In vivo model of cartilage degradation—effects of a matrix metalloproteinase inhibitor

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Abstract

Objectives—To develop a model of cartilage degradation that (i) enables the testing of synthetic, small molecular weight matrix metalloproteinase (MMP) inhibitors as agents to prevent cartilage erosion, (ii) permits the direct assay of the principal constituents of the extracellular matrix (collagen and proteoglycan) in both the non-calcified articular cartilage and the calcified cartilage compartments, and (iii) is mediated by a chronic, granulomatous tissue that closely apposes intact articular cartilage, and in this respect resembles the pannus-cartilage junction of rheumatoid arthritis.

Methods—Femoral head cartilage was obtained from donor rats, wrapped in cotton and implanted subcutaneously into recipient animals. After a two stage papain digestion procedure, the proteoglycan and collagen contents were measured by assaying for glycosaminoglycans and hydroxyproline, respectively, in both the non-calcified cartilage that comprises the articular surface layer and the calcified cartilage compartment. The incorporation in vitro of [³⁵S]-sulphate into glycosaminoglycans was assayed as a measure of proteoglycan biosynthesis. An osmotic minipump was cannulated to the implanted femoral head cartilage and synthetic MMP inhibitors (MI-1 and MI-2) were infused continuously over a 14 day period.

Results—The implanted, cotton wrapped femoral head cartilages provoked a granulomatous response that resulted in the removal of collagen and proteoglycan from the cartilage matrix. The removal of proteoglycan and collagen was exclusively from the non-calcified articular cartilage, whereas the proteoglycan and collagen content of the calcified compartment increased during the experiments. MI-1 reproducibly reduced the degradation of proteoglycan and collagen in implanted femoral head cartilage.

Conclusions—We have described an in vivo model of cartilage degradation that permits the measurement of proteoglycan and collagen in both non-calcified articular cartilage and calcified cartilage compartments. The model can be used to test the effects of agents of unknown systemic bioavailability and pharmacokinetic profile by infusing them directly to the site of cartilage degradation. The removal of cartilage extracellular matrix

by granulomatous tissue was inhibited by an MMP inhibitor, thus proving the involvement of this family of proteinases in cartilage catabolism in this model.

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The destruction of articular cartilage is a major pathological event in both rheumatoid and osteoarthritis, leading ultimately to the loss of joint function. The primary functions of articular cartilage—to provide a friction free surface for joint articulation and to dissipate joint loading forces—are dependent upon the integrity of the cartilage matrix. The extracellular matrix of cartilage consists predominantly of a gel like solution of polyanionic proteoglycans embedded in a meshwork of type II collagen fibres. The swelling pressure exerted by the proteoglycans confers on cartilage its resistance to compression.¹ The precise mechanisms by which articular cartilage is degraded in joint disease have yet to be elucidated, but the matrix metalloproteinase (MMP) family (the stromelysins, gelatinases, and collagenases) is strongly implicated. Between them, these proteinases have the capacity to degrade all the proteinaceous components of articular cartilage.

We have developed an in vivo model of cartilage degradation that allows, over a two week period, the infusion of synthetic MMP inhibitors directly to cotton wrapped femoral head cartilages, and thus we have been able to determine the involvement of metalloproteinases in mediating the catabolism of matrix components. In addition, we have increased the resolving power of the system by assaying the proteoglycan and collagen content of the implanted femoral heads in both the non-calcified articular, and calcified cartilage compartments.

Materials and methods

PREPARATION OF COTTON WRAPPED FEMORAL HEAD CARTILAGES

Cotton squares of approximately 1 cm² surface area and weighing 5 (SEM 0.2) mg were cut from unravellable dental cotton rolls before ethylene oxide sterilisation. All animal procedures were in accordance with the Animals (Scientific Procedures) 1986 Act, and also subject to an internal ethical review. For most experiments, six week old female Wistar rats (Charles River) were killed, shaved, and then immersed in a solution of 0.5% Hibitane in 70% ethanol (Hibitane-ethanol). The

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femoral head cartilage caps were dissected using aseptic techniques and placed in Minimum Essential Medium containing Hanks's salts (MEM) (Gibco). The cartilage caps were then transferred to a laminar flow hood, wrapped tightly in sterile cotton squares (previously soaked in MEM), and held in microtitre wells on ice for no longer than two hours until implantation into recipient animals.

