Sulfasalazine blocks the release of proteoglycan and collagen from cytokine stimulated cartilage and down-regulates metalloproteinases

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Objective. To investigate the effect of SSZ on the release of GAG and collagen fragments from bovine nasal cartilage and MMP and ADAMTS (a disintegrin and metalloproteinase domain with thrombospondin motifs) proteinases from human articular chondrocytes (HACs) stimulated with IL-1α and oncostatin M (OSM).

Methods. SSZ was added to bovine nasal explant cultures stimulated to resorb with IL-1α and OSM, and the release of GAG and collagen has been determined. Collagenolytic activity was measured using the radio-labelled collagen bioassay. HACs were treated with IL-1α and OSM with and without SSZ, and MMP-1 and -13 and ADAMTS-4 and -5 were measured for protein and gene expression by ELISA and RT–PCR, respectively.

Results. SSZ blocked GAG and collagen fragment release from bovine cartilage, and reduced active and total collagenase activity in a dose-dependent manner. SSZ transcriptionally blocked MMP-1, -13 and ADAMTS-4, and reduced the protein levels of MMP-1 and -13 in a dose-dependent manner following stimulation of HACs with IL-1α and OSM.

Conclusion. This study shows for the first time that SSZ blocks release of proteoglycan and collagen fragments from resorbing cartilage and lowers the levels of proteoglycan and collagen-degrading enzymes. These results indicate that in addition to acting as an anti-inflammatory agent, SSZ may have a therapeutic role in protecting cartilage from damage in OA.

Key words: RA, OA, IL-1α, TNF-α, MMPs, Oncostatin M, SSZ, GAG, Hydroxyproline.

Introduction

In OA and RA, progressive loss of cartilage matrix ultimately leads to loss of joint function. The cartilage matrix consists of proteoglycan and collagen, and both are specifically cleaved during cartilage breakdown by metalloproteinases [1]. The MMPs are a family of enzymes in extra-cellular matrix cleavage, which can be detected in sera, SFs, synovial tissue and cartilage from patients with OA and RA; some members of this family are responsible for the cleavage of collagen [2].

Pro-inflammatory cytokines, IL-1α and TNF-α, are key molecules involved in promoting synovial proliferation and cartilage damage in disease, and are known to up-regulate MMPs [3]. Oncostatin M (OSM) is a cytokine that is known to induce joint inflammation and cartilage damage in animal models [4]. Raised levels of OSM are detected in RA SF [4], and these levels correlate with joint inflammation and the markers of collagen and aggrecan degradation in RA. OSM, in combination with IL-1α, represents a potent reprogrammable stimulus for the release of cartilage collagen by MMPs in in vitro and in vivo models [4, 5].

A number of studies have looked at MMP and ADAMTS (a disintegrin and metalloproteinase domain with thrombospondin motifs) gene expression in human articular chondrocytes (HACs) in monolayer culture. One study using mRNA from HACs stimulated with IL-1β showed increases in the expression of MMP-1, -13 and ADAMTS-4 [6].

The DMARD, SSZ, is a combination of sulphapyridine (SP) and 5-aminosalicyclic acid (5-ASA). It is used widely in the treatment of inflammatory diseases such as RA and Crohn’s disease [7]. Whilst it is known that SSZ is cleared during metabolism, the mechanisms of action of SSZ and its metabolites are not totally understood [8]. It is known that <12% of the SSZ is absorbed by the small intestine, whereas the remainder is broken down by colonic bacteria to its constitutive units of SP and 5-ASA prior to absorption [8, 9]. It is also known to have an IC50 value of 625 μM when blocking nuclear factor κB (NF-κB) activation.

Clinical and experimental evidence has shown that SSZ can affect a number of cellular functions including cell proliferation [10], apoptosis [11], pro-inflammatory cytokine production, MMP secretion [12] and PGE2 release [13]. Many of these effects involve the NF-κB signalling pathway [14]. In many cells, NF-κB is an inflammatory mediator in which its inactive form binds to its inhibitor IκB in the cytoplasm. Upon activation, IκB is degraded and NF-κB translocates to the nucleus, activating inflammation. A study of inflammatory bowel disease showed that SSZ directly inhibits the IκB kinases IκB kinase (IKK)-α and -β [15].

Treatment of synovial fibroblasts with SSZ suppresses cell proliferation, and lowers mRNA levels of MMPs and inflammatory cytokines [11, 16]. When SSZ was added to IL-1β-stimulated rabbit chondrocytes in vitro, it suppressed GAG, PGE2 and collagenase release into the culture medium [13].

