Monosodium urate crystal induced macrophage inflammation is attenuated by chondroitin sulphate: pre-clinical model for gout prophylaxis?

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Abstract

Background: Chondroitin Sulphate (CS), a natural glycosaminoglycan of the extracellular matrix, has clinical benefit in symptomatic osteoarthritis but has never been tested in gout. In vitro, CS has anti-inflammatory and positive effects on osteoarthritic chondrocytes, synoviocytes and subchondral bone osteoblasts, but its effect on macrophages is unknown. The purpose of our study was to evaluate the in vitro effects of CS on monosodium urate (MSU)-stimulated cytokine production by macrophages.

Methods: THP-1 monocytes were differentiated into mature macrophages using a phorbol ester, pretreated for 4 hours with CS in a physiologically achievable range of concentrations (10–200 μg/ml) followed by MSU crystal stimulation for 24 hours. Cell culture media were analyzed by immunoassay for factors known to be upregulated during gouty inflammation including IL-1β, IL-8 and TNFα. The specificity of inflammasome activation by MSU crystals was tested with a caspase-1 inhibitor (0.01 μM-10 μM).

Results: MSU crystals ≥10 mg/dl increased macrophage production of IL-1β, IL-8 and TNFα a mean 7-, 3- and 4-fold respectively. Induction of IL-1β by MSU was fully inhibited by a caspase-1 inhibitor confirming inflammasome activation as the mechanism for generating this cytokine. In a dose-dependent manner, CS significantly inhibited IL-1β (p = 0.003), and TNFα (p = 0.02) production from macrophages in response to MSU. A similar trend was observed for IL-8 but was not statistically significant (p = 0.41).

Conclusions: CS attenuated MSU crystal induced macrophage inflammation, suggesting a possible role for CS in gout prophylaxis.

Background

Gout is the most common cause of arthritis in men after osteoarthritis. Its prevalence is on the rise and thought to affect around 4% of the total US population [1]. Patients have fewer flares when their serum uric acid is maintained below 6.0 mg/dl [2]. However, initiation of urate-lowering therapy can often lead to an increase in the frequency and severity of flares [3,4]. Consequently, both the European League Against Rheumatism (EULAR) and the American College of Rheumatology (ACR) have recommended the use of prophylactic agents when initiating urate-lowering therapy [5,6]. Oral colchicine, low-dose non-steroidal anti-inflammatory drugs (NSAIDs) and daily corticosteroids have all been recommended [7], but all are associated with intolerances or adverse effects [4]. Thus, the identification of new agents for treating or preventing gout flares would be of great clinical value.

Gouty inflammation is initiated when monosodium urate (MSU) crystals are taken up by macrophages or other cells in the joints [8]. This results in assembly of the NLRP3 inflammasome, a multimeric protein complex responsible for activating caspase-1, which in turn cleaves pro-IL-1β leading to production and secretion of active IL-1β [9]. Other factors are upregulated during gouty inflammation, including IL-8 and TNFα [8].

Chondroitin Sulphate (CS), a natural glycosaminoglycan of the cartilage extracellular matrix [10], is of clinical benefit in symptomatic osteoarthritis [11] but results are
mixed [12,13]. The effects of CS have never been tested in
gout. In vitro, CS has anti-inflammatory and positive
effects on osteoarthritic chondrocytes, synoviocytes
and subchondral bone osteoblasts [14], but its effect
on macrophages is unknown. On the other hand, in vivo,
CS given orally prevents hepatic NF-kB nuclear trans-
location, suggesting that systemic CS may elicit an anti-
inflammatory effect in many tissues besides the joint [14].
There is preliminary evidence in human beings that CS
may be of benefit in other diseases where inflammation is
an essential component such as psoriasis and atheroscler-
osis [14]. The purpose of our study was to evaluate the
in vitro effects of CS on MSU-stimulated cytokine produc-
tion by macrophages.

Methods
Cell culture
We established an in vitro cell culture system using the
human monocytic cell line, THP-1 (ATCC TIB-202),
grown in RPMI 1640 with HEPE and supplemented with
glucose, pyruvate, 2-mercaptoethanol, 10% FBS and peni-
cillin/streptomycin as recommended by ATCC. These
cells were grown to a density of 1.5 × 10^6 cells/ml in a
75 cm flask and then were induced to differentiate into
macrophages using 12-O-tetradecanoylphorbol-13-acetate (Enzo Life Sciences) at a concentration of
0.5 μM for 3 hours [15]. Following induction, cells were
washed with PBS and then plated into 12-well tissue cul-
ture plates at a density of 6 x10^5 cells/well and incubated
overnight in normal media. Prior to any activation studies,
cells were washed with PBS followed by the addition of
0.5 ml of serum free Opti-MEM per well.

