known to be responsible for much, if not all, of the destructive aggrecanolysis in cartilage which occurs in OA and RA joints (Sandy et al. 1992; Lohmander et al. 1993). ADAMTS-4 (aggrecanase-1) and -5 (aggrecanase-2) cleave primarily between Glu^{373}-Ala^{374} and generate G1-NITEGE which is truly destructive since it results in total loss of the GAG-binding domains of aggrecan (Sandy et al. 1991; Arner et al. 1999). Further aggrecanase activity was described at four more aggrecan sites between the G2 and G3 domain (Sandy et al. 1991; Arner et al. 1999; Ilic et al. 2000; Tortorella et al. 2000]. Moreover, high concentrations of ADAMTS-4 in incubations with bovine aggrecan have also been shown to cleave secondarily at Asn^{341}-Phe^{342} (Westling et al. 2002).

Some studies have already shown an influence of IL-1 on meniscal tissue GAG loss and biosynthetic activity (Shin et al. 2003; Wilson et al. 2006). A potent broad-spectrum inhibitor, which blocks MMP activity could protect meniscal explants from IL-1α -induced degradation (Wilson et al. 2006). However, the mechanism of action of the inhibitor and its effects on aggrecanase-mediated aggrecanolysis were not reported. It therefore remains unclear whether MMPs, aggrecanases (or other proteinases) are primarily responsible for aggrecanolysis in menisci in response to IL-1α treatment. We therefore established an in vitro-model which allows the isolation of meniscal tissue explants of defined geometry and anatomical location. Using this model we have investigated the effect of IL-1α on GAG loss, biosynthetic activity, aggrecan fragmentation, and expression and activity of matrix

metalloproteinases and aggrecanases. Our data suggest that IL-1-mediated degradation of meniscal proteoglycans is initiated by aggrecanase activity and concomitant up-regulation of MMP-2 and -3 proforms. According to the literature the latter must be important players in later stages of meniscal matrix destruction (McNulty et al. 2009).

Materials and Methods

Isolation and culturing of meniscal and articular cartilage explant disks

Meniscal explant disks were isolated from 16-24 months-old bovine menisci. Tissue cylinders (10 mm in diameter) were punched perpendicular to the meniscus bottom surface (Fig. 1a). 1 mm of the bottom surface (normally adjacent to the tibial articular cartilage surface) was removed and four to five smaller explant disks (3 mm in diameter x 1 mm thick) were isolated from this using a biopsy punch (Unipunch, Lamoral) and cultured in Dulbecco`s Modified Eagles medium (DMEM supplemented with 100 U/ml penicillin G, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B; Sigma) in a 37°C, 5% CO_2 environment after measurement of wet weight. Explants were randomized matched by their anatomical location, and three explant disks per well of a 24-well plate were cultured for 3 days in 1 ml medium with or without recombinant human IL-1α (R & D Systems). We used IL-1α instead of IL-1β, because it is known to bind to the IL-1 receptor type I (IL-1 RI) with a higher affinity (Scapigliati et al. 1989; Arend et al. 1994). Additionally, bovine cartilage has been described to be either insensitive to human IL-1β, or GAG release was at least higher when explants were exposed to IL-1α instead (Pattoli et al. 2005; Smith et al. 1989). For inhibitory studies a broad-spectrum MMP-inhibitor (No. 444225, Calbiochem, Germany) and different TIMPs (R & D Systems) were used (here, only one meniscal explant/well was cultured in 200 µl medium in 96-well plates). For some experiments articular cartilage explants of the same size were isolated from the same knee joints according to a method described elsewhere (Kurz et al. 2001), as control tissue. In brief, cartilage/bone cylinders (1 cm in diameter) were drilled out of the femoropatellar groove, and 1 mm thick sections of cartilage were cut parallel to the surface. The final disks were punched out of these sections and cultured and treated as described above for meniscal explants.