IMPLANTATION OF COTTON WRAPPED FEMORAL HEAD CARTILAGES INTO RATS

Eight week old male Wistar rats (Charles River) were anaesthetised using halothane (May and Baker). The stomach area was shaved and swabbed with Hibitane-ethanol. A single cotton wrapped femoral head cartilage was inserted subcutaneously to the left side of a ventral midline incision. The wound was sealed using a skin staple (Auto Suture UK Ltd), and the animal allowed to recover.

IMPLANTATION OF OSMOTIC MINIPUMPS

Osmotic minipumps (Alzet) were filled with test substances under sterile conditions according to the manufacturer's instructions. Experiments were conducted with 2ML2 pumps that infuse 5 μ l/h sustained over 14 days. A custom made cannula was attached to the pump flow regulator and the pumps were primed by holding them overnight in sterile saline at room temperature. The pump cannula consisted of 6.5 cm Tygon tubing of internal diameter (id) 0.25 mm, external diameter (ed) 0.76 mm, attached at one end to a 26 gauge needle (Becton and Dickinson) previously removed from its Luer fitting. Attached to the other end was 1.2 cm of Tygon tubing (id 0.76 mm, ed 2.29 mm) that permitted connection of the cannula to the flow regulator. A small collar was fashioned using the wider bore tubing and placed over the narrow bore tubing close to the needle. This was used as an attachment point for subsequent suturing of the needle into the cotton wrapped femoral head cartilages.

Rats (10 per group) were anaesthetised with a single intraperitoneal (ip) injection of a mixture of fentanyl (Sublimaze, Janssen) and medetomidine hydrochloride (Domitor, SmithKline Beecham) 0.3 mg/kg each agent. The animals were shaved on their right flanks, and the area swabbed with Hibitane-ethanol. A small skin incision was made, followed by blunt dissection using a trocar, to allow the osmotic minipumps to be implanted subcutaneously in the caudal dorsal region away from the site where the cotton wrapped femoral heads had been implanted 11 days previously. The cannula needle was inserted into the granuloma that had sequestered the femoral head cartilage by this stage, and sutured into position, great care being taken not to damage the cartilage implant. Skin incisions were closed using 3/0 Mersilk sutures (Ethicon) and skin staples. Anaesthesia was reversed and

analgesia provided with a single intramuscular injection of a mixture of nalbuphine hydrochloride (Nubain, Du Pont) 20 mg/kg and atipamezole hydrochloride (Antesedan, Smith-Kline Beecham) 2 mg/kg.

REMOVAL OF COTTON WRAPPED FEMORAL HEAD CARTILAGES

At various times after implantation, animals were killed with an ip injection of 0.5 ml pentobarbitone sodium (Euthetal, Rhone Merieux). The cotton wrapped femoral head cartilages were dissected, removed carefully from the associated granulomatous tissue, blotted, and their wet weights recorded. They were then dried at 60°C and their dry weights recorded. The granulomas were dried at 60°C, weighed, and the mean weight of the cotton subtracted.

BIOCHEMICAL ANALYSIS

All standard laboratory reagents were obtained from Sigma. Femoral head cartilages were processed using a two stage papain digestion procedure. First, to digest the non-calcified articular cartilage, the femoral head cartilages were digested for four hours at 65°C with 1 ml of a 20 U/ml papain solution (10–20 U/mg, Sigma) in 50 mmol/l sodium phosphate buffer, pH 6.5, containing 2 mmol/l *N*-acetylcysteine and 2 mmol/l disodium ethylenediaminetetraacetic acid (EDTA). Second, the remaining calcified cartilage was washed three times with distilled water, and then digested overnight with 1 ml of a 20 U/ml papain solution made up in 50 mmol/l sodium phosphate buffer, pH 6.5, containing 2 mmol/l *N*-acetylcysteine and 200 mmol/l EDTA. Samples from both the papain digests were assayed for hydroxyproline (OHP) and glycosaminoglycan (GAG), as measures of collagen and proteoglycan content, respectively. GAGs were assayed using the 1,9-dimethylmethylene blue binding assay,² using shark chondroitin sulphate (Sigma C3254) as a standard. OHP was assayed using the *p*-dimethylaminobenzaldehyde colorimetric assay.³ For both assays, the standard curve and experimental samples were incubated under identical conditions.

