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Glucosamine and chondroitin sulfate: biological response modifiers of chondrocytes under simulated conditions of joint stress

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Summary

Objective: To test the hypothesis that chondrocytes are more responsive to the chondroprotective agents, glucosamine (glcN) and chondroitin sulfate (CS), under *in vitro* conditions simulating *in vivo* joint stress.

Design: Synthetic and anticatabolic activities of bovine articular cartilage were assayed using 35-sulfate labeling and assaying the specific activity of glycosaminoglycans (GAGs) under the conditions of enzyme-induced matrix depletion, heat stress, mechanical compression and cytokine stress.

Results: The response of cartilage to simulated conditions of *in vivo* stress varies, depending on the type stress and age of the animal. Cartilage from aged animals was more responsive to stress and to glcN and CS. Pronase-induced matrix depletion and mechanical stress increased proteoglycan synthetic activity. Exposure to glcN and CS significantly enhanced this stress response from 85 to 191% and from 40 to 1000%, respectively. Heat stress and stromelysin digestion decreased synthetic activity, which was reversed or normalized on exposure to glcN and CS. Cartilage from young joints was somewhat refractory to the level of stress imposed and to treatment with glcN and CS.

Conclusion: The metabolic response of cartilage from aged animals to glcN and CS under simulated conditions of *in vivo* stress is significantly greater than that seen in nonstressed or young tissue. By enhancing the 'protective' metabolic response of chondrocytes to stress, glcN and CS may improve its ability for repair and regeneration. These observations suggest that these compounds function as biological response modifiers (BRMs), agents which boost natural protective responses of tissues under adverse environmental conditions.

Key words: Cartilage, Stress, Metabolism, Osteoarthritis.

Introduction

Osteoarthrosis (OA) is a multifactorial disorder of diarthrodial joints with aging, genetic and hormonal influences and no evidence of a primary metabolic defect in cartilage[1]. Exposure of articular cartilage to localized physical or chemical stresses, including mechanical[2,3], inflammatory[4], oxidative[5,6], and thermal[7] entities, may contribute to the depletion of matrix macromolecules. In fact, the hypermetabolic state seen early in OA is considered a reactive feedback response of chondrocytes to proteoglycan depletion in the matrix. Under these circumstances, cartilage lesions ultimately develop because the stress-induced 'hypermetabolic' response is dominated by a catabolic cascade and degradation of cartilage matrix[8]. Degeneration of cartilage is progressive, facilitated by continual excessive and/or aberrant stresses[9,10].

If the 'hypermetabolic' response seen in early OA is a protective, albeit ineffective, attempt to counteract the results of environmental stress, agents, which enhance the anabolic response and diminish the catabolic response may retard the progression of cartilage degeneration. Natural agents endowed with this ability have been termed biological response modifiers (BRM)[11]. The term BRM originally referred to agents that enhance the immuno-defensive response of hosts to pathogens or cancer. BRM designation also refers to agents that affect atherosclerosis[12] and rheumatoid arthritis[13], and include naturally derived compounds like proteins[14], polysaccharides[15] and glucans[16]. The definition implies that these agents promote the defense of the host against multiple stresses[17]. Our studies indicate that the symptomatic/disease modifying osteoarthritis agents (S/DMOADs) glucosamine (glcN) and chondroitin sulfate (CS) are candidates for this classification. Indirect support for this concept is provided by several *in vivo* rabbit studies. In one study, dietary supplements of glcN and CS preserved cartilage matrix and slowed down the progression of degenerative changes in a rabbit instability model of OA. In these animals, normal humeral cartilage metabolism was not altered and the beneficial response was attributed to their effect on chondrocytes from the stressed joints[9]. In another study, glcN administration to animals given intraarticular injections of chymopapain improved proteoglycan content in stressed cartilage, but had no effect on normal tissue[18]. A related study indicated that CS administered to rabbits prior to and following chymopapain injection had significantly higher proteoglycan content in the injected joint[19].

To further explore the relationship between stress and activities of glcN and CS, and to test the hypothesis that these agents function by intensifying protective cellular responses initiated by adverse environmental conditions, *in vitro* simulations of *in vivo* joint stress were used.

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Materials and methods

Six millimeter diameter bovine cartilage explants from normal radiocarpal joints of retired Holsteins (age 8–10 years) were conditioned to a metabolic steady state in DMEM/F-12+12% fetal bovine serum and penicillin/streptomycin for 6–10 days. In those experiments indicated, tissue was also obtained from young (15 months old) steers. When cell cultures were tested, cells isolated by overnight digestion with 400 U/ml collagenase were seeded at high density (60 000–80 000 cells/well) in polylysine-coated 96 well micro plates. Total volume per well was 250 µl and the media was changed daily. The cells were utilized after culturing for 10 days. The dosage of agents used in these studies was based on published data indicating a 5–15% bioavailability of CS and evidence of accumulation in tissues with continued dosing[20,21]. All the agents were tested after dissolving in DMEM/F-12 containing 0.5% fetal bovine serum.