Histology and immunohistochemistry

Explants were fixed overnight in 4% paraformaldehyde, embedded in paraffin, and serial sections (7 μm) were cut sagittally through the entire width of the explant disks. Sections were immobilized on glass slides, deparaffinized and stained either with toluidine blue or with immunostaining, where sections were heated for 2 ½ min in a digester (in 0.01 M citric acid, pH 6.0, 100°C), incubated overnight at 4°C with anti-NITEGE (17 ug/ml) in 1% BSA, PA1-1746, Affinity BioReagents, Golden, USA), rinsed in Tris-NaCl, incubated with the AlexaFluor 488 goat anti-rabbit IgG (1:500) for 1 h at room temperature, rinsed again and briefly incubated with bisbenzimide (Sigma), mounted with fluorescence mounting medium (Dako), and visualised using the Apotome (ZEISS) fluorescence microscope.

Measurement of glycosaminoglycans (GAGs) and biosynthetic activity

GAG content of papain-digested explants (see below) or media samples was determined by DMMB dye assay (Loening et al. 2000) using shark chondroitin-sulfate as standard. Values are presented as μg GAG per mg wet weight of the explants. For radiolabel incorporation, explants were placed in fresh culture medium containing 10 μCi/ml [$^{35}SO_4$]-sulfate or [^{3}H]-proline (Amersham Pharmacia) and 10% FBS for 6-8 h at 37°C under free-swelling conditions immediately after cytokine treatment, washed in PBS containing 0.5 mM proline (to remove unincorporated isotope) and digested overnight in 1 ml of papain solution [0.125 mg/ml (2.125 U/ml, Sigma), 0.1 M Na_2HPO_4, 0.01 M Na-EDTA, 0.01 M L-cysteine, pH 6.5] at 65°C. 200 μl of each sample were added to 2 ml scintillation fluid (Opti Phase Hi Safe 3, Perkin Elmer). Counts were measured using a scintillation counter (Wallac 1904. Turku, Finland), expressed in cpm/mg wet weight and normalized to the radiolabel incorporation of untreated control tissue, which was set to 100%.

Measurement of peak stresses

Biomechanical testing of the tissue was performed after IL-1-treatment using a computer-controlled loading device described elsewhere (Kurz et al. 2001). A single uniaxial unconfined compression with 10% strain was applied with a velocity of 0.1 mm/sec to groups of three meniscal explants and peak stresses were recorded (in MPa) during compression, and correlated with the GAG loss of the explants.

Real time RT-PCR

Quantitative transcriptase-polymerase reaction (real time RT-PCR) was performed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as reference gene to determine gene expression levels. Meniscal explants (approximately 100 mg from each group) were flash frozen, pulverized, and total RNA was extracted using the TRIZOL reagent (1 ml/100 mg wet weight tissue; Invitrogen, Carlsbad, CA, USA) followed by extraction with chloroform and isopropanol precipitation. RNA was quantified spectro-photometrically (OD_{260}/OD_{280} nm) and digested with DNase (65°C for 10 min; Promega). Real time RT-PCR was performed using the Qiagen QuantiTect SYBR® Green RT-PCR Kit (Qiagen) according to the manufacturer´s instructions. Bovine primers (0.5 µM) were: GAPDH (S: 5´-ATC AAG AAG GTG GTG AAG CAG G-3´; AS: 5´-TGA GTG TCG CTG TTG AAG TCG-3´; product size 101 bp); MMP-1 (S: 5´-GGA CTG TCC GGA ATG AGG ATC T-3´; AS: 5´-TTG AAT GCT CA AGG CCC A-3´; product size 91 bp); MMP-2 (S: 5´-GTA CGG GAA TGC TGA CGG GGA ATA-3´; AS: 5´-CCA TCG CTG CGG CCT GTG TCT GT-3´; product size 93 bp); MMP-3 (S: 5´-CAC TCA ACC GAA CGT GAA GCT-3´; AS: 5´-CGT ACA GGA ACT GAA TGC CGT-3´; product size 109 bp); ADAMTS-4 (S: 5´-GCT GCC CTC ACA CTG CGG AAC-3´; AS: 5´-TTG CCG GGG AAG GTC ACG-3´; product size 101 bp); ADAMTS-5 (S: 5´-AAG CTG CCG GCC GTG GAA GGA A-3´; AS: 5´-TGG GTT ATT GCA GTG GCG GTA GG-3´; product size 196 bp). Conditions were: reverse transcription

30 min, 50°C; PCR initial activation step 15 min, 95°C; denaturation 15 s, 94°C; annealing 30 s, 60°C; extension 30 s, 72°C; optional: data acquisition 30 s, melting temperature 70-78°C. Data were calculated using the $\Delta\Delta C_T$-method.

