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

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Summary

Objective: To test the effectiveness of glucosamine (GluNH2)-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 GluNH2 (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 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: GluNH2 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.

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

Abbreviations: GluNH2, 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, dimethylmethylen blue.

Introduction

Glucosamine (GluNH2) and chondroitin sulfate (CS) are two commonly used nutraceutical compounds that have been reported to have chondroprotective qualities. GluNH2 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.

Clinical trials on GluNH2 used in relief of osteoarthritis (OA) symptoms have shown that GluNH2 has a moderate treatment effect, whereas CS has a larger treatment effect2–4. Although slower acting than nonsteroidal anti-inflammatory drugs (NSAIDs), GluNH2 has been reported to be as effective as NSAIDs at relieving the symptoms of OA5,6. 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 used7–9. Some studies have shown no effect, possibly related to the source of GluNH2 used7–9 as discussed10. CS has also been shown to be efficacious for the treatment of mild to moderate OA11–13 and also as effective as NSAIDs in pain reduction11. Several studies also indicated a disease modification potential in knees and hands12,13.

Although CS and GluNH2 are now commonly used as a combination for treatment of OA, only a few studies have evaluated efficacy of the combination. Das and Hammad14 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 placebo14. 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
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”) is currently comparing GluNH₂ and GluNH₂ 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. 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. 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 GluNH₂ 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. 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 and 7D4. Animal trials have also shown this combination to exert a protective effect on cartilage degradation in various experimental models.

While the clinical effectiveness of GluNH₂ 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 GluNH₂ include stimulation of GAG synthesis, inhibition of cyclooxygenase independent anti-inflammatory properties, and inhibition of IL-1 stimulated gene expression or protease activity. Reported effects of CS include stimulation of synthesis of PG and inhibition of degradative enzymes and inhibition of IL-1 stimulated gene expression and production of pro-inflammatory genes including matrix metalloproteinases (MMPs). 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 GluNH₂ 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 and maximal concentrations of 3–36 μg/ml, depending on the species. In contrast, GluNH₂ in plasma has very low bioavailability, due to the apparent effect of first pass metabolism and maximal levels of unmetabolized GluNH₂ in dogs, horses, and humans of 2–11 μg/ml.

Radiotracer studies show high uptake and maximal levels of unmetabolized GluNH₂ in dogs, horses, and humans of 2–11 μg/ml. These levels are far below those often used in in vitro studies and apparently too low to serve as an effective, glucose-competing substrate for PG synthesis. These results draw into question the assumption that GluNH₂ and 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 GluNH₂, to have an affinity for articular cartilage and thus, serum levels of these agents may not be useful in predicting their efficacy after oral ingestion. Nonetheless, only recently have studies on GluNH₂ 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 physiologic concentration. There have also been limited studies that have tested whether specific combinations are more effective than the individual agents, although it has been postulated that combining GluNH₂ with CS yields a synergistic rather than additive effect.

In order to investigate whether or not GluNH₂ and CS as single agents or as a combination at concentrations close to those observed after oral ingestion are effective, we have tested the GluNH₂ hydrochloride and the CS used in the GAIT study, and also tested in other studies in a cartilage chondrolytic culture model in which the catabolic mediators, fibronection fragments (Fn-fs), are added to cartilage explants. The Fn-fs elevate catabolic cytokines and MMPs which cause transient suppression of PG synthesis and severe depletion of cartilage PG. 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 joint causes cartilage degeneration 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.

Materials and methods

All common chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO). [35S] sulfate was from ICN Biomedicals Inc. (Cosa Mesa, CA). Fetal bovine serum was from Gibco-Invtrogen (Carlsbad, CA). Rabbit polyclonal antibody to the hinge region of human MMP-3 (#ABB810) 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 (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 GluNH₂ and CS pharmacologic formulations tested (GluNH₂ hydrochloride (FCGHG49®) 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 uses.

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. However, for these studies, a more physiologically relevant mixture of Fn-fs was the major type of Fn-f studied. This mixture was derived by digestion of human 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. 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...
that a 1 μM solution of this mixture (500 μg/ml of total Fn-fs) had approximately the same rate constant as a 1 μM solution of the highly purified amino-terminal 29-kDa Fn-f. The mixture at 1 μM also suppressed PG synthesis to the same level as 1 μM 29-kDa Fn-f when assayed as described.

In order to add credibility to our use of the mixture, we tested whether a 29-kDa band found in the mixture was similar to the thrombin-generated 29-kDa Fn-f. The MMP-3 digest mixture was subjected to electrophoresis on 10% acrylamide gels, lightly stained with Coomassie Blue in fresh plastic containers. Bands of 29-kDa, 50–70-kDa and 140–160-kDa were detected after light staining with Coomassie. The 29-kDa band was excised, subjected to trypsin digestion and peptides analyzed by ESI-QUAD-TOF mass spectrometry using standard protocols. The band yielded a tryptic fragment of residues 58–67. Therefore, the small 29-kDa Fn-f in the MMP-3 digest is likely the same thrombin-generated 29-kDa Fn-f we have extensively studied.