STRESS INDUCTION BY MATRIX DEPLETION

Conditions of matrix digestion sufficient to elicit a substantial reduction of matrix proteoglycans were established using varying dosages of the proteolytic enzyme, pronase (EC3.4.24.31, Sigma–Aldrich, St. Louis, MO). Stromelysin (EC3.4.24.17) was used at much lower concentration (0.045–0.74 mU/ml). For the final experiment, explants exposed to 50 U/ml pronase for 3 h were cultured in the presence of 25, 100 or 400 µg/ml of Cosamin® DS (CDS), a mixture of glcN HCl (FCHG49®), CS (TRH 122®) and manganese ascorbate (500, 400 and 76 mg/g, respectively; Nutramax Laboratories®, Edgewood, MD), for 24 h. The second study tested the effect of adding 100 µg/ml CDS to tissues exposed to varying doses of stromelysin for 2 h. For comparison purposes, we also observed the effect of 3 h of exposure to 0.25 U/ml chondroitin ABC lyase (EC 4.2.2.4) or 10 U/ml hyaluronidase (EC 3.2.1.35) (Sigma–Aldrich, St. Louis, MO). All the tissues were pulse-labeled during the final 4 h with 5 µCi/ml 35-sulfate. In these studies, we monitored newly synthesized proteoglycans incorporated into the explants or pericellular matrix of monolayers. Hence, tissues (explants or monolayers) were thoroughly rinsed 5X with cold DMEM and twice with cold 95% ethanol to remove unincorporated radioisotope and labeled proteoglycan in media. Tissue were kept at 5°C in 100% ethanol overnight and were then lyophilized. Dry weights to the nearest microgram were obtained using a microbalance. Radiolabel uptake was determined by dissolving individual explants in 50 µl of 1 N NaOH at 40°C for 1 hour, addition of 200 µl of Optiphase SuperMix scintillation fluid and counting in a MicroBeta Trilux plate counter (Perkin–Elmer–Wallac Inc., Gaithersburg, MD). Statistical analysis of the differences between groups was accomplished using the Student *t*-test and Kruskal–Wallis nonparametric ANOVA.

STRESS INDUCTION BY HEAT STRESS

Chondrocyte monolayers conditioned to a metabolic steady state were cultured in the presence of DMEM/F-12 containing 12% fetal calf serum at 37 and 41°C for 2 and 17 h. At the end of the stress interval, the media was changed to DMEM/F-12+0.5% fetal calf serum containing 30 µg/ml glcN or 25 µg/ml CS or 12.4 ng/ml IGF-1, and the cultures were placed at 37°C for 18 h to allow for recovery. Pulse labeling with 5 µCi/ml 35-sulfate was done in the terminal 4 h of the recovery phase and label uptake assayed as previously described.

CYTOKINE STRESS

Chondrocyte monolayers prepared from aged joints and conditioned to a metabolic steady state in DMEM/F-12+12% fetal calf serum, were prelabeled with 35-sulfate for 72 h. Unincorporated isotope was washed out by repeated washings and incubating for 48 h with daily media changes. Control cultures containing plain DMEM/F-12 were compared with the cultures, to which a mixture of IL-1 beta (10 ng/ml) and plasminogen (0.35 µM) was added with and without 25 µg/ml CS. Release radioactivity was monitored daily for 72 h and the data expressed as a percentage of the total radioactivity incorporated.

STRESS INDUCTION BY MECHANICAL COMPRESSION

Explants were preconditioned by exposure to 250 µg/ml CDS for 10 days. A specially constructed MTS-driven device was used to expose multiple explants to a physiological level of unconfined static compression of 0.5 MPa for 24 h at 37°C. Explants were pulse-labeled with 35-sulfate as previously described. Individual explants were digested with 0.4% pronase at 60°C. and aliquots were used for: (a) assay of total glycosaminoglycan (GAG) by the DMB reaction[22], (b) isotope uptake and (c) chondroitin 4:6 sulfate ratios assessed after ABC lyase digestion and HPLC analysis of disaccharides[23]. The data were expressed as 4:6 ratio, specific activity GAG (cpm/µg GAG) and percent change from unpressurized controls.

STATISTICAL ANALYSIS

The mean, standard deviation and standard error of the mean (S.E.M.) were evaluated for each sample group. Means were compared using ANOVA and Student's *t*-test using the Bonferroni criteria for multiple group comparisons. An unpaired two-tailed Student's *t*-test was employed to test the percentage differences for statistical significance. Significance was accepted at $P<0.05$.

Results

Exposure of cartilage explants to pronase induces a time and dose-dependent upregulation of proteoglycan synthesis (Fig. 1). In tissue from aged animals, maximum stimulation in any single experiment occurred at 1 h using 50 U of enzyme (+660%), but the most consistent data over multiple experiments were obtained using 50 U for 3 h. All the subsequent experiments were done with this dose and time of exposure. Tissue from young animals was less responsive and required higher amounts of enzyme or longer digestion times to elicit an effect. Figure 2 demonstrates the degree of depletion of Safranin-O staining material obtained with 50 U and during the three tested time periods using tissue from aged animals.

The metabolic response to pronase, stromelysin, hyaluronidase and chondroitin ABC lyase varied significantly. While pronase significantly up regulated synthetic activity, ABC lyase and hyaluronidase had an inhibitory effect (−34 and −37%) (Table I). Addition of 100 µg/ml CDS after enzyme exposure did not reverse or enhance the inhibitory