Zymography analysis

Protein levels of MMPs were assayed in conditioned media by gelatin and casein zymography. Equal volumes of medium samples and loading buffer (2 mM EDTA, 2% (w/v) SDS, 0.02% (w/v) bromophenol blue, 20 mM Tris-HCl, pH 8.0) were mixed, subjected to electrophoresis using 0.1% (w/v) gelatin and 0.2% (w/v) casein as substrate in 4.5-15% gradient SDS-polyacrylamide gradient gels, washed in 2.5% (v/v) Triton X-100, rinsed in distilled water and incubated for 16 h at 37°C in 50 mM Tris-HCL (pH 8.5) containing 5 mM $CaCl_2$. Then, gels were stained for 20-30 min with 0.1% (w/v) Coomassie brilliant blue R250 (Serva, Heidelberg) at room temperature and destained with 10% (v/v) acetic/50% (v/v) methanol and with 10% (v/v) acetic acid/10% (v/v) methanol. MMPs were identified by molecular weights and substrate specificity as clear bands against a blue background of undigested substrate. Additionally, some samples were incubated with 1 mM APMA (4-aminophenylmercuric acetate, A 9563, Sigma) for 3 h at 37°C to activate matrix metalloproteinase-pro-forms prior to loading.

Western blot analyses

For isolation of aggrecan fragments from meniscal tissue, finely sliced explants were extracted for 48 h at 4°C in 4 M guanidine hydrochloride (10 mM MES, 50 mM sodium acetate, 5 mM EDTA, 0.1 mM AEBSF, 5 mM iodoacetic acid, 0.3 M aminohexanoic acid, 15 mM benzamidine, 1 µg/ml pepstatin, pH 6.8). Extracts were centrifuged and clear supernatants were dialysed against water for 24 h at 4°C. Conditioned culture mediums were supplemented with proteinase inhibitors (as above) and dialysed against water as

above. The dialyzed samples were adjusted to 75% ethanol and stored at -20 C° overnight. The precipitates were isolated by centrifugation, dried, dissolved in 50 mM sodium acetate, 50 mM Tris, 10 mM EDTA, pH 7.6 and incubated for 1-2 h at 37°C with protease-free chondroitinase ABC. Deglycosylated samples were analysed separately for aggrecan fragments with anti-G1 (Sandy et al. 1995) and anti-KEEE/anti-GELE (Sandy et al. 1995; Sandy et al. 2000) antibodies, as described by Sandy (2001). The SK-28 antibody was a very kind gift from Dr. Shimizu, Department of Orthopaedic Surgery, Gifu University Graduate School of Medicine.

Statistics

Quantitative data are presented as mean + SEM (if not otherwise indicated), n represents the number of independent experiments. Statistical analysis of data was made using the two-tailed Student's t-test. Differences were considered significant if $p \leq 0.05$.

Results

Histological characterisation of meniscal explants

Using a specific protocol for the isolation of tissue explants (Fig. 1a) we reproducibly obtained disks of a defined structure from a single anatomical location. One flat side of the disks represented the original bottom surface of the meniscus (Fig. 1b), resulting in a zonal structure of the disks: at the original surface a thin zone (about 30 µm) could be found with flat to oval cells (Fig. 1b and c) and fibres which appeared to be oriented in line with the surface. The rest of the tissue consisted of thick bundles of collagen fibres containing fibroblast-like cells (Fig. 1d), and these bundles were embedded in fibrous cartilage with chondrocytic cells. Negative charges in the matrix were concentrated around the chondrocytic cells, visualized by purple toluidine blue staining, suggesting the presence of proteoglycans.