In order to ensure that active proteinases in the Fn-f solutions did not account for cartilage degradation, their effects at 1 μM concentrations on cartilage in DMEM that had been freeze-thawed for three cycles to inactivate metabolic processes were measured. None of the Fn-fs solutions had a significant effect on enhancing PG degradation and release into the culture media over a 7 day period. Further, in order to ensure that no MMP-3 activity remained in the mixture, the MMP-3 digest was passed over reactive Reactive Red 120-agarose to remove MMP-3 and then subsequently treated with 10 mM ethylenediaminetetraacetic acid (EDTA), prior to a final dialysis against phosphate buffered saline to completely remove the 29-kDa thrombin-generated 29-kDa Fn-f. In order to ensure that trace inactive MMP-3 in the MMP-3 digest which may not have been totally removed by Reactive Red-agarose treatment would not interfere with our MMP blotting, the 29-kDa thrombin-generated 29-kDa Fn-f was utilized in experiments where media were to be blotted against anti-MMPs. All the Fn-f solutions were treated with detoxi-Gel prior to use, dialyzed to remove protease inhibitors and EDTA and sterile filtered. Assays of endotoxin with a kit from Sigma Chemical Co. showed levels <50 pg/mg of protein in any Fn-f solution.

EXPLANT CULTURES

Culturing of slices of bovine metacarpophalangeal cartilage from 18-month-old bovines was performed as described in DMEM containing 50 U/ml penicillin streptomycin in 10% serum/DMEM or in DMEM alone and with 20–60 mg cartilage per 1.5 ml/well. The cartilage was allowed to equilibrate for 2 days in DMEM alone prior to adjustment to the final culturing conditions. During the culture, the media were changed every other day for 10% serum/DMEM cultures but were not changed for kinetic assays of PG degradation in serum-free cultures. Each condition was replicated in triplicate for each culture.

ASSAYS OF KINETICS OF PG DEGRADATION

The kinetics of PG degradation and release into the media in serum-free cultures were studied in order to generate conditions in which the damaged cartilage could not effectively respond with enhanced anabolic activities that might have been contributed by growth factors in serum. These conditions provide tests of the effects on mostly catabolic activities. Briefly, cartilage was preequilibrated in DMEM for 2 days and then adjusted to various concentrations of the agents or a mixture. For the mixture, the concentrations given refer to the concentration of each agent. After 4 h, the cultures were adjusted to 1 μM Fn-f. The media were not changed during the culture. Each day, for up to 5 days, 50 μl aliquots of the culture media were removed, the media diluted 10-fold and PG content was assayed using the dimethylmethylene blue (DMB) assay as modified. The PG content was converted to μg PG/mg cartilage for each of three similar wells and the amounts graphed vs day in culture to determine rate constants of μg PG/mg cartilage/day by linear regression. Separate assays showed that Cs when added to the PG standards used to generate our standard curve or added to papain digests did not increase the colorimetric response, indicating that Cs that had been added to the media was not capable of interfering with the assay.

ASSAYS OF EFFECTS OF AGENTS ON PG CONTENT OF CARTILAGE IN 10% SERUM CULTURES

In order to test the effects of the agents on blocking Fn-f mediated PD degradation as measured by the PG content of cultured cartilage, conditions of 10% serum were used. These conditions are more physiologic than serum-free and are conditions in which damaged cartilage has serum growth factors in the media which would help mount an anabolic response to the damage as well as proteinase inhibitors which would decrease proteolytic damage. This latter culture system allowed us to measure effects of agents on homeostasis closer to steady state conditions than serum-free and thus, to measure the summation of anabolic and catabolic events. We have shown that in these long-term 21–28 day cultures, that cell viability as measured by DNA content does not decrease by more than 20%, an amount not statistically different than untreated cartilage.

Bovine cartilage was cultured in 10% serum/DMEM with the single agents or with a mixture and with or without 100 nM Fn-f for periods up to 21 or 28 days. For the mixture, the concentrations given refer to the concentration of each agent. The agents were added first, followed by addition of Fn-f 4 h later. Media were changed every other day. Every 7 days, the PG content of the remaining slices was measured by DMB assays of papain digests. Each condition was replicated in triplicate for each culture.

ASSAYS OF EFFECT OF AGENTS ON PG CONTENT OF DAMAGED CARTILAGE IN 10% SERUM CULTURES

In order to test the reparative activity of the agents, cartilage was damaged with 100 nM Fn-f prior to addition of agents. Briefly, bovine cartilage was cultured in 10% serum/DMEM with 100 nM Fn-f from days 0–7 to deplete PG. Media were changed every other day. At day 7, some of the cultures were adjusted to various concentrations of GluNH2 and Cs or mixtures but without Fn-f. For the mixtures, the concentrations given refer to the concentrations of each component. PG content was analyzed every 7 days. PG contents were determined on papain digests using the DMB assay as described. The PG contents were reported as μg PG/mg wet weight cartilage. Each condition was replicated in triplicate for each culture.

WESTERN BLOTTING AND CHEMILUMINESCENT DETECTION OF MMPs

In order to test whether potentially beneficial effects of the agents might occur through the ability to suppress expression of MMPs, Western blotting of conditioned media was...
employed. We have shown that MMP activity is involved in the ability of Fn-fs to decrease PG content of cultured cartilage \(^{60}\). An aliquot of conditioned serum-free media, after concentrating 10-fold, was applied to 7.5 or 10% acrylamide sodium dodecyl sulphate (SDS) gels run at 30 mA per gel and the resultant gels blotted onto nitrocellulose for 1 h at 100 V. The amount of protein applied in each lane was identical and typically about 5 \(\mu\)g, as measured with the modified Lowry protein assay. The blot was then treated with 3% BSA in 20 mM Tris buffer, pH 7.4, containing 140 mM NaCl (Tris buffered saline (TBS)) for 1 h at room temperature or overnight at 4 °C. The blot was then incubated with a 1–5000 fold dilution of anti-MMP-3 or 1–3300 for anti-MMP-13 for 1 h, washed three times with TBS containing 0.1% Tween 20 (TBST) for 5–10 min each. The blot was then reacted with HRP conjugated rabbit anti-sheep IgG for 1 h, washed three times with TBST and one last time with TBS. The blot was then reacted with Supersignal West Dural Extended Duration substrate (Pierce Chemical Co., Rockford, IL) for 5 min before being placed in a plastic membrane protector. The image was captured by a Lumi-Imager—Mannheim Lumi Analyst 3.0 software.