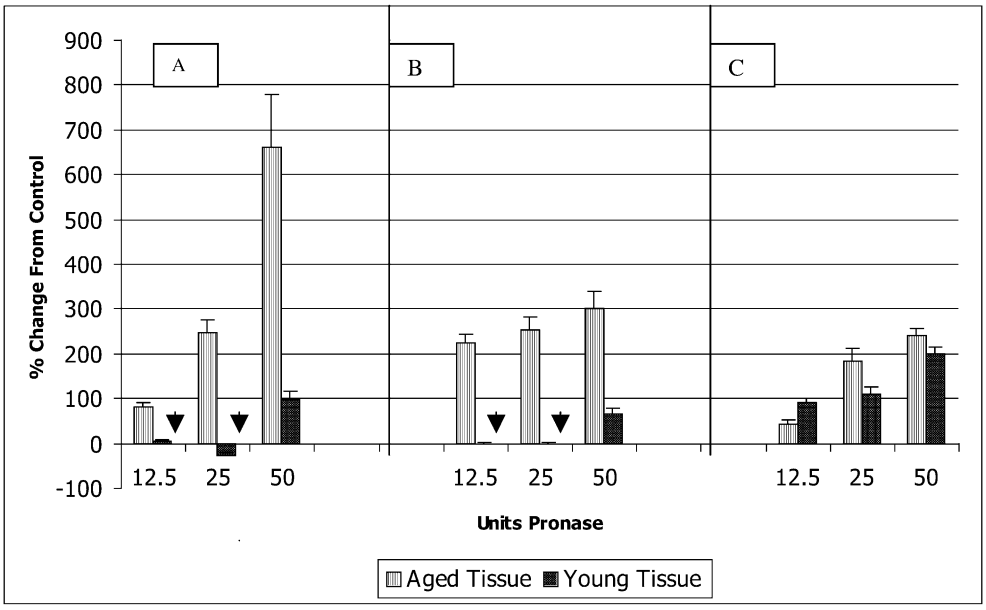

Fig. 1. Effect of dose and time of pronase exposure on 35-sulfate incorporation into explants of cartilage obtained from young and aged animals. (A, B, C) Explants exposed to pronase for 1, 2 and 3 h, respectively. Arrows indicate those specimens, which did not differ significantly from control values. Data expressed as percent change from control values and represent mean±S.E.M. for six to eight replicates.

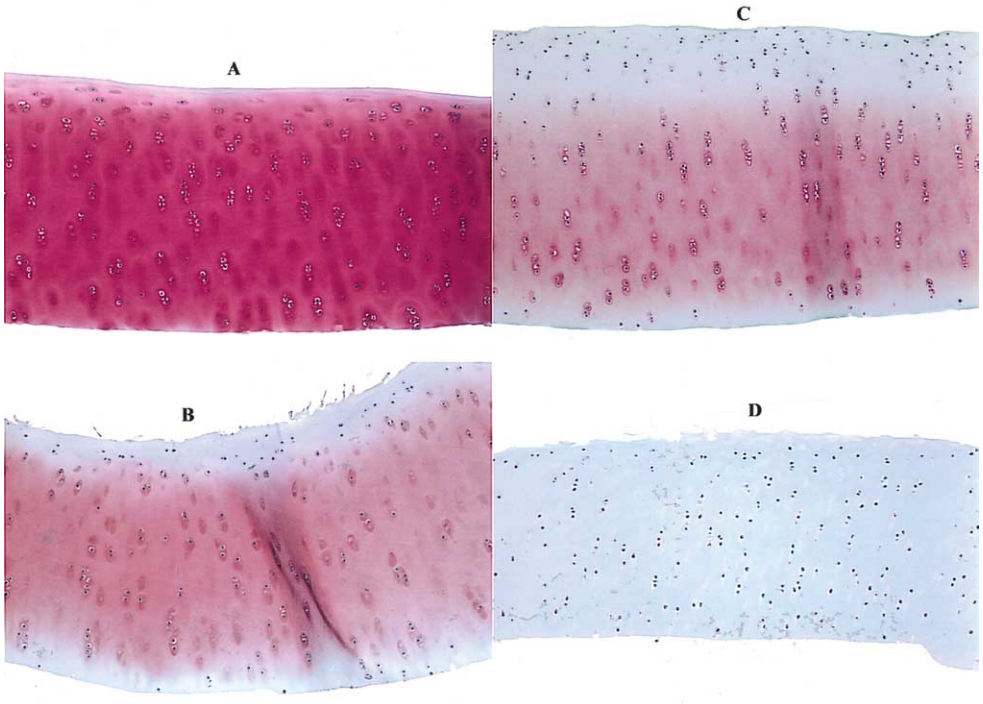

Fig. 2. Safranin-O-stained histologic sections of cartilage explants. (A) Control tissue; (B, C, D) explants exposed to 50 U pronase for 1, 2 and 3 h, respectively.

effect of ABC lyase or hyaluronidase. Interestingly, ABC lyase with pronase reduced the pronase stimulation. However, subsequent exposure to CDS increased the synthetic rate 34% above the CDS control and 60% above the pronase+ABC lyase rate (Table I). The response of pronase-treated cartilage explants from aged joints to CDS was dose-dependent with a maximum increase in synthesis of 183% obtained with 400 µg/ml (Fig. 3). At the low CDS dose of 25 µg/ml, no effect was noted. As previously noted, for the time interval studied, treatment of unstressed

Table I
35-Sulfate uptake by cartilage explants from aged animals following enzyme digestion: post-treatment response to CDS

Enzyme	−CDS	%	*P*-value	+CDS	%	*P*-value
Control	1425 (106)	–	–	1470 (90)	–	–
Pronase (50 U)	2640 (120)	+85	0.001	–	–	–
ABC lyase (0.25 U/ml)	905 (42)	−37	0.001	900 (70)	−39	0.001
Pronase+ABC lyase	1273 (70)	−11	n.s.	1965 (200)	+34	0.03
Hyaluronidase (10 U/ml)	935 (94)	−34	0.005	934 (60)	−36	0.001

Data presented as mean±S.E.M. of cpm/mg dry tissue weight ($N=8$). *P* values based on comparison with appropriate control (−CDS and +CDS).