Effect of IL-1 on GAG content, stiffness and biosynthetic activity of meniscal explants

Mean GAG content of freshly isolated meniscal explants was 14.2 $\pm$ 0.9 µg/mg wet weight (n=6). During 6 days of culture the amount of about 32% of the initial tissue GAGs (4.5 $\pm$ 0.2 µg/mg;n=6) were released into the media in controls. Stimulation with IL-1α induced a concentration-dependent increase in GAG release which was significant at 5 ng/ml IL-1 (Fig. 1e). At 10 ng IL-1/ml (chosen for all subsequent experiments) GAG release was enhanced about 1.8-fold over control (8.1 $\pm$ 0.6 µg/mg; Fig. 1f), and this release represented about 57% of the initial tissue GAG content. Tissue GAG content decreased by about 35% during IL-1-treatment which markedly affected the compressive tissue properties. Peak stresses during a 10% uniaxial compression (strain rate 0.1 s^{-1}) were about 0.8 $\pm$ 0.2 MPa (n=3) in control tissue and reduced by about 50% to 0.4 $\pm$ 0.1 MPa (n=3) after IL-1 treatment. This loss is consistent with a major loss of the tissue integrity,

further confirmed by the strong negative correlation between loss of GAG and loss of peak stress with a correlation coefficient of r=-0.85 (n=8). The biosynthetic activity of meniscal explants was also significantly inhibited by IL-1 treatment: Sulfate incorporation was reduced about 54% and proline incorporation about 26% compared to control (Fig. 1f). IL-1 did not alter the viability of the meniscal cells since there were no significant changes relative to control in the ethidium bromide/FDA staining or the numbers of apoptotic cells; apoptosis was evaluated by counting cells with nuclear blebbing in serial sections of the explants (data not shown). We conclude that the inhibitory effects of IL-1 on meniscal cells were not due to pro-apoptotic effects of the cytokine.

In a parallel study with animal-matched articular cartilage explants we found that the relative effects of IL-1 over control on both GAG release and biosynthetic activity were greater for articular cartilage than for meniscus. Basal GAG content in articular cartilage tissue was about 4-fold higher than in meniscal explants (57.8± 4.9 µg/mg), but only 10% of GAG (5.8 µg/mg ± 0.8; n=6) were released from control tissue during 6 days of culture. IL-1-treatment (10 ng/ml) increased GAG release about 2.8-fold (16.1 µg/mg ± 2.4, n=6), which represented about 28% of the initial GAG content. Sulfate and proline incorporations were decreased in articular cartilage by IL-1 about 84% (n=6) and 76% (n=6) respectively, suggesting that articular chondrocytes are more responsive to IL-1 than meniscal cells.

Effect of IL-1 on MMP and ADAMTS mRNA levels

To determine whether the IL-1-dependent GAG release could be due to increased expression of matrix-degrading enzymes we examined mRNA levels by quantitative RT-PCR. MMP-1, -2 and -3 mRNA levels were found to be expressed constitutively. IL-1 had no noticeable effect on MMP-1, but it increased MMP-2 expression about 3-fold, and MMP-3 expression about 20-fold relative to controls (Fig. 2a). The expression of ADAMTS-

4 mRNA was readily detected in controls, and there was a marked but variable up-regulation in gene expression after stimulation with IL-1 (Fig. 2b). In five separate experiments, ADAMTS-5 mRNA was either undetectable (2 cases) or detected at very low levels and IL-1 had no major effect on transcript abundance.

Effect of IL-1 on MMP and ADAMTS protein abundance

The IL-1α-dependent induction of MMP-2 and MMP-3 gene expression apparently results in increased protein secretion. The only bands visible in gelatin and casein zymography of culture supernatants after cytokine treatment were identified as MMP-2 and -3 proteins on the basis of molecular size and substrate specificity; however, predominantly the inactive pro-forms were detected (Fig. 2c and d). Pro-MMP-3 protein (~57 kDa, according to Manicourt et al. 1993) was present at very low levels in control supernatants but was markedly enhanced by IL-1, corresponding to the quantitative RT-PCR data. Incubation of samples with APMA (1mM for 3h at 37degrees) before zymography converted the pro-MMP-3 to the active form (45kDa). The zymographic gelatinase activity of pro-MMP-2 (~66 kDa) was constitutive and there was a slight IL-1-dependent increase of this protein, again consistent with the PCR data. On incubation with APMA before zymography there was a partial conversion to active MMP-2 which migrated at 62kDa.