When 10% serum conditioned media were concentrated 10-fold and subjected to blotting against anti-MMP-3, broad heavy smears of about 70-kDa in both nontreated and Fn-f treated samples, due to 100% serum, made visualization of MMP-3 bands difficult. As an alternative, a human MMP-3 ELISA kit was tested but no significant reaction was noted from any samples. As another alternative, media were first treated with Reactive Red 120-agarose to concentrate MMP-3 and separate it from the serum. A 50 \(\mu\)l suspension (50X) of agarose was mixed with 1.0 ml of nonconcentrated conditioned media for 30 min, followed by washing of the resin with TBS and treatment of the resin with denaturation buffer and reducing agent. This yielded a releasate that when subjected to blotting yielded elevated MMP-3 bands in Fn-f treated cultures and only baseline levels in non-Fn-f treated cultures.

ASSAYS OF EFFECTS OF AGENTS ON PG SYNTHESIS IN EXPLANTS

In order to test whether potentially beneficial effects of the agents might occur through the ability to reverse the PG synthesis suppression activity of Fn-fs \(^{34}\), assays of 35-S sulfate incorporation were employed. Bovine cartilage was cultured in 10% serum/DMEM cultures in the presence of various concentrations of the agents alone or as a mixture. For the mixture, the concentrations given refer to the concentration of each agent. Four hours after addition of agent, some cultures were adjusted to 100 nM Fn-f. Media were changed every other day. At various times, the media were adjusted to 10 \(\mu\)Ci/ml of 35S-sodium sulfate for 2 h, followed by a 2-h cold chase with 10% serum/DMEM. Slices were weighed and then extracted with 1 ml of 4 M guanidine-HCl, 0.1% Triton X-100, 10 mM EDTA, 100 mM sodium acetate, pH 5.5 for 16 h, followed by exhaustive dialysis of the extracts against 10 mM EDTA—water. For explants, the amount of label was expressed as dpm/mg wet weight cartilage and converted into percentage of values for control untreated cultures. Each condition was replicated in triplicate for each culture.

STATISTICAL TREATMENT

For kinetic assays of PG degradation, three wells were used per time point in each culture and 5 days analyzed by linear regression to obtain rate constants and s.e.m. values. An \(n\) value of 15 was used to compare slopes using the two-tailed unpaired Student’s t test. For assays of PG content and of PG synthesis rates, for each major culture, cartilage was pooled from three to four bovines and three culture wells were used for each condition. Each of the major cultures was performed four times 1 month apart and the final mean and final s.e.m. values from a total of 12 wells/datum were determined as \(n = 12\). In this way, the variance in the final analysis reflected as many parameters as possible, including but not limited to, cartilage weighing variation, assay variation, culturing condition variations and cartilage heterogeneity. Cartilage heterogeneity was inherent due to well to well variation in cartilage slice masses, shapes and original \(in situ\) joint locations, the latter of which could theoretically introduce variability in biochemical response. Once the final mean and s.e.m. values were calculated, control vs experimental data at selected time points were compared using two-tailed unpaired Student’s t tests of the 12 culture wells and associated s.e.m. values. This analysis allowed us to perform pairwise analysis of single time points without assumption of a mechanism that would be implied by analysis of data as kinetic curves. In order to concisely describe statistical trends in a subset of data, \(P\) values, representing the least significance within the set are given where possible and appropriate.

Results

GLuNH2 OR CS SEPARATELY OR IN A MIXTURE DID NOT DECREASE KINETICS OF DAMAGE

In the first test of effects of the agents, serum-free conditions were chosen such that proteinase inhibitors and growth factors present in serum would not be able to contribute a response to the damage and obscure visualization of blocking of catabolism. Aliquots of media were removed each day to measure PG contents so rate constants could be estimated. Figure 1 shows that the Fn-f mixture (MMP-3 generated) enhanced the rate of PG degradation and release into the media as compared to untreated cartilage. Further, 100 \(\mu\)g/ml of either GluNH2 or CS alone had no effect on the PG release kinetics. To test protective effects, the agents at 0.1–100 \(\mu\)g/ml singly or in mixtures (M) were added to cartilage, followed 4 h later by addition of the Fn-f mixture. The agents did not significantly decrease the enhanced rate caused by the Fn-f while a concentration of 100 \(\mu\)g/ml significantly increased the rate \((P = 0.002)\). Analysis of the PG remaining at the end of the experiments, confirmed that the agents did not significantly decrease the ability of Fn-fs to decrease PG content (data not shown).