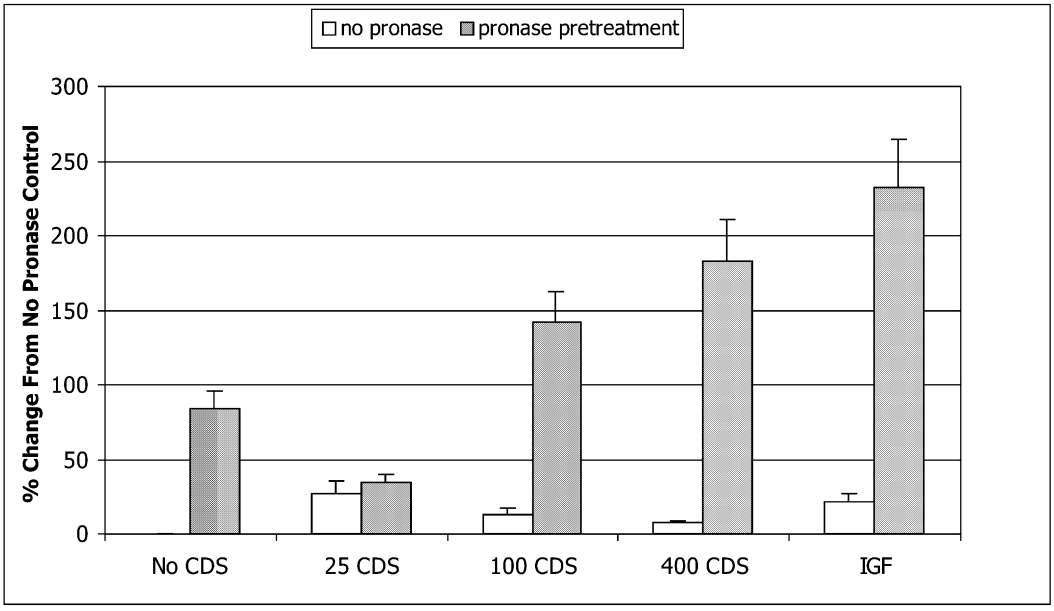

Fig. 3. Effect of CDS on incorporation of 35-sulfate by cartilage explants exposed to 50 U pronase for 3 h. Values representing four to six replicates are graphed as the percent change (±S.E.M.) from control explants cultured in DMEM/F-12 without enzyme treatment. All enzyme treatments significantly differed from control (no additions). Of the samples not exposed to enzyme, only 25 CDS was significantly higher (+27%) than control.

explants with high levels of CDS usually resulted in no change or inhibition of 35-sulfate uptake. Significant increases in synthetic activity were associated with lower doses (25 µg/ml=27–34%). Enzyme-treated tissue was also more responsive to IGF-1 (232 vs 22%). In contrast to pronase-induced upregulation, stromelysin gave a dose-dependent inhibition of synthetic activity (Table II). Under these conditions, exposure to 100 µg/ml CDS reversed this inhibition at all but the highest enzyme dose, producing approximately 40% stimulation.

The response of monolayer cultures of cells from aged joints to a heat stress of 41°C for 2 or 17 h is presented in Fig. 4. In control cultures, heat stress of 2 or 17 h induced a maximum 23% inhibition of 35-sulfate uptake in the recovery phase. However, response to IGF-1 increased from 145% at 37°C to 250 and 361% at 41°C at 2 and 17 h, respectively (Fig. 4). No response to glcN was seen at 37°C and for 2 h at 41°C, but a significant increase (+110%) occurred after 17 h at 41°C. Similarly, CS elicited an increased response (+70%), but only after 17 h at 41°C.

Preliminary studies in our laboratory and published data show little evidence of an anticatabolic effect of glcN. However, an analysis of CS activity on labeled cartilage exposed to IL-1+plasminogen indicates a 24 h lag phase before a reduction in catabolism is seen (Fig. 5). CS restored metabolism to normal by 48 h.

Application of a physiological level of static compressive stress to tissues from young animals inhibited GAG synthesis by 65% (Fig. 6). Preconditioning explants by exposure to a high level of CDS did not alter this response. However, synthetic activity in explants from aged animals subjected to compressive stress was stimulated by 40% over non-stressed tissue, which was further increased to 1000% after preconditioning with CDS. CDS conditioning also raised the CS 4:6 ratios in aged tissue by 34% (from 0.50 to 0.67) ($P<0.05$).

Discussion

Adverse tissue culture conditions can be used to stimulate cellular stress responses of chondrocytes. Easily applicable are mechanical, chemical and temperature stresses. The rationale for choosing the various simulations is based on literature indicating that each may play a significant role in cartilage biology. For example, enzyme-stressed tissue mimics an early stage of OA associated

Table II
CDS reversal of stromelysin-induced inhibition of 35-sulfate incorporation into cartilage explants obtained from aged animals

Treatments		35-Sulfate uptake (cpm/mg, S.E.M.)	% Change	*P*-value
Stromelysin (mU)	CDS (μg/ml)			
Control	–	1720 (178)	–	–
0.74	–	1065 (72)	−38	–
0.74	100	1155 (58)	−33	n.s.
0.37	–	1365 (66)	−21	–
0.37	100	1865 (60)	+9	*P*<0.002
0.184	–	1110 (105)	−35	–
0.184	100	1535 (220)	−11	*P*<0.05
0.045	–	1405 (40)	−12	–
0.045	100	1850 (30)	+8	*P*<0.004

Data presented as mean±S.E.M. of six replicates. The % change refers to the difference between the control (without enzyme or CDS) and samples. Significance evaluated on samples with and without CDS.