Western blotting of conditioned medium for ADAMTS-4 (Fig. 2e) showed primarily a 68kDa form which was about 2-3-fold more abundant in the IL-1 sample,and also a 53kDa form seen only in the IL-1 treated sample. These changes are consistent with the PCR data (Fig. 2b) and also suggest that IL-1 treatment of these explants increases the abundance of the diffusible active forms of ADAMTS-4 in the tissue, which are released to the medium along with the aggrecan fragments. Similar changes have been observed after IL-1

treatment of bovine articular cartilage (Patwari et al. 2005). Due to the absence of detectable ADAMTS-5 mRNA we did not examine these cultures for ADAMTS-5 protein.

Aggrecan cleavage in the meniscus

Since IL-1 treatment was accompanied by increased GAG release and concomitant increases in the abundance of MMP-2, MMP-3, and ADAMTS4, we next examined the evidence of either MMP-mediated and/or aggrecanase-mediated aggrecanolysis. We analyzed tissue extracts and conditioned medium by Western blot with anti-aggrecan antibodies which detect fragments which have been identified by size and immunoreactivity profiles in other systems (see Fig. 3a). The immunoreactive band notations used here (1,a,b,c,d,6,e,12,13,i) have been previously characterized and described in detail (Patwari et al. 2005; Sandy and Verscharen 2001; Oshita et al. 2004). Aggrecan fragment analysis showed that after 3 days there was an IL-1 dependent release of G1-NITEGE (fragment 6) into the medium with a concomitant decrease in the species in the tissue relative to control in the G1 blots. This is consistent with the known aggrecanase-mediated loss of G1-NITEGE from tissue to medium seen in cartilage explants at longer times (Patwari et al. 2005). G1-VDIPEN (fragment e) was also detectable in the tissue but no change in abundance or release to medium by IL-1 could be seen. Analysis of the medium compartment for aggrecan fragments terminating at their C-terminals with aggrecanase-generated neo-epitopes (GELE or KEEE) gave clear evidence for an IL-1 mediated aggrecanolysis within the CS-attachment region (fragments 12 and 13) as well as for the small (70kDa) KEEE-+ve/ fragment (fragment i). Interestingly, the tissue contained abundant fragments b and c (Oshita et al. 2004) with the m-calpain-generated C-terminal GVA708 which was detected with Ab SK-28, however, there was no evidence for an IL-induced change in abundance of these products.

In keeping with the IL-1-induced aggrecanase-mediated aggrecanolysis, immunostaining of meniscal sections for neoepitope NITEGE showed this product to be slightly detectable in chondrocytic cells in control tissue (Fig. 3b), but abundant in IL-1 treated tissue in association with the matrix network surrounding the collagen fibers in tissue areas containing fibrous cartilage (Fig. 3c). The IL-1-dependent increase in NITEGE signal observed by immunohistochemistry appears to conflict with the Western analysis of parallel samples (Fig. 3a). This suggests that the two methods are detecting different pools of product, with the Western blot evaluating the extractable pool only and the immunohistochemistry detecting the tissue pool with exposed (unmasked) neo-epitope only.

Inhibitor studies also support a role for ADAMTS-aggrecanases

We investigated whether the IL-1-induced GAG release could be influenced by tissue inhibitors of metalloproteinases (TIMP-1, -2 and -3). TIMPs 1-3 are known as potent inhibitors of MMPs, but TIMP-3 has the unique additional ability to inhibit the aggrecanases ADAMTS-4 and -5 (Kashiwagi et al. 2001; Gendron et al. 2003). Addition of TIMPs did not affect the GAG loss in control cultures (not shown), but the IL-1-induced GAG release was significantly reduced (about 65%) by TIMP-3 (0.1 µM) whereas TIMP-1 and TIMP-2 at this concentration slightly increased the IL-1-induced GAG loss (Fig. 4a). TIMP-3 also totally inhibited immunohistochemical detection of extracellular NITEGE accumulation in IL-1-treated samples, consistent with TIMP-3 inhibition of ADAMTS-aggrecanase activity in these explants (Fig. 4b and c).

