OsteoArthritis and Cartilage (2006) **14,** 793–806

doi:10.1016/j.joca.2006.02.003

Osteoarthritis and Cartilage

International Cartilage Repair Society

OARSI OSTEOARTHRITIS RESEARCH SOCIETY INTERNATIONAL

Mixtures of glucosamine and chondroitin sulfate reverse fibronectin fragment mediated damage to cartilage more effectively than either agent alone

G. A. Homandberg Ph.D.*, D. Guo M.S., L. M. Ray C.L.S. and L. Ding B.S.

Department of Biochemistry and Molecular Biology, University of North Dakota School of Medicine and Health Sciences, Box 9037, Grand Forks, ND 58202, USA

Summary

Objective: To test the effectiveness of glucosamine ($GluNH_2$)-HCl, chondroitin sulfate (CS) and mixtures in protecting cartilage exposed to fibronectin fragments (Fn-fs), an exposure known to enhance catabolic cytokines and matrix metalloproteinases (MMPs).

Methods: Pharmacologic formulations of $GluNH_2$ (FCHG49®) and CS (TRH122®) (Nutramax Laboratories, Inc.) were added at 1, 10 or 100 μg/ml singly or in mixtures to bovine cartilage cultures in serum or serum-free conditions with or without Fn-f. Proteoglycan (PG) release into media and remaining cartilage PG content were measured by dye binding analysis and effects on PG synthesis by assays of 35-sulfate incorporation. Effects on MMP-3 and -13 expression were measured by Western blotting of conditioned media.

Results: In serum-free conditions, the agents singly or as mixtures did not block Fn-f mediated matrix degradation. In serum, single agents were weakly effective at 100 μg/ml, while the mixture of each agent at 0.1 μg/ml decreased PG loss by about 50% by day 7 and at 1 μg/ml restored nearly 50% of the PG after 7 days in Fn-f pretreated cartilage. However, both agents singly and as mixtures at 0.1–100 μg/ml decreased MMP release. In serum, the single agents at 1–10 μg/ml weakly reversed Fn-f mediated PG synthesis suppression, while the mixtures were 100% effective at 1 μg/ml.

Conclusions: $GluNH_2$ and CS act synergistically in reversing damage and promoting repair at concentrations found in plasma after oral ingestion of these agents. Reversal of PG synthesis suppression correlates more with these activities than suppression of MMP-3 or -13 expression.

Key words: Glucosamine, Chondroitin sulfate, Fibronectin fragment, Cartilage repair, Cartilage damage, Matrix metalloproteinases, Proteoglycan.

Abbreviations: $GluNH_2$ glucosamine, CS chondroitin sulfate, GAGs glycosaminoglycans, OA osteoarthritis, NSAIDs nonsteroidal anti-inflammatory drugs, PG proteoglycan, MMP matrix metalloproteinase, MMP-3 stromelysin-1, Fn fibronectin, Fn-f fibronectin fragment, ECL enhanced chemiluminescent, HRP horse radish peroxidase, DMEM Dulbecco's modified Eagle's medium, SD standard deviation, S.E.M. standard error of mean, DMB dimethylmethylene blue.

Introduction

Glucosamine ($GluNH_2$) and chondroitin sulfate (CS) are two commonly used nutraceutical compounds that have been reported to have chondroprotective qualities. $GluNH_2$ is an amino monosaccharide precursor that is incorporated, either directly or after conversion to galactosamine, into the disaccharide unit of glycosaminoglycans (GAGs) found in proteoglycans (PGs) in the cartilage matrix. CS is a long-chain, sulfated polymer of up to 40-kDa of repeating, partially sulfated disaccharide units of galactosamine sulfate and glucuronic acid and represents the majority of GAGs in articular cartilage[1].

Clinical trials on $GluNH_2$ used in relief of osteoarthritis (OA) symptoms have shown that $GluNH_2$ has a moderate treatment effect, whereas CS has a larger treatment effect[2–4]. Although slower acting than nonsteroidal anti-inflammatory drugs (NSAIDs), $GluNH_2$ has been reported to be as effective as NSAIDs at relieving the symptoms of OA[5,6]. Efficacy depends on the measurement methodology, with the Lequesne Index showing improvement over placebo in many studies but no improvement when the WOMAC Index is used[7–9]. Some studies have shown no effect, possibly related to the source of $GluNH_2$ used[7–9] as discussed[10]. CS has also been shown to be efficacious for the treatment of mild to moderate OA[11–13] and also as effective as NSAIDs in pain reduction[11]. Several studies also indicated a disease modification potential in knees and hands[12,13].