In this study, we investigated if SSZ could block cartilage breakdown in an in vitro cartilage model. We show that SSZ acts in a chondroprotective manner, blocking the release of GAG and collagen from resorbing cartilage by regulating members of the MMP and ADAMTS families of proteinases.

Materials and methods

Chemicals were obtained from the following suppliers: IL-1α was a generous gift from GlaxoSmithKline (Stevenage, UK), was stored at −70°C, and used at a final concentration of 1 ng/ml in bovine explant experiments and 0.02 ng/ml in HAC experiments. OSM, a generous gift from Prof. John Heath (University of Birmingham, UK), was stored at −70°C and diluted in culture medium to a final concentration of 10 ng/ml in all experiments. All other chemicals were commercially available analytical grade reagents obtained from Calbiochem (Nottingham, UK) or BDH (Poole, UK), or have been previously described [4]. SSZ was obtained from Calbiochem and made up to a stock concentration of 100 mM with dimethyl sulphoxide. The final concentrations used ranged from 1 to 1000 μM, taking into account the IC50 value (IC50 = 625 μM). The study was approved by the Newcastle and North Tyneside Local Research Ethics Committee.
Cell culture
HACs were derived from articular cartilage obtained from consenting OA patients following knee replacement surgery. Enzymatic digestion of tissue and culture was conducted as previously described [4]. After stimulation, cells were incubated for 24 h and the medium was harvested for assay. All chondrocytes used were primary cultures.

Cartilage degradation assay
Bovine nasal septum cartilage was dissected as previously described [4]. Cartilage discs were treated with SSZ at concentrations 1, 10, 100, 625 and 1000 μM with or without IL-1α and OSM. Culture supernatants were harvested at Day 7 and replenished with identical treatments. Day 14 culture supernatants were harvested and any remaining cartilage was digested with papain [4].

Papain digestion of cartilage chips
Cartilage chips were placed in capped LP3 tubes and digested overnight at 65°C with papain (4.5 mg/ml), cysteine-hydrochloric acid (HCL) (5 mM) and EDTA (5 mM) in 550 µl phosphate buffer. After digestion, 450 µl of phosphate buffer and 0.02% sodium azide was added to each tube and stored at 4°C.

Sulphated GAG assay
A range of GAG standards (bovine chondroitin sulphate) from 0 to 40 mg/ml were prepared in phosphate buffer. Supernatants and digest samples (n = 4) were diluted in phosphate buffer and plated alongside the standards at 40 µl in duplicate into a 96-well plate. Two hundred and fifty microlitres of 1,9-dimethylethylene (DAB) solutions 1, 10, 100, 625 and 1000 µl in a centrifugal evaporator for 2 h. After drying, the residue was hydrolysed for 20 h at 105°C. The hydrolysates were dried in a Micro-beta counter (Perkin Elmer, Beaconsfield, UK). Absorbance was read at 560 nm. The amount of hydroxyproline released into the medium was calculated as a percentage of the total GAG present.

Enzyme assays
For experiments using HACs, MMP-1 and -13 were assayed by specific ELISAs (n = 4) [4].

RNA extraction
RNA was purified using a Qiagen RNeasy mini kit (Qiagen, Crawley, UK). Culture medium was removed from the cells grown in a monolayer. Under RNase free conditions according to the manufacturer's instructions, total RNA was extracted from the cells. Isolated RNA samples were immediately stored at −80°C. The concentration and purity of RNA was determined following measurement of the absorbance at 260 and 280 nm.

Synthesis of cDNA
cDNA was synthesized from 1 µg of total RNA using Superscript II reverse transcriptase (Invitrogen, Paisley, UK) and random hexamers in a total volume of 20 µl according to the manufacturer's instructions. cDNA was stored at −80°C until used in downstream PCR.

TaqMan low-density arrays
The Micro Fluidic Card (Applied Biosystems, Warrington, UK) functions as an array of reaction vessels for real time PCR, consisting of a series of 384 interconnecting wells divided into eight sets of assays. Each of the wells contains dried TaqMan primers and probes for one mRNA target. Sequences for MMP primers and probes are as described in [17] and sequences for ADAMTS primers and probes are as described in [18]. To control against amplification of genomic DNA, primers were placed within different exons close to an intron/exon boundary, with the probe spanning two neighbouring exons if possible. The BLAST was used to ensure gene specificity for all the primer and probe sequences. cDNA of 100 ng was added to each fill reservoir, then the card was centrifuged and sealed to ensure that the wells of the Micro Fluidic Card are isolated after the sample loading. The PCR reactions were performed on the ABI Prism 7900HT SDS and gene expression was measured and analysed using the relative quantification ΔC_T programme [19]. A housekeeping gene was selected where variation in expression was no more than 1 C_T value. Gene expression was then normalized against 18S mRNA and expressed as fold induction compared with control.