Macrophage activation studies
Different concentrations of Monosodium Urate (MSU)
crystals (Enzo Life Sciences) in a physiological range
(concentrations of serum uric acid that are possible in
humans, i.e. up to 20 mg/dl) [16,17] were initially tested
to establish conditions for inducing pro-inflammatory
cytokines from activated macrophages. MSU crystals
(2.5 to 20 mg/dl) were added to the differentiated cells
grown in Opti-MEM and incubated for 24 hours in 10%
CO2. Cell culture media were then removed and stored
at -80°C until analyzed by immunoassay for IL-1β (high-
sensitivity assay R&D Systems), and TNFα and IL-8 (run
as part of a human proinflammatory 9-plex by Mesoscale
Discovery, MSD). All samples yielded measurable concen-
trations; 23 of 46 values for IL-8 were out of range high
but could be readily extrapolated as they were within the
linear range of the assay. The intra and inter-assay coeffi-
cients of variation (CV) for IL-1β were 2.85% and 4.87%
respectively as reported by the manufacturer. However, no
CVs were reported by the manufacturer for the MSD kit.

The intra-assay CVs were 4.85% for TNFα, and 2.77% for
IL-8 according to our calculations.

In order to identify the component of IL-1β production
attributable to inflammasome activation, a commercially
available cell-permeable caspase-1 inhibitor (EMD Milli-
pore catalog#400011, sequence: Ac-AAVALLALLA
PYVAD-CHO) was used. Cells were pre-treated with vari-
ous concentrations of the inhibitor (0.01-10 μM) for six
hours prior to stimulation with MSU crystals (20 mg/dl).
This high concentration of MSU was tested to provide a
stringent test of caspase inhibition. After stimulation of
macrophages for 24 hours as described above, the cell
culture media were analyzed for IL-1β.

CS inhibition studies
To test for anti-inflammatory effects of CS, macrophages
were pretreated with highly purified bovine chondroitins
4 and 6 sulfate of ≥98% purity, and with an average mo-
olecular weight of ~15–16 kDa (Bioibérica, Barcelona,
Spain) for 4 hours prior to the addition of MSU crystals
(10 mg/dl). A range of doses of CS (10–200 μg/ml) that
approximate physiological conditions [18,19] were tested.
Culture media were collected at 24 hours and IL-1β,
IL-8 and TNFα concentrations were analyzed as above.
The IL-1β data represent the aggregate of 7 total rep-
licates over 4 independent experiments; the IL-8 and
TNFα data represent 4 total replicates from 4 separate
experiments.

Endotoxin assay
To test for the presence of endotoxin in the experimental
reagents utilized for these experiments, we used Pyrogene
Recombinant Factor C Endotoxin Assay (Lonza) accord-
ing to the manufacturer’s instructions. This assay utilizes a
recombinant Factor C, which when activated by endotoxin
binding reacts with a fluorogenic substrate to produce a
fluorescent signal in direct proportion to the amount of
endotoxin in the sample.

Statistical analysis
Fold activation of cytokines was determined comparing
the negative controls (no added MSU) to MSU with
results expressed as mean % control. CS effects on
MSU-induced cytokine concentrations were expressed as a
mean percent of the MSU only condition (set to 100%).
Statistical significance was determined by one-way ANOVA
with Dunnett’s post-hoc test. Linear trend analyses of
these data were performed to assess for a CS dose re-
response. Analyses were performed using GraphPad Prism
software (San Diego, CA). Linear trend analyses were
performed using JMP 9 (SAS). Results were considered
significant for p < 0.05.
Results and discussion

All cell culture reagents, including the MSU and CS, were tested for the presence of endotoxin by our laboratory or the manufacturer, and all were found to contain less than 0.03 EU/ml endotoxin. Increasing concentrations of MSU crystals led to increasing IL-1β production (Figure 1). Specifically, MSU concentrations of 10 mg/dl and greater increased IL-1β production by macrophages; thus, concentrations of 10–20 mg/dl were used for subsequent experiments.

To stringently assess the mechanism of IL-1β production in macrophages in response to MSU, cells were stimulated with a high concentration of MSU, 20 mg/dl, with pre-incubation with varying concentrations of a caspase-1 inhibitor. IL-1β production was fully inhibitable by the caspase-1 inhibitor in a dose dependent manner confirming inflammasome activation as the source of this cytokine (Figure 2).