GLuNH2 OR CS OR MIXTURES ALONE SLOWED Fn-f MEDIATED MMP-3 RELEASE

Since the data in Fig. 1 suggested that the agents did not decrease PG catabolism, we investigated next whether this lack of blocking correlated with lack of effect on expression of a major MMP involved in Fn-f mediated cartilage damage, MMP-3 \(^{60}\). Conditioned media from similar serum-free cultures as in Fig. 1 were tested for MMP-3 content, except the 29-kDa Fn-f was used instead of the Fn-f mixture in order to avoid possible detection of trace amounts of MMP-3 used to generate the mixture. As shown in Fig. 2, at day 3, various conditions and concentrations of the agents in the absence of the 29-kDa Fn-f did not enhance...
MMP-3 release (G, C and M correspond, respectively, to GluNH₂, CS and mixtures) as compared to that of the Fn-f control (lane F) and untreated controls (Ct). The numbers refer to mg/ml of agent and in the case of the mixture, the concentrations of each component. These experiments were performed with three different cartilage preparations with similar observations.

However, the agents paradoxically appeared to slow Fn-f mediated MMP-3 release (Fig. 3). Each test of the agent corresponds to a well in which the 29-kDa Fn-f was added 4 h after test agent. Aliquots of conditioned media were assayed for PG content as a function of time and plotted as µg PG/mg wet weight cartilage. Each condition was in triplicate and each datum was plotted for each of 5 days, resulting in an n value of 15 for linear regression. Linear regression was used to estimate rates and s.e.m. values of best fit curves.

Four cartilage harvests were used for the analysis.

MMP-3 release (G, C and M correspond, respectively, to GluNH₂, CS and mixtures) as compared to that of the Fn-f control (lane F) and untreated controls (Ct). The numbers refer to µg/ml of agent and in the case of the mixture, the concentrations of each component. These experiments were performed with three different cartilage preparations with similar observations.

GluNH₂ OR CS ONLY WEAKLY BLOCKED Fn-f MEDIATED PG DEPLETION IN 10% SERUM, WHILE MIXTURES WERE MORE EFFECTIVE

Fig. 1. Effect of GluNH₂ and CS and mixtures on Fn-f mediated kinetics of PG release into media in serum-free cultures. Cartilage was cultured in DMEM with 0.1, 1, 10 or 100 µg/ml GluNH₂ (G in figure) or CS (C in figure) or equal amounts in a mixture (M) also in presence or absence of 100 nM of an MMP-3 generated Fn-f mixture. The Fn-f, agent combination is shown with a plus sign. Agents were added 4 h prior to addition of Fn-f. Aliquots of conditioned media were assayed for PG content as a function of time and plotted as µg PG/mg wet weight cartilage. Each condition was in triplicate and each datum was plotted for each of 5 days, resulting in an n value of 15 for linear regression. Linear regression was used to estimate rates and s.e.m. values of best fit curves.

Fig. 2. Effect of GluNH₂, CS and mixtures on MMP-3 release into media. Cultures were established as in Fig. 1, except the thrombin-generated 29-kDa Fn-f was used rather than the MMP-3 digest. At day 3, media were recovered, dialyzed against water and concentrated 10³/C². Samples were subjected to electrophoresis, blotted and blots probed with anti-MMP-3. Shown in panel A are effects of 1, 10 and 100 µg/ml GluNH₂ (G in figure) on MMP-3 release as compared with a positive control 29-kDa Fn-f treated well (F) and untreated control (Ct). Panel B shows similar studies with CS (C in figure) and panel C shows effects of the mixture (M in figure). Panel D shows a side by side comparison of the higher concentrations.

Fig. 3. Effect of GluNH₂ and CS and mixtures on Fn-f mediated MMP-3 release (G, C and M correspond, respectively, to GluNH₂, CS and mixtures) as compared to that of the Fn-f control (lane F) and untreated controls (Ct). The numbers refer to mg/ml of agent and in the case of the mixture, the concentrations of each component. These experiments were performed with three different cartilage preparations with similar observations.

GluNH₂ OR CS ONLY WEAKLY BLOCKED Fn-f MEDIATED PG DEPLETION IN 10% SERUM, WHILE MIXTURES WERE MORE EFFECTIVE

The previous observations suggested that the agents were not powerful enough to block catabolism under serum-free conditions in the absence of serum growth factors and protease inhibitors. We next tested whether the agents could block Fn-f mediated cartilage PG depletion in the presence of serum which would provide protease...
inhibitors to limit damage and anabolic growth factors to allow cartilage mediated compensation to the damage. In this serum culture system, the PG content of the remaining cartilage is measured and this reflects not only catabolism but the summation of catabolism and anabolism over a culture period of up to 21 days.

Figure 5(A) shows that when cartilage was adjusted to 1, 10 or 100 µg/ml GluNH₂ alone without Fn-fs, PG content was unaffected. The apparent decrease in PG content with 100 µg/ml of agent at days 14 and 21 was insignificant. However, when Fn-fs were added 4 h after addition of the agents, the 100 µg/ml concentration appeared to decrease Fn-f mediated PG loss by days 7, 14 and 21 (all < 0.01) as compared to Fn-f treatment. A weaker protective effect was observed for 1 and 10 µg/ml at day 7 (P < 0.01 for both) but only for 10 µg/ml at day 21 (P = 0.007). Concentrations of 0.01 and 0.1 µg/ml were not tested because of the weaker effect of 1 µg/ml.