Toxicity assays
Media from bovine nasal cartilage assays were measured for the release of lactate dehydrogenase (LDH) from the cells using the Cyto-Tox-ONE kit (Promega, Southampton, UK). HACs were assayed for caspase 3 and 7 activity using the Caspase-Glo 3/7 Assay system (Promega).

Statistical analysis
Statistical significance was determined by either by one-way analysis of variance, Bonferroni post hoc test or Student's t-test. Probability values <0.05 were considered as statistically significant. All experiments were repeated three times.

Results

Effect of SSZ on the release of GAG, collagen and collagenolytic activity from bovine nasal cartilage treated with IL-1α and OSM

Previous studies have shown that the IL-1α in combination with OSM can reproducibly stimulate the release of GAGs and collagenase activity was measured in units per millilitre, where 1 U can degrade 1 µg of collagen per min at 37°C.
collagen from bovine nasal cartilage in explant culture [20]. In this study, we used this combination as the cytokine stimulus to reproducibly promote cartilage degradation and the release of cartilage GAGs at Day 7 and collagen at Day 14 and investigated the effect of SSZ in this in vitro system. In the presence of IL-1α and OSM alone, ~48.1% of the GAG was released at Day 3, whereas ~95% cartilage collagen fragments were released at Day 14. In the presence of SSZ, a significant reduction of GAG release was seen at concentrations of 625 and 1000 μM (Fig. 1A), and a dose-dependent reduction of collagen release was seen at all concentrations (Fig. 1B).

Earlier studies have shown that the increase of collagen fragments released from bovine nasal cartilage is MMP dependent as it can be completely blocked by TIMP-1 or -2. Conditioned medium was assayed in the presence and absence of aminophenylmercuric acetate to measure total and active collagenolytic activity (Fig. 1C). IL-1α and OSM stimulated the production of collagenase activity at Day 14 (active and total) to 4.2 ± 1 U/ml and 25.7 ± 10.5 U/ml, respectively. A dose-dependent reduction was seen after the treatment with SSZ from 1 to 1000 μM.

Measurement of MMP-1 and -13 in conditioned media from HACs following stimulation with IL-1α + OSM ± SSZ

To determine if SSZ could block production of either MMP-1 or -13 from HACs in culture, cells were treated with IL-1α + OSM ± SSZ. Levels of MMP-1 and -13 were increased after stimulation with IL-1α + OSM to 1629.6 ± 186 (Fig. 2A) and 17.6 ± 4.3 ng/ml (Fig. 2B), respectively. The addition of SSZ at concentrations 1–625 μM showed a significant inhibition of both MMP-1 and -13 in the conditioned medium with SSZ at 625 μM reducing levels to 168.5 ± 55.7 and 2.2 ± 0.3 ng/ml for MMP-1 and MMP-13, respectively.

The effect of SSZ on gene expression of MMP-1 and -13, and ADAMTS-4 and -5 in HACs

To quantify the effect of SSZ on gene expression levels of MMP-1 and -13, and ADAMTS-4 and -5 during cartilage breakdown, average C_T values were taken and 2^{-ΔΔC_T} was calculated giving an arbitrary value. Standardization was carried out by plotting each gene as a fold induction compared with control.

In the experiment shown, IL-1α and OSM caused an increased MMP-1 release (10.8 ± 3) compared with control of 1 ± 0.2 (Fig. 3A). Addition of SSZ at 625 and 1000 μM caused significant...
reduction to 2.4 ± 0.9 and 3 ± 1, respectively. The level of expression for MMP-13 was induced by IL-1β and OSM to 3 ± 1 compared with control levels of 1 ± 0.5 (Fig. 3B); SSZ at 625 and 1000 μM in the presence of IL-1β and OSM showed significant inhibition of MMP-13 expression to 0.8 ± 0.2 and 0.6 ± 0.2, respectively.

ADAMTS-4 (Fig. 3C) and -5 (Fig. 3D) expression in IL-1β- and OSM-stimulated HACs resulted in a significant increase compared with control. The addition of SSZ caused a significant reduction in ADAMTS-4 expression at a dose of 625 μM, whereas there was no significant inhibition in ADAMTS-5 expression.