To assess the effect of CS on MSU stimulated cytokine production, we pre-incubated THP-1 macrophages in the absence and presence of CS for 4 hours followed by stimulation with MSU 10 mg/dl. This concentration was chosen as it reliably induced IL-1β production, is representative of hyperuricemia, and is associated with a high incidence of gout [20]. IL-1β, TNFα and IL-8 were induced a mean 7-, 4- and 3-fold respectively by MSU. CS significantly inhibited IL-1β (p = 0.0029) and TNFα (p = 0.0174) production from macrophages in response to MSU (Figure 3). These results were also significant by linear trend analysis (p = 0.001 and p = 0.009 for IL-1β and TNFα respectively). Although IL-8 was similarly inhibited by CS (Figure 3), this trend was not statistically significant by ANOVA (p = 0.4147) but was significant by linear trend analysis (P = 0.05). The linear trend analyses demonstrate a reduction of inflammation by CS in a dose-dependent manner.
Three cytokines associated with gouty inflammation, IL-1β, TNFα and IL-8 were all induced by exposure of activated macrophages to MSU crystals. Macrophage exposure to CS for 4 hours prior to MSU led to a significant dose-dependent decline in production of both IL-1β and TNFα. Many of the anti-inflammatory effects of CS are thought to affect the transcription of various cytokines, such as IL-1β and TNFα. In particular, they are thought to affect various kinases, which in turn block the translocation of NFκB to the nucleus [14]. Others have suggested that NFκB could also affect IL-8 production [21]. Martinon et al. demonstrated that MSU crystals lead to IL-1β production through activation of the inflammasome [22]. TNFα is also upregulated by MSU crystals in an experimental animal model of gouty arthritis, but blocking IL-1β (either pharmacologically or genetically) lessened this response [23]. In addition, IL-8, a chemotactic factor responsible for neutrophilic infiltration, was upregulated when MSU crystals were injected in the joints of rabbits; this neutrophil response and the
gout related synovitis were attenuated with the use of an anti-IL-8 antibody [24].

Based on the literature, the concentrations of CS used in these experiments are comparable to those suggested to be within a physiologically achievable range [18,19]. The data presented here suggest there may be a role for CS in preventing flares of gout due to initiation of uric acid lowering agents. To gain potential insights into whether CS might play a role in treatment of active gout flares, future studies are needed to test the effects of CS added coincidentally or after MSU stimulation. Given the low side effect profile of CS, it represents an intriguing treatment option for these scenarios in gout. In particular, CS might synergize with other established treatments for gout thereby making it possible to lower doses or discontinue traditional therapies, particularly in the subset of individuals with relative contraindications to the traditional therapies including allopurinol, NSAIDs and colchicine in the context of renal insufficiency.

A number of meta-analyses have found oral CS to be both safe and well tolerated [12,13]. However, one must take into account both the purity and source (i.e. bovine or shark etc.) as other in vitro studies have shown that in vitro anti-inflammatory properties of CS can vary based on the preparation [25–27]. Further studies in humans will be needed to determine if CS has a role as a treatment option for patients with gout.

A limitation of our study was the use of THP-1 cells derived from a human monocytic cell line. They are often employed in the laboratory setting because of their ease of use. However, it would be of benefit to repeat these experiments in primary peripheral monocytes or primary synovial macrophages. Although we established that IL-1β, produced by macrophages in this system, was a product of inflammasome activation, these experiments do not establish the exact target of CS inhibition. CS may be blocking NFκB activation, as established by others [18], which would block pro-IL-1β transcript expression. Alternatively, CS could be acting outside of the cell by blocking the interaction of MSU or extracellular matrix fragments with cell surface receptors on macrophages [28]. Still the anti-inflammatory effects of CS and other sulphated glycosaminoglycans may be mediated through sequestering of cytokines as suggested based on NMR and fluorescent spectroscopy as well as computational simulation studies [29]. Finally, CS could have pleiotropic effects on the cell, some of which have yet to be elucidated.

Conclusions

CS decreased MSU-mediated cytokine production from activated macrophages. In particular, IL-1β and TNFα were lowered in a dose-dependent manner by CS. Given the role of these cytokines in initiating gouty inflammation, CS may have a role as a prophylactic agent in the treatment of gout.

Ethics statement

This paper represents a series of in vitro cell culture experiments using an immortalized cell line (ATCC TIB-202). These studies did not involve any human or animal experiments. Therefore, we did not need the approval of any research ethics committee or institutional review board.

Abbreviations

ATCC: American type culture collection; CS: Chondroitin sulphate; FBS: Fetal bovine serum; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, a commonly used organic ion buffering agent; IL-1β: Interleukin 1β; MSU: Monosodium urate; PBS: Phosphate buffered saline; RPMI: Roswell park memorial institute, medium commonly used in cell culture; TNF: α-tumor necrosis factor α.

Competing interests

EM and JV are employees of Bioibérica, a commercial producer of chondroitin sulphate. EWO, TVS and VBK have all received some research funding from Bioibérica.

Authors’ contributions

TVS and VBK were responsible for the experimental design. EWO and TVS were responsible for performing the experiments. EWO, TVS and VBK were supplied the chondroitin sulfate for these experiments. EM and JV were additionally supported by P01 AR050245 and P30 AG-028716. Bioibérica supplied the chondroitin sulfate for these experiments. EM and JV are employees of Bioibérica and provided input into the design and manuscript preparation and revision. All authors approved the final version for submission.

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