CS was tested in a similar fashion as shown in Fig. 5(B). In this case, CS alone at any concentration had no significant effect on PG content. However, CS at all concentrations appeared to diminish Fn-f mediated decreases in PG content by day 14 and by day 21 and there also appeared to be a repair response. When compared to Fn-f treatment, 1, 10 and 100 µg/ml decreased Fn-f activity, at day 7 (P = 0.01, 0.06, and 0.005, respectively), at day 14 (P < 0.004) and at day 21 (P < 0.0004). Concentrations of 0.01 and 0.1 µg/ml did not have a significant effect (data not shown).

In contrast, Fig. 5(C) shows that mixtures of the agents at 0.1, 1, 10 and 100 µg/ml very effectively blocked Fn-f mediated PG depletion at day 7 (P < 0.001, 0.0005, and 0.0002, respectively). A concentration of 0.01 µg/ml was ineffective (data not shown). For day 14, concentrations of 1, 10 and 100 µg/ml were still effective (P < 0.005), as well as for day 21, (P < 0.001). Concentrations of 1 µg/ml (shown) and 0.01 µg/ml had no significant effect (data not shown).
MIXTURES COMPLETELY RESTORED PG CONTENT IN DAMAGED CARTILAGE

In the next type of experiment, cartilage was damaged first by culture with Fn-f for 7 days, in order to test for the ability of agents to enhance restoration of lost PG. The Fn-f was removed at 7 days and agents added. Figure 6(A) shows that treatment of nondamaged cartilage with 10 or 100 μg/ml GluNH2 alone did not have a significant effect on decreasing PG content by day 14. However, by day 21, 10 μg/ml did decrease PG content by 11% with similar effects of 100 μg/ml ($P < 0.0005$ for both). At day 28, 10 and 100 μg/ml decreased PG content ($P < 0.003$ for both) as compared to untreated control. Apparently, GluNH2 had a small but significant effect, by itself. Adjustment of Fn-f damaged cartilage cultures to 100 μg/ml GluNH2 at day 7, did not significantly increase PG content by day 14 or 21, as compared with Fn-f treatment but did so by day 28 ($P = 0.005$). However, concentrations of 1 or 10 μg/ml had no significant effect at any time points. Concentrations of 0.01 and 0.1 μg/ml were not tested because of the weaker effect of 1 μg/ml.

Figure 6(B) shows that CS alone, when added to nondamaged cartilage, had no significant effect on PG content at any time points. Further, adjustment of Fn-f damaged
cartilage cultures to 1, 10 or 100 μg/ml CS at day 7, did not significantly increase PG content by day 14. However, by day 21, 10 and 100 μg/ml CS did enhance PG content as compared to Fn-f treatment alone (P = 0.01 and 0.001, respectively). By day 28, the effects were greater (P < 0.0001 for both). Concentrations of 0.01 and 0.1 μg/ml were not tested because of the weaker effect of 1 μg/ml.

Figure 6(C) shows that mixtures of the two agents at 1–100 μg/ml when added to nondamaged cartilage did not significantly enhance PG content at any time point as shown in Fig. 6(C). However, adjustment of Fn-f damaged cartilage cultures to 10 or 100 μg/ml of the mixture at day 7, significantly enhanced PG content by day 14 as compared to Fn-f treatment alone (P < 0.0001). At day 21, the effects were still significant for 10 and 100 μg/ml (P < 0.0001 for both). A concentration of 1 μg/ml was also effective (P = 0.003). At day 28, the effects for 1, 10 and 100 μg/ml were still significant (P < 0.0004 for all). Concentrations of 0.01 and 0.1 μg/ml had no significant effect (data not shown).

THE AGENTS DECREASED RELEASE OF MMP-3 IN SERUM CONDITIONS

Conditioned media from the serum cultures tested for blocking activity were probed for MMP-3 at day 7 of the culture period. This time point was chosen since we have shown that release of MMP-3 is maximal during this period as is Fn-f mediated cartilage matrix degradation. The purified thrombin-generated 29-kDa was used for these studies and a Reactive Red 120-agarose capture method was required to isolate the MMP-3 antigen from serum components as described in Materials and methods. MMP-13 expression was not tested because of an apparent failure of the agarose to separate MMP-13 away from serum components. Figure 7 shows that as compared to the serum-free data described earlier, the serum cultures allowed the agents to decrease MMP-3 release into the media to a much greater extent. The separate agents and mixture [Fig. 7(A,C,E)] did not enhance MMP-3 except for a slight increase in 0.1 μg/ml CS treated cultures [Fig. 7(A)]. When the agents were tested for their ability to block Fn-f, CS strongly blocked even at concentrations as low as 0.1 μg/ml, with a suggestion of lesser blocking as CS concentrations were increased. A concentration of 0.01 was also tested and found not to decrease MMP-3 expression (data not shown).

Similar results were observed with GluNH2 treatment as well as treatment with the agent mixture (M). Note also the same anomaly we described earlier for GluNH2 in serum-free conditions and for all three conditions in serum and for CS in panel B. The blocking effect is decreased with higher concentrations of agents. This analysis for MMP-3 release was tested with three different cartilage preparations with similar results.

MIXTURES REVERSED Fn-f MEDIATED PG SYNTHESIS SUPPRESSION

Since GluNH2 and CS blocked MMP release better than the agent mixture but were relatively inactive in blocking of PG degradation or in promotion of PG restoration, the possibility that the mixture worked through reversal of Fn-f mediated suppression of PG synthesis was investigated. A complication of this analysis is that Fn-f treated cartilage responds to the damage first with suppression of PG synthesis; however, after days 5–7, the PG synthesis rates slowly increase to untreated control levels and above. Thus, the potential effects of the agents on promoting repair can only be tested early in the culture within the first week.