Although CS and $GluNH_2$ are now commonly used as a combination for treatment of OA, only a few studies have evaluated efficacy of the combination. Das and Hammad[14] conducted a randomized, placebo-controlled study with 93 knee OA patients, using as the primary outcome the Lequesne Index of Severity of OA of the Knee (ISK) and found that the combination with added manganese ascorbate showed significant improvement over the placebo[14]. In a smaller study of subjects with degenerative joint disease of the knee or lower back, Leffler *et al.*[15] used the same combination and showed significant effects

*Address correspondence and reprint requests to: Gene A. Homandberg, Ph.D., Department of Biochemistry and Molecular Biology, University of North Dakota School of Medicine and Health Sciences, 501 N. Columbia Road, EJ Med Res Bldg, Grand Forks, ND 58203, USA. Tel: 1-701-777-6422, Fax: 1-701-777-2382; E-mail: ghomandberg@medicine.nodak.edu

Received 1 December 2005; revision accepted 2 February 2006.

over placebo on patient assessment of treatment effect, visual analog scale for pain, and physical examination score. A large, ongoing study (the Glucosamine–chondroitin Arthritis Intervention Trial, "GAIT")[16] is currently comparing CS and $GluNH_2$ hydrochloride, alone and in combination, to a nonsteroidal cyclooxygenase-2 inhibitory drug (NSAID) using the same source of CS used in these two smaller studies[14,15]. To date, this study, which is using the WOMAC Index, has shown that the combination is substantially more effective than either agent alone in relieving symptoms of knee OA, even surpassing the NSAID in effectiveness[16]. Interestingly, only those patients with moderate to severe knee pain had a better response with the combination than with celecoxib. It remains to be seen whether or not these results will be evident in a larger set of patients.

The specific combination of $GluNH_2$ hydrochloride and low molecular weight CS, with added manganese ascorbate, has also been studied clinically in animals and with *in vitro* studies. The mixture has been shown *in vitro* to act synergistically in stimulating the production of PGs in the articular cartilage, while inhibiting the activity of the degradative enzymes that act on articular cartilage[17]. In a study of surgical reconstruction of canine cruciate ligament, this formulation taken orally, stimulated cartilage metabolism as measured by an increase in CS epitopes 3B3 and 7D4[18]. Animal trials have also shown this combination to exert a protective effect on cartilage degradation in various experimental models[17–20].

While the clinical effectiveness of $GluNH_2$ and CS is controversial and warrants further studies, the possible mechanism(s) of action also remain a matter of controversy and ongoing research. Reported *in vitro* effects of $GluNH_2$ include stimulation of GAG synthesis[21,22], inhibition of cyclooxygenase independent anti-inflammatory properties[23,24] and inhibition of IL-1 stimulated gene expression[25–31] or protease activity[32]. Reported effects of CS include stimulation of synthesis of PG[12,13,17,33], inhibition of degradative enzymes[34–36] and inhibition of IL-1 stimulated gene expression and production of pro-inflammatory genes including matrix metalloproteinases (MMPs)[28,29,32]. Thus, each agent has been reported to have both proanabolic and anti-catabolic or anti-inflammatory activities.