[³⁵S]-SULPHATE RADIOLABELLING OF PROTEOGLYCAN

Femoral head cartilages were placed in sterile tubes and washed twice with 1 ml of Dulbecco's Modification of Eagle's Medium (DMEM) (Gibco) before being incubated at 37°C for three hours in 1 ml of DMEM containing 165 KBq of [³⁵S]-sulphate (specific activity = 1.1 TBq/mg) (Amersham). The femoral head cartilages were washed three times with 3 ml of DMEM containing 1 mg/ml sodium sulphate, and were then digested with papain as described above. One hundred microlitres of 5% cetyl pyridinium chloride in 0.3 mol/l potassium chloride solution was added to 500 μ l of papain digest and the mixture allowed to stand for 30 minutes at

room temperature to precipitate the glycosaminoglycans. After centrifugation (3000 *g* for five minutes), the supernatants were removed and the precipitates dissolved in 600 μ l of concentrated formic acid by heating to 70°C for 10 minutes. Aliquots were taken for liquid scintillation counting as a measure of GAG biosynthesis.

MMP INHIBITORS AND ASSAYS

3-(S)-Mercapto-6-methyl-4-(S)-[[[1-(S)-[(methylamino) carbonyl]-2-(3-indolyl)ethyl]-amino]carbonyl] heptanoic acid methyl ester (MI-1) and 3-(R or S)-mercapto-6-methyl-4-(R or S)-[[[1-(S)-[(methylamino) carbonyl]-2-(3-indolyl)ethyl]amino]carbonyl] heptanoic acid methyl ester (MI-2) incorporate thiol moieties that are predicted to act as ligands for the zinc cation resident within the active site of matrix metalloproteinases. They were synthesised at SmithKline Beecham.⁴ Propylene glycol vehicle (5 μ l/h) or a matrix metalloproteinase inhibitor (80 nmol/h) was infused into femoral head cartilages for 14 days, starting 11 days after the implantation of cotton wrapped femoral head cartilages. On day 25, the femoral head cartilages were assayed for GAG and OHP content using the two stage papain digestion procedure. IC₅₀ values for the inhibitors *versus* collagenase and stromelysin were determined using the diffuse fibril assay,⁵ and casein assay,⁶ respectively.

STATISTICAL ANALYSIS

All data are represented as mean (SEM). Groups were compared using a two tailed Student's *t* test. Error bars that are not visible in the figures lie within the data point symbols.

Results

GAG AND OHP ASSAYS

Using standard papain digestion conditions,² in most experiments a significant proportion of the femoral head cartilage remained visibly intact after an overnight incubation, despite data indicating that the release of GAG and OHP under these conditions was complete by four hours (fig 1). When femoral head

Figure 1 Hydroxyproline (OHP) (●) and glycosaminoglycan (GAG) (○) contents of femoral head cartilages digested by papain. (*n* = 5).

Effect of EDTA on papain digestion of femoral head cartilage

Digestion conditions	GAG per femoral head cartilage (μg)	OHP per femoral head cartilage (μg)
First digestion with papain solution	443 (33)	428 (19)
Second digestion with papain solution containing 200 mmol/l EDTA	79 (8)	81 (14)

Values are mean (SEM) (*n* = 5). GAG = Glycosaminoglycan; OHP = hydroxyproline.

cartilages were subjected to a second digestion using papain buffer supplemented with 200 mmol/l EDTA, the resistant tissue was completely digested, resulting in the further release of GAG and OHP (table).

We also demonstrated (data not shown) that incubation of the femoral head cartilages overnight in 200 mmol/l EDTA digestion buffer (lacking papain) removed the x ray radio-opacity of the femoral head cartilage caps. Together, these data indicated that calcified cartilage—effectively, the inner surface of the dissected femoral head cartilage cap⁷—was resistant to standard papain digestion conditions. This property was exploited using the two stage papain digestion procedure to distinguish between the non-calcified articular cartilage layer that was fully degraded after four hours of incubation using standard papain digestion conditions, and the calcified cartilage compartment that could be digested with a subsequent overnight digestion in 200 mmol/l EDTA-papain solution.