To test effects of the agents on PG synthesis in the absence of Fn-f, cartilage was treated first with the agents alone and the cultured slices then incubated at various times with 35S sulfate to measure rates of incorporation as a relative measure of PG synthesis. Figure 8(A) shows that the higher concentrations of 10 μg/ml CS appeared to enhance PG synthesis at days 5 and 7 (P < 0.004 for both), while 100 μg/ml significantly enhanced PG synthesis.

Fig. 7. Effect of GluNH2, CS and mixtures on MMP-3 release in 10% serum conditions in the presence of the 29-kDa Fn-f. Cartilage was cultured as in Fig. 5. Agents were added to cartilage in 10% serum cultures and after 4 h, Fn-f added. Media were changed at days 3 and 5. Media that had been conditioned from days 5–7 were collected on day 7 and treated with Reactive Red 120-agarose to trap MMP-3 and leave serum proteins behind. Without use of Reactive Red-agarose, visualization of MMP-3 was problematic. The washed resin was subjected to denaturation buffer and releasate blotted against anti-MMP-3. Panels A, C and E show untreated control (C) and B, D and F show samples with both Fn-f and agent present.
The mixture was more effective at later times with 100 mg/ml with significant effects at day 14 (P = 0.004). Concentrations of 0.01 and 0.1 µg/ml were ineffective (data not shown). The GluNH₂ curves were similar to untreated control data.

Next the effect of GluNH₂ on blocking Fn-f mediated PG synthesis suppression was tested. Figure 8(B) shows that the MMP-3 generated Fn-f mixture suppressed PG synthesis by day 2 (P < 0.0001), followed by a reparative response of the cartilage as shown by slowly increasing PG synthesis rates, as we have reported earlier. However, preincubation with 1 µg/ml GluNH₂ for 4 h, followed by addition of Fn-f, reversed Fn-f activity by day 2 (P = 0.0068) with significant reduction through day 5 (P < 0.001) and day 7 (P < 0.01). A concentration of 10 µg/ml was more effective at days 2 and 5 (P < 0.0007 for both) while 100 µg/ml was even more effective at days 2, 5 and 7 (P < 0.0005 for all). By day 10, the differences between Fn-f treatment and the 100 µg/ml GluNH₂ treatment were not significantly different, suggesting that the effects of GluNH₂ are not great enough to cause persistent blocking. Concentrations of 0.01 and 0.1 µg/ml were ineffective (data not shown).

The effects of CS were tested next. Figure 9(A) shows that CS weakly reversed PG synthesis suppression caused by the Fn-f. Concentrations of 1 µg/ml (P = 0.01), or 10 µg/ml (P = 0.0001) or 100 µg/ml (P = 0.0002) significantly decreased Fn-f activity at day 2. The effect of 1 µg/ml was lost by day 5, while 10 µg/ml showed a partial effect (P = 0.021) and 100 µg/ml was completely effective at day 5 (P < 0.0001). The 100 µg/ml concentration continued to decrease Fn-f activity through day 10 (P < 0.0001). Concentrations of 0.01 and 0.1 µg/ml were ineffective (data not shown).

Fig. 8. Effect of GluNH₂, CS and mixtures in absence of Fn-f (panel A) and of GluNH₂ in presence of MMP-3 generated Fn-f digest (panel B) on PG synthesis. Cartilage in 10% serum was cultured in presence of 1, 10 or 100 µg/ml test agent and mixture and at various times, cartilage subjected to labeling with 35S-Sulfate. After labeling, cartilage was extracted with guanidine-HCl, the extracts dialyzed and cpm/mg wet weight cartilage measured. In panel A, only those lines that are distinct from the control are labeled. In panel B, the effects of the various concentrations of GluNH₂ in the presence of Fn-f were measured. A control of Fn-f alone (F(d0–21)) is also shown. All values were normalized to untreated controls (100%).

Fig. 9. Effect of CS (panel A) and mixtures (panel B) in presence of MMP-3 generated Fn-f and effect of mixtures in previously dam-aged cartilage (panel C) on PG synthesis. For panels A and B, cultures were established as described in Fig. 8, panel B. For panel C, cartilage was first damaged with Fn-f for 7 days, the Fn-f removed and then mixtures added at various concentrations at day 7. A control of Fn-f alone (F) is also shown. All values were normalized to untreated controls (100%).
In contrast, Fig. 9(B) shows that mixtures of the agents at 10 or 100 µg/ml were all totally effective in blocking or decreasing Fn-f mediated PG synthesis suppression for up to day 14 (p < 0.0004 at all days). The effects were also greater than the sum of the individual effects at days 7, 10 and 14 (p = 0.007 at all days). A concentration of 1 µg/ml had a lesser effect for days 2, 5, 7 or 10 (p < 0.006 for all days). A 0.1 µg/ml concentration was indistinguishable from Fn-f treatment (shown), as well as a 0.01 µg/ml concentration (data not shown). The major difference between the agent mixture and the single agents is that the mixture at 10 and 100 µg/ml totally blocked Fn-f mediated PG synthesis suppression at any time point during the culture, while the single agents only had a short term effect.