Discussion

Aggrecanase-mediated aggrecanolysis in the meniscus

Our data clearly indicate that IL-1-mediated aggrecanolysis in meniscal explant cultures (resulting in loss of compressive properties) is due to the action of ADAMTS-aggrecanases, especially in the first days of culture. This conclusion is supported by the finding that IL-1 treatment causes loss of GAG from the tissue as well as accumulation and release of the aggrecanase-specific product G1-NITEGE373. This process is completely blocked by TIMP-3, a potent inhibitor of ADAMTS-aggrecanases. These findings and conclusions are consistent with many studies of this kind performed with articular cartilage or chondrocytes (Lark et al. 1995; Gendron et al. 2003; Nagase and Kashiwagi 2003).

The relative importance of ADAMTS4 and ADAMTS5 in joint aggrecanolysis is under intensive investigation, and the consensus view is that both proteases appear to be involved in articular cartilage degradation (Nagase and Kashiwagi 2003). However, little is known about the expression and activity of aggrecanases in the meniscus. ADAMTS-4 mRNA expression was found in degenerated human menisci, and both, ADAMTS-5 and - 4 are expressed in rabbit menisci after OA induction (Ito et al. 2000; Bluteau et al. 2001). In the present investigation there was mRNA expression of ADAMTS-4 detected which increased with IL-1 treatment whereas ADAMTS-5 mRNA expression was low or not detectable, suggesting that ADAMTS-4 may represent a key player in meniscal aggrecanolysis. Activation of ADAMTS-4 appears to be a complex process. ADAMTS-4 is synthesized as a latent precursor protein and needs cleavage of the N-terminal pro-domain for activation (Tortorella et al. 2005). This generates the p68 form, which can be presented in association with MT4-MMP on the cell surface (Gao et al. 2004; Patwari et al. 2005). After further extracellular C-terminal truncation of ADAMTS-4 the p53 and p40

forms are produced, which have enhanced activity against the IGD (interglobular domain) compared to the p68 form. p68 cleaves aggrecan within the CS-2 domain but apparently has only limited activity for the Glu373-Ala374 bond (Gao et al. 2002; Kashiwari et al. 2004). Here we demonstrate that both, the p68 and the p53 form of ADAMTS-4, are detectable in IL-1-stimulated meniscal tissue, suggesting further activation of the enzyme.

Studies with tissue inhibitors of matrix metalloproteinases (TIMPs) also support the hypothesis that aggrecanases are involved in IL-1-dependent meniscus destruction. TIMPs are able to inhibit the active forms of almost all matrix metalloproteinases by binding to the C-terminal site of these enzymes (Baker et al. 2002). However, in addition to the effect on matrix metalloproteinases, TIMP-3 inhibits ADAMTS-4 and -5 activity, while TIMP-1 and TIMP-2, however, have no effect on aggrecanases at concentrations $\geq$ 1 µM (Apte et al. 1995; Knäuper et al. 1996; Will et al. 1996; Butler et al. 1997; and 1999; Hashimoto et al. 2001; Kashiwagi et al. 2001; Gendron et al. 2003; Zhao et al. 2004). Consistent with an important role for ADAMTS-4 and/or -5 we show that only TIMP-3 inhibited the IL-1-induced GAG release from meniscal tissue. Additionally, IL-1-dependent NITEGE-formation was blocked by TIMP-3 in our study. The addition of TIMP-1 and -2 had no such inhibitory effects which suggests that ADAMTS-4 and/or -5 might be involved in the IL-1-dependent GAG release. On the other hand, TIMP-3 also regulates the activity of members of the membrane-bound ADAM (a disintegrin and metalloproteinase) family, e.g. ADAM-10, -12 and -17, TACE (Amour et al. 1998 and 2000; Loechel et al. 2000). Therefore, these proteinases should also be studied in further investigations of meniscal aggrecanolysis. ADAM-10 and -17 are also expressed in articular cartilage and even up-regulated in OA and in response to IL-1, and might therefore also play a role in the meniscus (Chubinskaya et al. 2001). The aggrecanases which seem to account for the degradation of aggrecan in bovine and human articular cartilage are ADAMTS-4 and/or -5

and our study suggests that these enzymes, and here especially ADAMTS-4, play a major role in IL-1-induced proteoglycan degradation in the meniscus. This may be related to the suggestion that ADAMTS-4 is also the major IL-1-stimulated aggrecanase in bovine articular cartilage (Tortorella et al. 2001; Patwari et al. 2005; Song et al. 2007). However further work with specific inhibitors for ADAMTS-4 and -5 is needed to confirm this.