One complication in interpretation of relevant published studies is that many of these studies have used concentrations of CS and $GluNH_2$ far higher than the levels reported to occur after oral ingestion. Typical concentrations of the agents in plasma after oral ingestion are in the tens of μg/ml. For example, pharmacokinetic studies indicate a bioavailability of CS up to 5–70%, with some of the CS being absorbed as partially degraded components[37] and maximal concentrations of 3–36 μg/ml, depending on the species[17,38–41]. In contrast, $GluNH_2$ in plasma has very low bioavailability, due to the apparent effect of first pass metabolism[38,42–45]. Radiotracer studies show high uptake[23,24,38,42–45] and maximal levels of unmetabolized $GluNH_2$ in dogs, horses, and humans of 2–11 μg/ml[38,42–45]. These levels are far below those often used for *in vitro* studies and apparently too low to serve as an effective, glucose-competing substrate for PG synthesis[23,24,38,42–47]. These results draw into question the assumption that $GluNH_2$ and/or CS act by providing additional substrates for PG synthesis. However, it should also be noted that CS given in a low mass form has been shown, like $GluNH_2$, to have an affinity for articular cartilage[36] and thus, serum levels of these agents may not be useful in predicting their efficacy after oral ingestion. Nonetheless, only recently have studies on $GluNH_2$ shown effects on chondrocytes at low μg/ml concentrations, and only one study has shown clear effects of CS on inhibition of IL-1 action at the reported physiological concentration[28,29,31]. There have also been limited studies that have tested whether specific combinations are more effective than the individual agents[17], although it has been postulated that combining $GluNH_2$ with CS yields a synergistic rather than additive effect[48,49].

In order to investigate whether or not $GluNH_2$ and CS as single agents or as a combination at concentrations close to those observed after oral ingestion are effective, we have tested the $GluNH_2$ hydrochloride and the CS used in the GAIT study[16] and also tested in other studies[16,17,28,29], in a cartilage chondrolytic culture model in which the catabolic mediators, fibronectin fragments (Fn-fs), are added to cartilage explants[50]. The Fn-fs elevate catabolic cytokines[51,52] and MMPs[51,53] which cause transient suppression of PG synthesis and severe depletion of cartilage PG[54]. Since the damaged bovine cartilage does not spontaneously restore PG after the Fn-fs are removed, this model is also useful for tests of effects on cartilage repair (reviewed in Ref. 55). The relevance of this model is based on our observations that Fn-fs are found in OA synovial fluids and cartilage, that injection of Fn-f into rabbit knee joints causes cartilage degeneration[56,57] and that these Fn-fs are easily generated under cartilage damage conditions initiated by other catabolic mediators such as addition of MMP-3 or IL-1 to cultured cartilage[58].

Materials and methods

All common chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO). [^{35}S] sulfate was from ICN Biomedicals Inc. (Cosa Mesa, CA). Fetal bovine serum was from Gibco-Invitrogen (Carlsbad, CA). Rabbit polyclonal antibody to the hinge region of human MMP-3 (#AB810) was from Chemicon Corporation (Temecula, CA). Rabbit polyclonal antibody to the hinge region of human MMP-13 (#M4052) and Reactive Red 120-agarose were from Sigma Chemical Co. (St. Louis, MO). Horse radish peroxidase (HRP) conjugated sheep anti-rabbit IgG (A0545) was from Sigma Chemical Co. (St. Louis, MO). The modified Lowry protein assay kit and the Enhanced Chemiluminescent (ECL), the Super Signal Chemiluminescent Substrate kit for HRP, were from Pierce Chemical Co. (Rockford, IL). The Quantikine Human MMP-3 enzyme linked immunosorbent assay (ELISA) kit was from R & D Systems, Inc. (Minneapolis, MN). The $GluNH_2$ and CS pharmacologic formulations tested ($GluNH_2$ hydrochloride (FCHG49®) and CS (TRH122®)) were from Nutramax Laboratories, Inc. (Edgewood, MD). Solutions were made up fresh at the start of the experiment and kept frozen for up to 21 days in between use.

ISOLATION OF Fn-fs

An amino-terminal 29-kDa thrombin-generated Fn-f is the most characterized of the Fn-fs in terms of cartilage chondrolytic activities[55]. However, for these studies, a more physiologically relevant mixture of Fn-fs was the major type of Fn-f studied. The mixture was derived by digestion of human plasma fibronectin with 1 μg/ml MMP-3 and contains Fn-fs of 29-kDa, 40–60-kDa and 120–160-kDa, and has been described elsewhere[59]. This mixture has activities indistinguishable from the thrombin-generated 29-kDa. In comparisons between the two different types of Fn-fs, assays of the effects of the mixture on rates of PG degradation in bovine metacarpophalangeal cartilage in DMEM cultures showed