Measurement of toxicity levels in bovine nasal cartilage and HACs due to the addition of SSZ

LDH is released from cells with damaged membranes. The LDH can be measured in the culture medium giving a measure of cell damage. Media of Day 14 from bovine nasal cartilage assay was measured to assess if SSZ can cause cell damage. Control samples released 0.1 ± 0.001 A490 absorbance levels and treatment with SSZ had absorbance levels equal to that of the untreated cells.

During apoptosis, caspase 3 and 7 at high levels are known to play an effector role in mammalian cells. To determine whether the inhibitory effect seen with SSZ was due to apoptosis, HACs were treated with IL-1β + OSM ± SSZ, with staurosporine (1 μM) used as a positive control to induce apoptosis. Cells treated with positive control staurosporine produced a high level of caspase 3/7 activity (109.364 ± 2453.5 U). Untreated control cells produced much low levels of activity of 57 709.3 ± 1559.2 U (Fig. 4). Caspase activity levels in cells treated with SSZ remained well below the levels of the positive control; however, a significant increase was seen on unstimulated cells treated with SSZ at 100 μM, but higher concentrations were not significant.

Discussion

In this study, we investigated whether SSZ, a drug known to be effective in the prevention of inflammation, has chondroprotective
properties. Previous studies showed that dose-dependent SSZ suppresses the release of GAGs and collagenase activity from rabbit chondrocytes treated with IL-1β [13]. SSZ is able to significantly inhibit RA synovial fibroblast proliferation and IL-1 and -6 production, while also inhibiting the overexpression of c-fos [11]. Here we show, for the first time, that SSZ prevents IL-1β- and OSM-initiated GAG and collagen fragment release from an in vitro bovine explant model as well as a decrease in active and total collagenase activity in the conditioned media.

Few studies have shown the effect of SSZ on gene expression, with no study looking at the effect of SSZ on IL-1- and OSM-stimulated chondrocytes or cartilage. This study investigated the effect of SSZ on protein and gene expression in stimulated chondrocytes showing that SSZ blocks the up-regulation of MMP-1 and -13 at a transcriptional and translational level, suggesting that SSZ blocks the production of these collagen-degrading proteinases. We also demonstrate that SSZ could possibly block the breakdown of aggrecan by its ability to inhibit ADAMTS-4 transcriptionally.

SSZ is commonly introduced at an early stage in RA and is given at an oral dose of 3 g/day. Less than 12% of the SSZ is absorbed by the small intestine [9], whereas the remainder is broken down by colonic bacteria to its constitutive units of SP and 5-ASA prior to absorption [8]. It is also known that the half-life of the drug in vivo is ~10 h [9]. The low absorption level and half-life of SSZ accounts for the detectable serum concentration of 6 μg/ml (15 μM) [10]. Whilst higher concentrations of SSZ were required to block proteoglycan release, concentrations below the levels found in patient serum were sufficient to block collagen release. As the breakdown of collagen is considered to be the irreversible step in cartilage destruction, this effect of SSZ is important.

One study looking at the effect of SSZ on intracellular signalling pathways suggests that inhibition of NF-κB is due to direct suppression of IkB-α phosphorylation and its subsequent degradation, suggesting the ability of SSZ to block IKK or an upstream signal [15]. The effect of SSZ on cartilage was not due to apoptosis [21–23]. NF-κB has been shown to be crucial in the induction of inflammatory responses and resulting in the chronic activation of the immune system. The cytokine cascades, which occur as a stress response to inflammation, lead ultimately to NF-κB activation and thus contribute to diseases such as RA [24]. This suggests that by inhibiting NF-κB activation, SSZ can prevent inflammation in RA. This may also be true in OA, where it has recently been shown that the synovium is often inflammed [25]. Therefore, it may be possible that SSZ is potentially able to slow disease progression in both RA and OA.

This is the first time that SSZ has been shown to block cytokine-induced collagen breakdown in cartilage explant culture. It is also the first evidence that SSZ can block MMP-1 and -13 and ADAMTS-4 produced from cytokine stimulated HACs. This effect of SSZ upon chondrocytes and cartilage suggests that it may have a potential therapeutic role in preventing cartilage damage in both inflammatory joint disease such as RA and non-inflammatory disease such as OA.

**Rheumatology key messages**

- SSZ blocks cartilage collagen breakdown in an in vitro model.
- SSZ down-regulates both MMP-1 and -13, the major collagenases found in cartilage.
- SSZ could block cartilage collagen loss in OA.

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