MMP and m-calpain expression in the meniscus

We have also demonstrated that the meniscus is a potentially abundant source for secreted MMP-2 and -3. This indicates that meniscal cells might be responsible for at least some of the increased MMP levels which have been described in OA animal models, human OA articular cartilage, as well as in the synovial fluid of RA and OA patients (Lohmander et al. 1993; Pelletier et al. 1993; Billinghorst et al. 1997; Yoshihara et al. 2000; Bluteau et al. 2001). We found no evidence however that these MMPs are involved in early IL-1-induced aggrecanolysis in the bovine meniscus. Both enzymes were expressed as pro-forms and would therefore require activation to be involved in subsequent degradative processes. This is most likely since inhibition of total MMP activity prevented the negative influence of IL-1 or TNF-α on repair mechanisms in a 2-week-porcine meniscal injury model (McNulty et al. 2009). Therfore, we postulate that aggrecanases and MMPs are active in a sequential manner: degradation of meniscal matrix starts with the ADAMTS enzymes followed by MMP activities. This is supported by the finding that aggrecan protects collagen fibres from enzymatic destruction in articular cartilage (Pratta et al. 2003).

The aggrecan species fragments *b* and *c* (Fig. 3a) described here as natural components of the mature meniscus were previously described to accumulate with age in bovine

cartilage and meniscal extracts (Sandy et al. 1995). In the present paper we have demonstrated that these are not influenced by IL-1. These fragments are suggested to be products of calpain-mediated cleavage of aggrecan at Ala719-Ala720, which islocated between the G2 domain and KS-rich region. Calpains are neutral Ca^{2+}-dependent cysteine proteinases (Suzuki et al.. 2004). M-calpain was suggested to be involved in the knee joint diseases OA and RA since it could be detected in chondrocytes and synovial cells as well as in the synovial fluid of OA patients (Yamamoto et al. 1992; Szomor et al. 1999). Immunostaining of m-calpain (not shown) indicates the presence of this enzyme in meniscal tissue. This suggests that calpain-mediated aggrecanolysis is part of the physiological aggrecan turnover in the bovine meniscus but probably not involved in accelerated degradation in the presence of inflammatory mediators. M-calpain-mediated intracellular processing of aggrecan has recently been described as an abundant process in articular chondrocytes (Maehara et al. 2007) and our data suggest that the same might be the case for meniscal cells.

The novel finding of G1-NITEGE accumulation in fibrocartilaginous tissue surrounding collagen bundles is consistent with its association with an aggrecan-rich hyaluronan matrix. This conclusion was previously drawn from the co-isolation of G1-NITEGE and full-length aggrecan in CsCl A1 fractions prepared from normal bovine meniscus samples of different ages (Sandy et al. 1995).

Conclusion

IL-1 treatment of meniscal tissue causes loss of proteoglycans, loss of compressive properties, inhibition of cellular biosynthesis and up-regulation of both, matrix metalloproteinases and ADAMTS4. In this study, we have shown in a newly established in-vitro model that aggrecanase activity, and here most likely ADAMTS4, is responsible for

IL-1 mediated aggrecanolysis in the meniscus during the first 3-6 days of culture. MMP-2 or -3, which are also up-regulated on the mRNA and protein level, seem not to be involved in this early meniscal aggrecan degradation. In summary, our data suggest that pharmacologic inhibition of ADAMTS-4 activity may effectively prevent the early phases of meniscal destruction and/or promote meniscal defect repair mechanisms.