The proportion of collagen and proteoglycan in the non-calcified articular cartilage and calcified compartments was first assessed in normal animals over a four week period (from age six weeks to age 10 weeks (fig 2). There

Figure 2 Distribution of collagen and proteoglycan in non-calcified articular (□) and calcified (▨) cartilage compartments. Data are represented as stacked columns; the total amount of collagen or proteoglycan is therefore given by the height of the column at each time point. (*n* = 5–11).

was an increase in collagen content in the non-calcified articular cartilage compartments, predominantly between weeks 7 and 8, whereas the collagen content of the calcified cartilage compartment increased progressively over the period studied. The overall effect of these changes was to produce an increase in total collagen content between weeks 7 and 8, followed by a period when the proportion of collagen within the calcified compartment increased, although the total collagen content of the femoral heads remained unchanged. In contrast, the amount of proteoglycan in the non-calcified compartment declined steadily between weeks 7 and 9 although, as with collagen, the proportion of proteoglycan in the calcified cartilage compartment was gradually augmented; these changes resulted in an overall reduction in the total proteoglycan content of the femoral heads.

EFFECTS OF COTTON INDUCED GRANULOMA FORMATION

Subcutaneous implantation of cotton wrapped femoral head cartilages induced a granulomatous response (fig 3A) that resulted in the degradation of collagen and proteoglycan from the non-calcified articular cartilage compartment of the femoral head cartilage (fig 3C). Proteoglycan loss was evident after 11 days, with a much less pronounced degradation of collagen following three days later. The contents of both components increased in the calcified cartilage compartment in a fashion similar to that observed in normal animals. Despite a 74% reduction in the proteoglycan content of the non-calcified articular cartilage by the end of the experiment (corresponding to a 47% reduction in the total proteoglycan content), there was no marked change in the cartilage wet weight. The dry weight of the femoral head cartilage increased slightly over the course of the experiment (fig 3B), presumably because matrix and other proteins were being deposited within the femoral head as a consequence of the cotton induced granulomatous response. Increasing the magnitude of the granulomatous response by wrapping the femoral head cartilage in a greater amount of cotton (15 mg compared with 5 mg routinely) had no effect on the rate of removal of cartilage constituents (data not shown).

Killed cartilage was initially degraded more rapidly than live cartilage (fig 4), but from day 17 the extent and rate of degradation of both live and killed cartilage was identical. The increase in collagen and proteoglycan contents in the calcified cartilage compartment was the same in live, killed, and age matched control femoral head cartilages.

PROTEOGLYCAN SYNTHESIS IN COTTON WRAPPED IMPLANTED CARTILAGE

After an initial suppression, the rate of proteoglycan biosynthesis in implanted femoral head cartilages recovered to near normal values after seven days, before subsequently declining to

26% of the rate in age matched controls (fig 5). As might be expected from the data shown in figure 2, the rate of proteoglycan synthesis in age matched femoral head cartilages declined over the course of the experiment to 77% of the day 0 value. There was no detectable proteoglycan synthesis in the calcified cartilage compartment (data not shown).

EFFECTS OF MMP INHIBITORS

MI-1 was shown to be equipotent in vitro against stromelysin and collagenase (see Methods), with an IC_{50} of 2.5×10^{-9} mol/l. MI-2, a less potent diastereoisomer of MI-1, had an IC_{50} of 7.3×10^{-7} M versus collagenase.⁴ Preliminary experiments (data not shown) demonstrated that there was no loss of collagen and proteoglycan from implanted femoral head cartilages before day 11; therefore, to maximise the duration of exposure of inhibitor to the processes mediating cartilage degradation, infusion of MI-1 and MI-2 was started from

Figure 3 Subcutaneous implantation of cotton wrapped femoral head cartilages. A: Dry weights of cotton associated granulomas. B: Wet weights (Δ) and dry weights ($\blacktriangle$) of femoral head cartilages after removal of the granulomatous capsule. C: Femoral head cartilage content of glycosaminoglycan (GAG) (circles) and hydroxyproline (OHP) (squares) in the non-calcified articular cartilage layer (open symbols) and calcified (filled symbols) cartilage compartments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared with respective day 0 values. ($n = 5$).