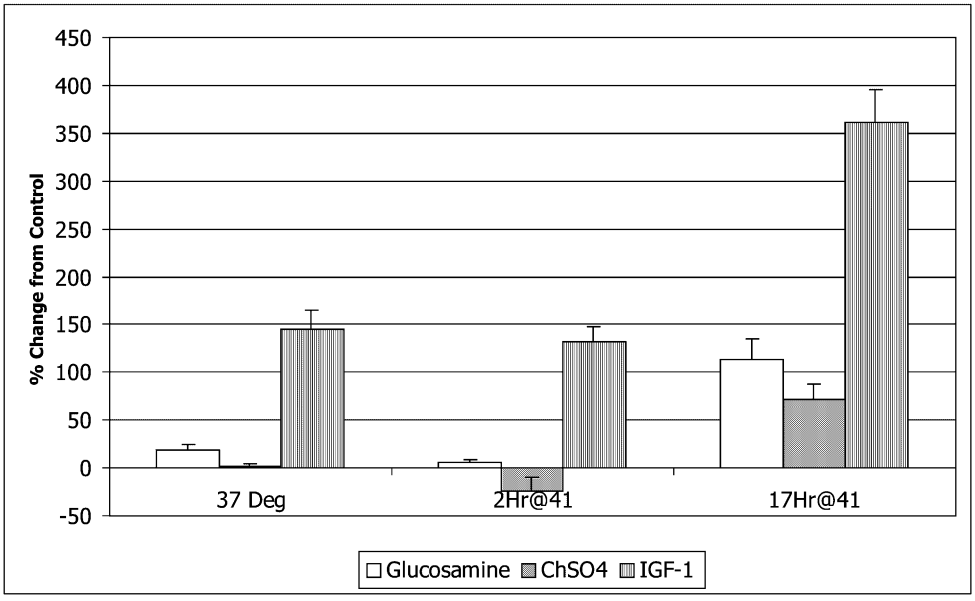

Fig. 4. Response of chondrocyte monolayers to glcN, CS and IGF-1 following heat stress. Data represent mean ±S.E.M. of six to eight replicates. All IGF-1 values and 17 h at 41°C values are significantly different at *P*<0.001.

with depletion of Safranin-O staining material and hypermetabolism of chondrocytes[8]. In similar studies, trypsin-induced matrix depletion of bovine cartilage stimulated proteoglycan synthesis at low levels of trypsin (5 μg/ml) but inhibited proteoglycan synthesis at high levels (20 μg/ml)[24]. These data are consistent with other reports using papain or trypsin[25,26].

In none of the cases cited was there any evidence that the response of chondrocytes to matrix depletion was of sufficient magnitude to enhance cartilage regeneration. When used as a stimulant *in vivo*, exposure of chondral lesions in rabbits to trypsin alone did not result in hyaline cartilage regeneration[27], but in the presence of blood did affect repair. Our data indicate that glcN and CS substantially enhance the proteoglycan synthetic response of chondrocytes under *in vitro* conditions, simulating matrix depletion.

In the enzyme stress studies, unstressed tissue responded maximally to low levels of glcN plus CS, higher levels having no effect. In previous studies, we found that high levels of CS alone can, at times, inhibit synthetic activity in cartilage explants or monolayers. Similar results were found by Collier and Ghosh[28] and Verbruggen *et al.*[29]. The mechanism for this biphasic dose response is unknown, but there are several possibilities. CS may act as a hormetic substance, low levels being protective and high levels toxic[30]. Alternatively, CS activity could be regulated by receptor-binding phenomena where stressed tissue allows for higher levels of agents to be biologically effective as a result of alterations in membrane conformation or accessibility to membrane receptors. If surface receptors are a requirement for CS action, normal tissue may reach saturation of surface receptors so that elevated levels of CS have no intracellular effect, but may exert an influence on permeability by virtue of surface binding. High doses of glcN, exceeding 2500 μg/ml, have also been found to be toxic to chondrocytes, but these are considered to be pharmacological levels and are not seen with oral dosing[31].

Stromelysin (MMP-3) is considered to be one of the major proteoglycan-degrading enzymes in cartilage[32]. It has a broad range of substrate specificity, and multiple inflammatory stimuli induce its expression in cartilage[33,34].