We next tested whether this blocking effect would also occur in experiments in which the cartilage was damaged first by the Fn-f, the Fn-f removed and the agents added. Figure 9(C) shows that the Fn-f by itself had significant effects (p < 0.0001). After removal of the Fn-f at day 7 and addition of the mixtures, 1, 10 and 100 µg/ml concentrations reversed the effects of the Fn-f at day 14 (p = 0.01, 0.0007 and 0.0002, respectively). Therefore, the proanabolic activity of the mixture can either occur during the Fn-f mediated damage or after maximal damage has been inflicted.

Discussion

GluNH₂ and CS have been shown to have pain-alleviating effects when taken orally. One basic question has been whether or not these agents also modify damaged cartilage for the progression of damage. Whether or not an agent has disease-modifying effects in OA depends on the demonstration that it can slow progression, with progression measured by a surrogate for structural change. Currently the only accepted surrogate in OA is joint space narrowing (JSN), measured by X-ray. Effects on JSN have been shown for GluNH₂ and Cosamin DS, when used in long-term treatment, was able to retard radiographic progression in patients with OA of the knee. This same measurement is being made in the ongoing GAIT study using the same source of CS. Michel et al. recently showed that the same CS (CondroSulf) present in Cosequin and Cosamin DS, when used in long-term treatment, was able to retard radiographic progression in patients with OA of the knee. This same measurement is being made in the ongoing GAIT study using the same source of CS. Thus, our objective was to test whether in our explant model, effects of these agents on protection of the cartilage matrix could be demonstrated. It should be noted that we have shown some differences in how Fn-fs damage human cartilage, as compared with bovine cartilage, an observation that might suggest some caution in predicting that the blocking activity of the agent mixture would be as significant on human cartilage as we demonstrate here for bovine cartilage.

With the initiation of this study, there were few observations that demonstrated that these agents were effective in blocking cartilage damage or repairing cartilage in vitro. However, there have been many studies of effects of these agents on cartilage tissue or other cells. Unfortunately, none of these studies were of the effect on the most relevant parameters, such as matrix content. Our objective was to test the effects of the single agents and combinations in a well defined in vitro damage model consisting only of cartilage and Fn-f. While the overall effects must be weighed in the final analysis in human subjects, analysis in a single type of tissue system provides us an opportunity to more clearly observe certain chondrolytic or anti-chondrolytic effects. We utilized a powerful damage system, one that employs Fn-fs that have the capability of removing half of the total PG within 7 days in culture. Thus, agents that block Fn-f mediated damage would have to have very potent anti-catabolic or proanabolic activities.

Our initial studies were of serum-free conditions in which the anabolic effects mounted by the tissue would have been lesser than in serum because of the absence of growth factors or protease inhibitors. These conditions allowed us to study effects mainly on catabolism. We did not observe significant effects of the agents on the kinetics of PG degradation in the presence of Fn-f, although higher concentrations of the mixture appeared to increase the rate of PG release. These data should be treated with caution since other experiments showed that the mixture enhanced PG synthesis and this newly synthesized PG maybe have been released into the media and contributed to the rates of PG release we measured. This contribution to the rate would have obscured the observation of a blocking effect.

In terms of effects on MMP-3 and -13 release in serum-free conditions, we did observe an early slowing of the release of MMPs, but this effect was mostly lost by 3 days. The temporary nature of this loss might have been due to exhaustion of the added GluNH₂ or CS, since these agents were added only once, at the beginning of the culture. This observation of effects on MMP release is consistent with other observations of the ability of these agents to decrease MMP expression. However, it should be noted that we found effects at µg/ml concentrations, much lower than typically reported. As examples, GluNH₂ at 100 µg/ml suppressed MMP release from normal chondrocytes and synovocytes but not from OA chondrocytes. GluNH₂ at 1–150 µM suppressed MMP-3 mRNA levels 18–65% in human chondrocytes and GluNH₂ at 2.5 mg/ml decreased MMP-3 protein release. Further, both GluNH₂ and CS were found to decrease MMP gene upregulation in IL-1 challenged bovine cartilage. Our data are also consistent with the reported ability of CS or GluNH₂ to block cytokine activities, since cytokines are involved in Fn-f mediated MMP upregulation.

It should also be noted that we showed that in our serum-free conditions in which no serum contained protease inhibitors would be present, that the agents did not block the activity of the MMPs involved in matrix PG degradation in our Fn-f model, although they have been proposed to act as MMP inhibitors. However, the data in Fig. 2 in which less proMMP-13 but more lower mass MMP-13 was apparent when the culture was treated with the mixture would be consistent with some type of inhibition of proteases involved in proMMP-13 activation.

Subsequent studies with serum present allowed us to study the combined effects of catabolism and anabolism. We found that while the agents singly or in combination did not block Fn-f mediated PG degradation in serum-free conditions, in the presence of serum the agents were more effective likely due to the presence of growth factors in serum which may have facilitated the restoration of PG or the maintenance of steady state levels of PG. The single agents showed weak activity, while the mixture was very active at low concentrations. When tested for reparative activities, a similar trend was observed. Since serum also contains protease inhibitors, the serum may have also decreased the proteolytic potential enough such that the enhanced anabolic effects of the test agents were more capable of compensation. Another possible explanation for the differences between our serum-free and serum
conditions may be that the low protein conditions of serum-free conditions may have reduced the activity of the test agents through additional binding interactions that otherwise might have been lessened by the presence of serum proteins.