Figure 4 Glycosaminoglycan (GAG) and hydroxyproline (OHP) content in the non-calcified articular cartilage layer (open symbols) and calcified (filled symbols) cartilage compartments of cotton wrapped live (circles) and killed (squares) femoral head cartilages, and non-implanted femoral head cartilages from normal age matched controls (triangles). ** $p < 0.01$ compared with killed cartilage at the same time point. ($n = 5$).

this time point. MI-1 inhibited the removal of proteoglycan from non-calcified articular cartilage by 51% ($p < 0.001$ compared with control), and collagen removal was inhibited by 65% ($p < 0.05$ compared with control), whereas MI-2, a less potent diastereoisomer of MI-1, inhibited proteoglycan and collagen removal by 22% and 29%, respectively (both NS) (fig 6). In two replicate experiments (data not shown), MI-1 inhibited the removal of proteoglycan by 44% and 63%, and the removal of collagen by 33% and 47% (all statistically significant), from the non-calcified articular cartilage. MI-1 and MI-2 did not

Figure 5 Glycosaminoglycan (GAG) synthesis in implanted and age matched femoral head cartilages, measured using [35 S]-sulphate incorporation into previously implanted cotton wrapped femoral head cartilages (○) and non-implanted femoral head cartilages removed from age matched animals (△). As a control, one group was killed by three cycles of freeze-thawing (●). ** $p < 0.01$ compared with day 0 value of live cartilage. ($n = 5$).

Figure 6 Effects of MI-1 and MI-2 on cartilage degradation (measured as glycosaminoglycan (GAG) and hydroxyproline (OHP) content and expressed as change in OHP and GAG contents during the 14 day pump infusion period). ■ = GAG content of non-calcified articular cartilage layer; □ = GAG content of calcified cartilage layer; ▨ = OHP content of non-calcified articular cartilage layer; ▩ = OHP content of calcified cartilage layer. * $p < 0.05$; *** $p < 0.001$ compared with the corresponding measurement for control cartilage implants.

affect the levels of proteoglycan or collagen in the calcified cartilage compartment.

There was no difference between the changes in the GAG and OHP contents of implanted femoral head cartilages in the vehicle control groups and groups not cannulated with osmotic minipumps (data not shown).

Discussion

There is a clear need for more effective treatments for the human arthritides. Currently, non-steroidal anti-inflammatory drugs (NSAIDs) comprise the mainstay of treatment for rheumatoid and osteoarthritis. While these offer symptomatic relief, there is little evidence that NSAIDs abrogate the underlying disease process manifested by the erosion of articular cartilage. It has been suggested that some agents, particularly gold containing compounds, halt the progression of disease, though the evidence is far from unequivocal⁸ and these compounds are not well tolerated by patients.

Numerous studies in a variety of species and adopting different experimental procedures have so far failed to provide a model reflecting closely every aspect of human arthritic disease;⁹ indeed, it is debatable whether such a goal is feasible. In the absence of such a model, our approach has been to focus on a single aspect of the pathological cascade of rheumatoid disease that is relevant and appropriate to the assessment of agents designed specifically to prevent cartilage degradation. Thus our

objectives were (i) to explore the likely pathological significance of matrix metalloproteinases using a specific, low molecular weight MMP inhibitor; (ii) to measure directly the removal of collagen and proteoglycan, the principal constituents of the cartilage implants, and (iii) to use a model system in which granulomatous tissue tightly sequesters intact articular cartilage and which in this respect resembles the close apposition of pannus tissue to articular cartilage where the most aggressive destruction of cartilage is seen to occur in rheumatoid arthritis.¹⁰⁻¹²

To provoke the development of a closely apposing granulomatous tissue, the femoral head articular cartilage was tightly wrapped in cotton before implantation. The importance of close tissue to substrate contact in mediating cartilage degradation has been demonstrated in other studies,¹³⁻¹⁴ in which the encapsulation of rat femoral head cartilage by a cotton induced granulomatous tissue greatly augmented cartilage degradation in mice. Indeed, there are a number of *in vivo* models that are variants of the model we have described here, incorporating different features designed to promote the rapid degradation of implanted cartilage. Thus the use of inflammogens, and the implantation of xenogeneic, cut, or non-articular cartilage are all strategies that have been used to accelerate the removal of extracellular matrix components from implanted cartilage,¹³⁻¹⁷ thereby expediting experimental investigations. However, those model systems designed to maximise the rate of cartilage degradation may involve pathophysiological processes that are different from those that operate in human disease, in which cartilage erosion occurs relatively slowly. Thus cut or non-articular cartilage may have an increased susceptibility to proteinases that would not normally gain access to the cartilage matrix in the human arthritides. The collagen matrix is organised very differently at the surface of articular cartilage compared with the deeper layers,¹⁸ and this may confer an increased resistance to proteolytic attack. We opted, therefore, to use intact, allogeneic articular femoral head cartilages to preserve the integrity of the cartilage matrix, and we avoided the use of inflammogens (other than the cotton used to wrap the femoral head cartilage), so that the implanted cartilage was degraded at a moderate rate that was nevertheless amenable to experimental manipulation.