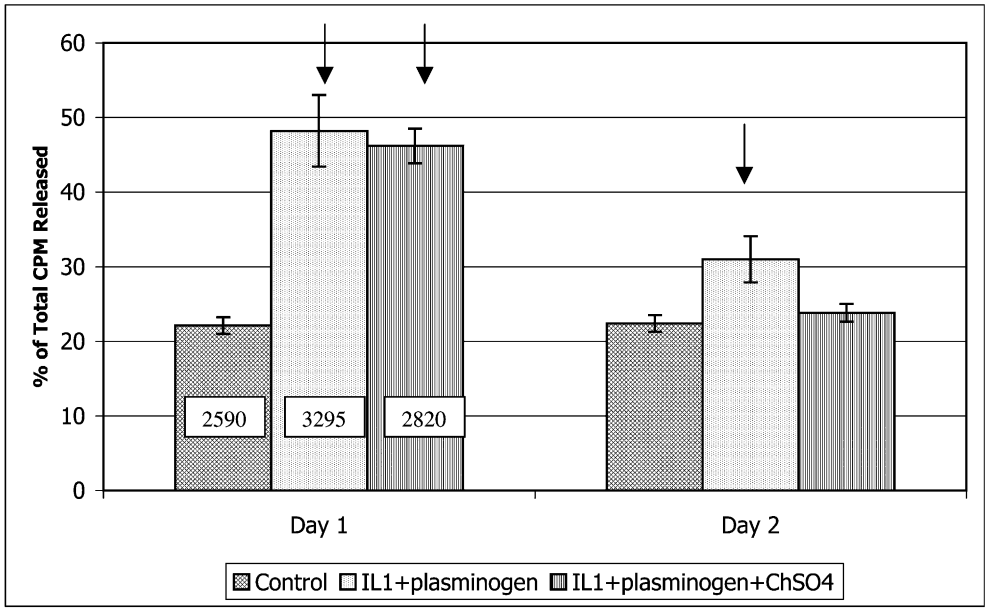

Fig. 5. Anticatabolic effect of CS on IL-1/plasminogen-stimulated cultures of chondrocyte monolayers. Data from eight replicates expressed as percent of total counts incorporated (±S.E.M.), which were released. Numbers reflect the total amount of radioisotope (cpm/mg dry tissue weight) in the explants at the start of the study. Arrows indicate those values, which are significantly different at $P<0.05$.

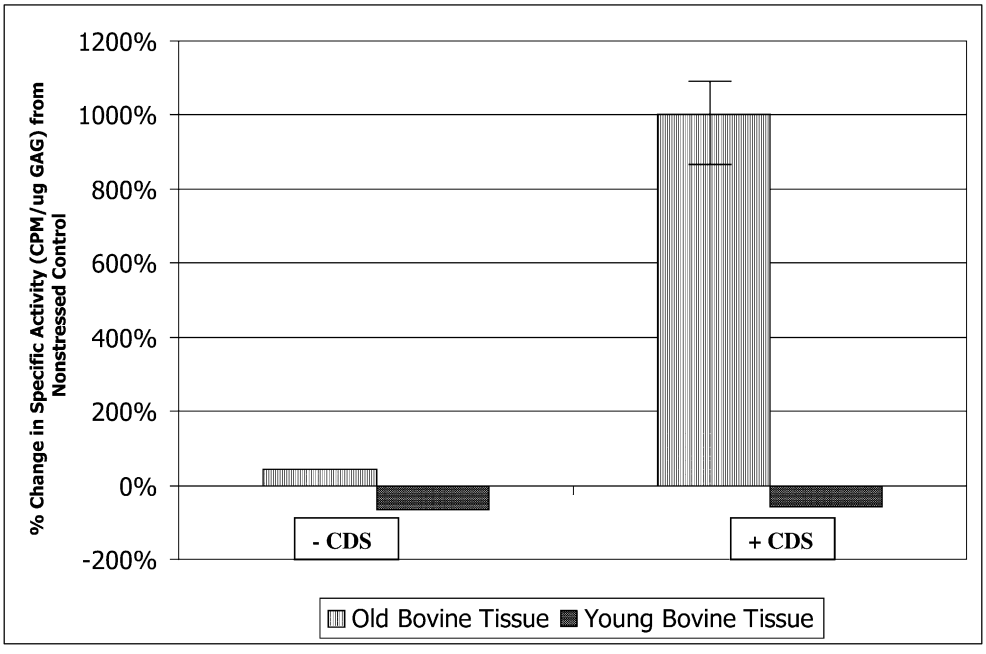

Fig. 6. Effect of preconditioning cartilage from young and aged bovine joints with glcN plus CS on metabolic response to static pressure. Data expressed as percent change (±S.E.M.) in specific activity of GAG (cpm/μg GAG) of 10 replicates. Young tissue inhibited by −65% with no change after CDS conditioning. Aged tissue uptake stimulated by 40% without conditioning and 1000% after conditioning.

Since the units of enzyme activity are not equilivent on the basis of activity, it is difficult to compare their dose–response profile (12.5 U pronase was stimulatory while 0.74 mU stromelysin was inhibitory). The loss of Safranin-O staining after stromelysin exposure was minimal compared with that of pronase (not shown). Even at this low dose, which may be more physiological, the inhibition of synthetic activity was reversed by CDS, suggesting that the net effect of CDS exposure favored synthetic activity.

Our report on the stress response to static compression is considered to be preliminary since the data were derived from a single experiment. Anabolic activity in cartilage from aged animals was elevated in response to static pressure, whereas cartilage from young animals was depressed.

Conditioning the tissue with CDS enhanced the aged tissue synthetic response to a level 1000% greater than non-conditioned tissue. Similar effects were noted by Nerucci *et al.*[35] with mechanical and cytokine (IL-1) stresses, and both studies confirm that stressed tissue is more responsive to CS. A recent study also found that glcN could reverse the decrease in proteoglycan synthesis elicited by static compression[36]. Using 0.5 MPa for 24 h on tissues from immature animals, Guilak *et al.* also got 60% inhibition of proteoglycan synthesis using similar conditions[37]. Based on our data, the inhibition of synthesis by static stress obtained by these authors was probably due to culturing of tissue obtained from young animals.