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Figure legends

Fig. 1 (a) Schematic illustration of the isolation of meniscal explants. (b) Light micrograph of a cross section of a meniscal explant after toluidine blue staining. The original meniscus bottom surface is facing down and covered by a thin surface zone (marked by a dashed line) followed underneath by fibrocartilaginous tissue, whereas the upper part of the meniscal explant mostly consists of collagen bundles which are surrounded by fibrocartilaginous tissue (small black arrows); bar = 200 µm. (c) Higher magnification of boxed area from (b) at the original surface of the meniscus with a thin zone (dashed line) where the cells are flat to oval. The cells are spindle-shaped and oriented parallel to the surface, whereas in deeper tissue layers they show an appearance of round-shaped fibrochondrocytes (white arrows); bar = 50 µm. (d) Higher magnification of boxed area from (b) from a deeper meniscus layer. Bundles of collagen fibrils are seen in cross section. In the collagen bundles lie small fibroblastic-like cells (black arrowheads), whereas in the fibrocartilaginous tissue, which surrounds the collagen bundles (small black arrows) the cells have a round appearance (white arrows) and are surrounded by purple staining, indicating negative charges in the matrix; bar = 50 µm. (e and f): Influence of interleukin-1α (IL-1α) on bovine meniscal explants. Values are shown as mean + SEM. (e) Cumulative GAG release into the culture medium during 6 days of incubation (n=6; *p < 0.05). (f) GAG loss, GAG content and biosynthetic activity of meniscal tissue measured after incorporation of [^{35}SO$_4$]-sulfate and [^{3}H]-proline for 6-8 h after 6 days of IL-1-treatment. Stimulated samples were normalized to the mean value of the unstimulated controls which were set as 100%; (n=6; *p < 0.05)

Fig. 2 Interleukin-1α (IL-1)-dependent expression of matrix-degrading enzymes in bovine meniscal explants. (a and b) RNA was extracted after 3 days of IL-1-treatment for quantitative RT-PCR, normalized to untreated controls, and presented as data points (circles) of n=5 for MMP-1, ADAMTS-4 and -5, and n=10 for MMP-2 and -3 independent experiments. Horizontal bars indicate mean values. A: Matrix metalloproteinase (MMP) expression. (b) Aggrecanase expression (ADAMTS = a disintegrin and metalloproteinase with thrombospondin motifs). ADAMTS-5 could only be detected in 3 of 5 experiments. (c and d) Culture supernatants were assayed by casein (c) and gelatin (d) zymography for activity of MMPs after 3 days of IL-1α-incubation. The bands with proteolytic activity corresponded presumably to pro-MMP-3 (~55 kDa) and pro-MMP-2 (~66 kDa), and were the only bands visible in the gel (Con = control). Some samples had been incubated with APMA (4-aminophenylmercuric acetate) in order to activate enzyme pro-forms prior to loading. (e) Western blot analysis of ADAMTS-4 expression in meniscal explants after 6 days showing an IL-1-dependent increase of the isoform p68 and induction of the isoform p53

Fig. 3 Aggrecan fragments in meniscal tissue (T) or culture supernatants (M) after 3 days of incubation in the absence (C=control) or presence of interleukin- α (IL-1). (a) Aggrecan fragments were analysed by Western blotting using anti-G1, ant-SK-28, or combined anti-KEEE/GELE neoepitope antibodies. The identification of the fragments was performed according to Sandy and Verscharen (2001). (b and c) Immunohistochemical detection of the distribution of aggrecan neoepitope NITEGE in bovine meniscal tissue. In addition to bisbenzimide staining of cellular nuclei (arrowheads) there was little or no staining in the matrix of control explants but some cellular staining (b), while there was a strong staining of NITEGE in the matrix of fibrocartilaginous areas of explants incubated with IL-1 (c). Bars = 50 μm

Fig. 4 Effects of TIMP-1, -2 and -3 (0.1 µM) on IL-1-induced release of glycosaminoglycans (GAG) (a) and immunostaining of the aggrecan neoepitope NITEGE (b and c) after 3 days of incubation with or without interleukin-1α (IL-1). (a) The white bar represents the IL-1-dependent GAG loss without TIMP treatment; mean values + SD, n=5; *p < 0.001. (b and c) There is no signal for NITEGE detectable in the matrix under the influence of TIMP-3. Nuclei of meniscal cells are visible by bisbenzimide staining