In order to provide some information on how the agents might be decreasing Fn-f mediated catabolism, the serum cultures were tested for MMP expression. Consistent with our studies in serum-free conditions, the serum conditions also showed an effect on decreasing MMP expression, with the single agents appearing to be more effective than the mixture. Since the mixtures were more effective than the single agents in blocking PG degradation and in restoring PG, the effects on MMP-3 release cannot explain the efficacy of the mixture. It is possible that the agents might be more effective on other proteases involved in cartilage damage, however, we have demonstrated that MMP-3 is largely responsible for PG degradation in our Fn-f cultures.50 It should also be noted that we measured MMP-3 and -13 in the media. We do not yet know if these agents also blocked MMP-3 transcription.

In terms of PG synthesis, the effects of the agents were first tested in the absence of Fn-f. CS by itself appeared to enhance PG synthesis early in culture but not at later time points, while the mixture was more effective later. GluNH₂ did not show significant effects by itself. When the agents were tested for their ability to reverse Fn-f mediated PG synthesis suppression, the single agents at higher concentrations did have some effect on Fn-f activity. CS at 100 μg/ml did totally block the effect of the Fn-f, while GluNH₂ appeared to be weaker. However, the mixture at 1 μg/ml was nearly as effective as 10 μg/ml CS and the mixture at 1—100 μg/ml totally blocked Fn-f activity. Our observation of the ability of CS to stimulate synthesis of PG is consistent with other reports.12,13,17,33 Since the mixture which was more effective at blocking PG depleting and restoring PG was also more effective at enhancing PG synthesis than were CS or GluNH₂, it is possible that a major explanation for efficacy of the mixture is through reversal of Fn-f mediated PG synthesis suppression.

The concept that suppressed MMP release does not lead to blocking of Fn-f mediated damage, while reversal of PG synthesis suppression does is interesting. This supports the notion we published earlier in which we showed that while treatment of both human knee and ankle cartilage with Fn-f resulted in enhanced MMP expression and elevated aggrecan degradation neoepitopes, only knee cartilage shows a decrease in PG content.67 Further, we found that PG synthesis suppression in knee cartilage is more sensitive to Fn-f than in ankle cartilage.67 We proposed that the major damaging mechanism of Fn-f toward human knee cartilage is through suppression of PG synthesis and not through elevated MMPs.67 In comparison, we have shown that in bovine cartilage, antibodies to MMP-3 slow Fn-f mediated damage.60 We have also shown that MMP inhibitors block Fn-f mediated damage to bovine cartilage (unpublished). Perhaps these differences are due to species differences as we have discussed.65

Enhance MMP activity may be more responsible for Fn-f mediated decreases in cartilage PG content in bovine cartilage than in human knee cartilage. However, the data presented here with bovine cartilage suggest that blocking of MMP-3 or -13 expression by GluNH₂ and CS is not sufficient for blocking of cartilage PG losses. It is also possible that the single agents have secondary negative effects that minimize the beneficial effects of blocking MMP release, while the mixture may have fewer of these side effects.

Our data show that the mixture was much more powerful than the single agents and that concentrations of down to 1 μg/ml of each agent in the mixture could totally block Fn-f mediated PG decreases in cartilage PG content. The effects were partial at 0.1 μg/ml but lost at 0.01 μg/ml. Observation of blocking at such a low concentration may address the preliminary conclusions that the agents by themselves cannot be effective after oral ingestion because of the dilution in the circulatory system. It should be noted that our demonstration of activity in the 0.1—1 μg/ml range is well within the windows reported for measured plasma levels in different species of CS of 5—40 μg/ml17,36—41 and of GluNH₂ of 2—20 μg/ml.43,45—48

Our observations are in accord with other observations17 and proposals48,49 that the mixture is much more effective than the single components. Our data showed that (1) 0.1 μg/ml of the mixture was as effective as 100 μg/ml CS in blocking Fn-f mediated damage; (2) 1 μg/ml of the mixture was more effective than 100 μg/ml GluNH₂ or CS in promoting restoration of PG; and (3) 1 μg/ml of the mixture was more effective than 10 μg/ml CS in blocking Fn-f mediated PG synthesis suppression. Thus, there is at least one order of magnitude difference in the potencies between the single agents and the mixture.

Discussion of the dose dependencies of these agents has to also consider observations that these agents may have an affinity for cartilage23,36. Thus, the serum levels of these agents may greatly underestimate the cartilage bound concentrations. We should also point out that we only preincubated cartilage with these agents for 4 h prior to addition of Fn-f. Since some of our experiments were up to 21 days duration, some concentration into the tissue might have occurred. Thus, we cannot be sure that our final tissue concentration is the same as the initial. This might also explain the trend to show slightly beneficial effects of these agents later in culture.

Regardless of the mechanism, this work is among the first to show the effects of these agents in an explant cartilage degeneration model in which a far downstream correlate of cartilage damage, PG depletion and restoration were studied. While the enhanced PG synthesis is consistent with cartilage repair, it should be noted that increased PG synthesis also occurs in early OA as well as during conditions in which the Fn-f causes minimal damage to cartilage.54 Thus, the increased PG synthesis we observed may not actually lead to stable repair. Further studies will be needed to confirm stable restoration of PG and other matrix components, restoration of structural integrity of the matrix and cessation or decreases of enhanced catabolic pathways. Our observations of the greater effects of the mixtures, suggest that single formulations may be less effective as nutraceutical compounds. Lastly, the concentrations of agents we found to be effective are actually lower than the concentrations measured in plasma of different species after oral ingestion of the agents17,38—46, further suggesting that these compounds might be capable of altering chondrocyte metabolism even with dilution into the circulatory system.

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