The cytokine stress culture system was adapted from that described by Oleksyszyn *et al.*[38]. This system differed from usual procedures in that it required a 72-h period of isotope exposure providing more uniform labeling of proteoglycans and exposure to IL-1 plus plasminogen to effect greater catabolic activity. Using bovine cartilage, the catabolic effect of the cytokines disappears by 72 h in spite of daily media changes, but the data did allowed us to observe that CS restored proteoglycan breakdown to normal levels by 48 h.

The use of adverse conditions on chondrocytes to stimulate a cellular stress response was also reported by Benton *et al.*[39]. This author found an increase in protein synthesis by equine chondrocytes exposed to 42°C. The response was thought to be a component of cellular protective mechanisms. Mitrovic *et al.*[7] studied the effect of hyperthermia on chondrocyte metabolism and observed decreased synthesis of proteoglycans at temperatures above 38°C, a loss of metachromasia in cartilage, and increased catabolism of matrix macromolecules. Our observations also suggest that increases in incubation temperature are inhibitory, but uniquely demonstrate that when reequilibrated to 37°C, the chondrocyte has increased sensitivity to growth factors like IGF-1 and the S/DMOADs glcN and CS. This increase sensitivity resembles an overshoot phenomenon and is substantially greater than could be accounted for by simple recovery from the temperature-induced inhibition.

There are significant differences in the response of young vs aged cartilage to glcN and CS, in both unstressed and stressed environments. Tissues taken from young animals are generally refractory to metabolic changes elicited by glcN and CS and appear to resist responding to stress-induced perturbations. The differences observed may be a function of matrix mechanical properties between tissues and/or cell sensitivity to external factors. In view of the data presented, it is a reasonable speculation that a therapeutic regimen of glcN, CS and manganese ascorbate may have significant protective effects in aged joints exposed to transient or chronic stress. In fact, a recent study demonstrates that cartilage from osteoarthritic joints is more responsive to polysulfated GAG than tissue from normal joints[40].

In summary, glcN and CS are considered to be 'chondroprotective' agents, more recently classified as S/DMOADs, based on their activity in diminishing symptoms of OA and retarding progression of cartilage lesions. Their ability to modify (enhance) the response of chondrocytes to various stresses allows them to be included in the general class of BRM. Hence, a new therapeutic approach to OA may be termed 'stress resistance engineering', which encompasses the use of S/DMOADs as BRMs[41]. This approach aims to increase the natural stress responses of tissues, which may contribute to increased cell survival, retardation of progression of cartilage lesions and possibly regeneration of cartilage matrix.

References

1. Ghosh P, Smith M. Osteoarthritis, genetic and molecular mechanisms. Biogerontology 2002;3:85–8.
2. Vincent T, Hermansson M, Bolton M, Wait R, Saklatvala J. Basic FGF mediates an immediate response of articular cartilage to mechanical injury. Proc Natl Acad Sci USA 2002;99:8259–64.
3. Meyer GF, Ratcliffe BC, Mow VC. The effects of matrix compression on proteoglycan metabolism in articular cartilage explants. Osteoarthritis Cartilage 1994; 2:91–101.
4. Mazzetti I, Grigolo B, Pulsatelli L, Dolzani P, Silvestri T, Roseti L, *et al.* Differential roles of nitric oxide and oxygen radicals in chondrocytes affected by osteoarthritis and rheumatoid arthritis. Clin Sci 2001; 101:593–9.
5. Femor B, Weinberg JB, Pisetsky DS, Misukonis MA, Banes AJ, Guilak F. The effects of static and intermittent compression on nitric oxide production in articular cartilage explants. J Orthop Res 2001; 19:729–37.
6. Fernandes JC, Martel-Pelletier J, Pelletier JP. The role of cytokines in osteoarthritis pathophysiology. Biorheology 2002;39:237–46.
7. Mitrovic D, Gruson M, Ryckewaert A. Local hyperthermia and cartilage breakdown. J Rheumatol 1981; 8:193–203.
8. Mankin HJ. The metabolism of articular cartilage in health and disease. In: Burleigh PMC, Poole AR, Eds. Dynamics of Connective Tissue Macromolecules. Amsterdam: North-Holland Publisher 1975;189–215.
9. Lippiello L, Woodward J, Karpman R, Hammad TA. *In vivo* chondroprotection and metabolic synergy of glucosamine and chondroitin sulfate. Clin Orthop 2000; 381:229–40.
10. Moskowitz RW, Davis W, Sammarco J. Experimentally induced degenerative joint lesions following partial meniscectomy in the rabbit. Arthritis Rheum 1973; 16:397–405.
11. Young AS, Koech DK. Biological response modifiers: cytokines in health and disease and their possible therapeutic value. Afr J Health Sci 1994;1:6–12.
12. Pang ZJ, Chen Y. Polysaccharide krestin enhances manganese superoxide dismutase activity and mRNA expression in mouse peritoneal macrophages. Am J Chin Med 2000;28:331–41.
13. Jenkens JK, Hardy KJ. Biological modifier therapy for the treatment of rheumatoid arthritis. Am J Med Sci 2002;323:197–205.
14. Zhang HT, Morisaki T. Protein-bound polysaccharide PSK inhibits tumor invasiveness by down-regulation of TGF-beta 1 and MMPs. Clin Exp Metastasis 2000; 18:343–52.
15. Kim KI, Shin KS, Jun WJ, Hong BS, Shin DH, Cho HY, *et al.* Effects of polysaccharides from rhizomes of *Curcuma zedoaria* on macrophage functions. Biosci Biotechnol Biochem 2001;65:2369–77.
16. Yan J, Vetvicka V. Beta-glucan, a specific biologic response modifier that uses antibodies to target tumors for cytotoxic recognitions by leukocyte complement receptor type 3. J Immunol 1999;163:3045–52.
17. Quillin P. Nutrients as biological response modifiers. Nat Med J 1998;12:5–11.