Recently, researchers have focused on the role of the MMPs in mediating the tissue destruction seen in arthritic disease. There is now a considerable body of evidence that suggests that this family of enzymes is crucially involved in mediating the damage to cartilage that is the hallmark of arthritis.¹⁹⁻²¹ Among the family of MMPs, collagenase is likely to be a key proteinase in mediating irreversible damage to the collagen network of articular cartilage, because it is the only proteinase that is able to cleave native fibrillar collagen in its helical region. Stromelysin has a broader substrate specificity, and is able to degrade proteoglycan core protein.²² Thus treatment with

a synthetic, small molecular weight inhibitor of MMPs should abolish the progressive destruction of cartilage in joint diseases.

There have been numerous reports on the successful use of MMP inhibitors in preventing matrix degradation *in vitro*. MMP inhibitors with some selectivity for inhibiting collagenase versus stromelysin and gelatinase were tested in an *in vitro* assay of interleukin-1 α (IL-1 α) stimulated cartilage degradation.²³ It was found that the collagenase selective inhibitor, Ro 31-7467, prevented collagen degradation, but not proteoglycan removal. Ro 31-4724, which is a potent inhibitor of both collagenase and stromelysin, was able to prevent the removal of both collagen and proteoglycan. Dose dependent inhibitions of the release of proteoglycan were obtained with three metalloproteinase inhibitors using retinoic acid or IL-1 stimulated rabbit articular cartilage as a model system.²⁴ Using IL-1 α to stimulate the release of proteoglycans from pig articular cartilage *in vitro*, it was shown that a small molecular weight collagenase inhibitor, but not the tissue inhibitor of metalloproteinases (TIMP), prevented matrix degradation.²⁵ Recently, however, it was demonstrated that TIMP and TIMP-2 were able to inhibit the degradation of collagen from bovine nasal cartilage stimulated with IL-1,²⁶ though there was no effect on the release of proteoglycan. In the same system, a synthetic matrix metalloproteinase inhibitor, BB87, was able to inhibit both collagen and proteoglycan removal. These reports suggest that synthetic inhibitors may be more effective in preventing cartilage damage than are endogenous, proteinaceous inhibitors of the MMPs. In a similar system, a hydroxamate-containing metalloproteinase inhibitor, U24522, reduced the release of proteoglycan from human articular cartilage stimulated with IL-1 β .²⁷ U24522 also markedly reduced the immunohistochemical staining mediated by antibodies raised to epitopes that are only revealed on degraded collagen, and that are absent on the native triple helical molecule. Other workers have shown that lipophilic inhibitors of cysteine endoproteinases are able to inhibit the IL-1 induced resorption of bovine cartilage *in vitro*²⁸⁻²⁹ although, in similar systems, MMP inhibitors have also been shown to be effective.²³⁻²⁵⁻³⁰ The possibility remains that cysteine proteinases may participate in the activation of MMPs.³⁰

While *in vitro* test systems can provide useful information, they are unable to reflect fully the panoply of cytokines, enzymes, and cellular elements that are likely to influence the production and effects of the matrix metalloproteinases *in vivo*. Thus assessing the efficacy of MMP inhibitors in *in vivo* models of cartilage destruction is an important step towards realising the potential of these agents as new therapies for human disease and, to date, there have been very few demonstrations of the utility of MMP inhibitors in relevant animal models. MMP inhibitors were used successfully in the adjuvant arthritis model to prevent the destructive secondary phase of that

disease,³¹ but quantitative data of the type we have presented here were not reported.

To assess fully and accurately the effects of the degradative process mediated by the granulomatous tissue, and the effects of MI-1 in ameliorating the erosion of cartilage matrix, we have developed a two stage papain digestion procedure that enables assay of proteoglycan and collagen in both the non-calcified articular cartilage layer⁷ and the calcified cartilage compartments. This assay procedure revealed that collagen and proteoglycan were degraded exclusively from non-calcified articular cartilage. In addition, we demonstrated a progressive increase in the proportion of proteoglycan and collagen in the calcified compartment in live and killed implanted cartilage, and showed that this process occurred identically in comparable tissues from age matched controls. We ascribe this increase to a process of progressive calcification of cartilage that is not, of necessity, chondrocyte mediated. Using [³⁵S]-sulphate incorporation into GAGs to measure the biosynthesis of proteoglycan, we showed that, after an initial suppression, living femoral head cartilage was temporarily able to restore its synthetic capability to control levels before eventually succumbing to the effects of the encapsulating granulomatous tissue. This *de novo* proteoglycan synthesis probably contributed to the increased resilience of live cartilage, as indicated by our finding that live cartilage was initially degraded less rapidly than killed cartilage, although the rates and extent of degradation converged after 17 days. Thus, in this model, the chondrocytes within the implanted cartilage did not augment the process of matrix destruction—a finding corroborated by others.^{13 15 16}

To control for any non-specific effects of MI-1 infusion, such as toxicity of the compound, we infused MI-2, a much less potent diastereoisomer of MI-1, into the femoral head granuloma. MI-2 infusion resulted in a weak inhibition of matrix degradation that did not differ significantly from that in the control group. Without testing a diastereoisomer of MI-1 that is completely devoid of MMP inhibitory activity (none exists with this class of compound), it is impossible to differentiate unequivocally between non-specific effects and genuine, but weak, intrinsic MMP inhibitory activity. However, as the inhibitory effects of MI-1 were substantial, reproducible, and significantly different from those in the control group, it is clear that MMPs are involved in the degradation of cartilage matrix in this model. As MI-1 did not completely abrogate the degradation of cartilage, other modalities for cartilage destruction may play a role. However, we suspect that an important element contributing to the failure of MI-1 to inhibit more profoundly the removal of matrix components was insufficient access of the compound to all of the sites of cartilage breakdown. In a system in which collagen-gelled cotton buds were implanted into rats, we demonstrated that MI-1, infused at the same concentration as used in this study, completely abolished the degradation of collagen

mediated by the encapsulating granulomatous tissue.³² Importantly, in this system, the collagen substrate was adhered to a loose cotton matrix that facilitated the penetration of infused compounds. Although cartilage degradation may have been more substantially inhibited by increasing the concentration of MI-1 infused into the femoral head cartilage granuloma, this option was precluded by the insolubility of the compound.

The two stage papain digestion allows a more sensitive assessment of the effects of MMP inhibitors than would be possible if the total GAG and OHP contents of the cartilage were measured. Thus if the data of figure 6 are recalculated based on the *total* GAG and OHP contents, the apparent efficacy of MI-1 would be approximately 20% lower. This is not, however, an accurate representation of the susceptibility to inhibition by MMP inhibitors of the degradative process mediated by the granulomatous tissue. Increasing amounts of the total GAG and OHP content of the cartilage were found within the calcified compartment between days 11 and 25 (as shown by the open GAG and vertically lined OHP bars in figure 6); as these compartments are not affected by the granulomatous tissue, these changes are irrelevant to any consideration of the importance of the MMPs to matrix degradation. Thus the methods described here optimise the power of the model to delineate the effects of interventions using pharmacological agents with antiarthritic potential, such as MI-1.

In conclusion, we have developed a model of cartilage degradation in which the levels of proteoglycan and collagen can be monitored, quantitatively and *in vivo*, in both the non-calcified articular cartilage layer and the calcified cartilage compartments. The implanted femoral head cartilage can be readily cannulated, and agents infused continuously over a two week period, directly to the site of cartilage degradation; thus agents with unknown pharmacokinetic profiles or systemic bioavailability can be tested. *In vivo* models also offer the opportunity to assess compounds administered by clinically relevant routes, for example orally, though in the absence of MMP inhibitors with adequate bioavailability by this route, we have not been able to perform investigations of this nature. We have demonstrated that a specific metalloproteinase inhibitor inhibited the removal of collagen and proteoglycan from cartilage matrix. Until inhibitors that selectively inhibit individual members of the MMP family have been tested in this model, it will be difficult to describe unequivocally the parts played by each of these proteinases in mediating cartilage degradation.

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