18. Oegema TR, Deloria LB, Sandy JD, Hart DA. Effect of oral glucosamine on cartilage and meniscus in normal and chymopapain-injected knees of young rabbits. Arthritis Rheum 2002;46:2495–503.
19. Uebelhart D, Thonar EJ, Zhang J, Williams JM. Protective effect of exogenous chondroitin 4,6-sulfate in the acute degradation of articular cartilage in the rabbit. Osteoarthritis Cartilage 1998;6:6–13.
20. Adebowale A, Du J, Liang Z, Leslie JL, Eddington ND. The bioavailability and pharmacokinetics of glucosamine hydrochloride and low molecular weight chondroitin sulfate after single and multiple doses to Beagle dogs. Biopharm Drug Dispos 2002; 23:217–25.
21. Volpi N. Oral bioavailability of chondroitin sulfate (chondrosulf) and its constituents in healthy male volunteers. Osteoarthritis Cartilage 2002;10:768–73.
22. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta 1986;883:173–7.
23. Zebrower ME, Kieras FJ, Brown WT. Analysis by high performance liquid chromatography of hyaluronic acid and chondroitin sulfates. Anal Biochem 1986; 157:93–9.
24. Bartholomew JS, Handley CJ, Lowther DA. The effects of trypsin treatment on proteoglycan biosynthesis by bovine articular cartilage. Biochem J 1985; 227:429–37.
25. McElligott TF, Potter JL. Increased fixation of sulfur-35 by cartilage *in vitro* following depletion of the matrix by intravenous papain. J Exp Med 1960;112:743–51.
26. Lee DA, Bentley G, Archer CW. Proteoglycan depletion alone is not sufficient to stimulate proteoglycan synthesis in cultured bovine cartilage explants. Osteoarthritis Cartilage 1994;2:175–85.
27. Lack W, Bosch P, Lintner F. Influence of trypsin on the regeneration of hyaline articular cartilage. Acta Orthop Scand 1986;57:123–5.
28. Collier S, Ghosh P. Evaluation of the effect of antiarthritic drugs on the secretion of proteoglycans by lapine chondrocytes using a novel assay procedure. Ann Rheum Dis 1989;48:372–81.
29. Verbruggen G, Cornelissen M, Elewaut D, Broddelez C, DeRidder L, Veys EM. Influence of polysulfated polysaccharides on aggrecans synthesized by differentiated human articular chondrocytes. J Rheumatol 1999;26:1663–71.
30. Calabrese EJ, Baldwin LA. Hormesis: U-shaped dose responses and their centrality in toxicology. Trends Pharmacol Sci 2001;22:285–91.
31. DeMattei M, Pellati A, Pasello M, DeTerlizzi F, Massari L, Gemmati D, *et al.* High doses of glucosamine–HCl have detrimental effects on bovine articular cartilage explants cultured *in vitro*. Osteoarthritis Cartilage 2002;10:816–21.
32. Armstrong AL, Barrach HJ, Ehrlich MG. Identification of the metalloproteinase stromelysin in the physis. J Orthop Res 2002;20:289–94.
33. Van Meurs J, Van Lent P, Holthuysen A, Lambrou D, Bayne E, Singer I, *et al.* Active matrix metalloproteinases are present in cartilage during immune complex-mediated arthritis: a pivotal role for stromelysin-I in cartilage destruction. J Immunol 1999;163:5633–9.
34. Wertheimer S, Katz S, Rowan K, Lugo A, Levin A, Levin W, *et al.* Stromelysin expression in IL-1 beta stimulated bovine articular cartilage explants. Inflamm Res 1995;Suppl 2:S119–20.
35. Nerucci F, Fioravanti A, Cicero MR, Collodel G, Marcolongo R. Effects of chondroitin sulfate and interleukin-1 beta on human chondrocyte cultures exposed to pressurization: a biochemical and morphological study. Osteoarthritis Cartilage 2000; 8:279–87.
36. Guilak F, Meyer BC, Ratcliffe A, Mow VC. The effects of matrix compression on proteoglycan metabolism in articular cartilage explants. Osteoarthritis Cartilage 1994;2:91–101.
37. Gouze JN, Grodzinsky AJ. Glucosamine inhibits compression-induced changes in chondrocyte biosynthesis and gene expression in alginate gel system Abstract. Trans Orthop Res Soc 2001;26:0554.
38. Oleksyszyn J, Augustine AJ. Plasminogen modulation of IL-1 stimulated degradation in bovine and human articular cartilage explants. Inflamm Res 1996; 45:464–72.
39. Benton HP, Cheng TC, MacDonald MH. Use of adverse conditions to stimulate a cellular stress response by equine articular chondrocytes. Am J Vet Res 1996;57:860–5.
40. Sevalla K, Todhunter RJ, Vernier-Singer M, Budsberg SC. Effect of polysulfated glycosaminoglycan on DNA content and proteoglycan metabolism in normal and osteoarthritic canine articular cartilage explants. Vet Surg 2000;29:407–14.
41. Lippiello L, Idouraine A, Albers R. Stress resistance engineering in cellular trauma: applications to articular cartilage Abstract. Ann Biomed Eng 1994;22(Suppl 1):37.