Cartilage degradation by polymorphonuclear leucocytes: in vitro assessment of the pathogenic mechanisms

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Abstract

Polymorphonuclear leucocytes (PMNs), which predominate in inflammatory synovial fluid, can degrade cartilage. This was measured by a novel in vitro model; PMNs were incubated for up to one hour with 2 or 3 μ m sections of cartilage and the glycosaminoglycan loss determined by microdensitometry after alcian blue staining. Glycosaminoglycan loss could be as a result of damage from reactive oxygen species, proteolytic enzymes, or a combination of the two. The relative contributions of these mechanisms were evaluated using selective inhibitors. The results show that activated PMNs will degrade cartilage and that this degradation is due to proteolytic enzymes and not reactive oxygen species. There is a specificity involving elastase but not other serine proteases. It is suggested that enzyme inhibition may play a part in reducing PMN mediated cartilage damage.

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The rheumatoid synovium is characterised by chronic inflammation with an infiltration of mononuclear inflammatory cells. An outgrowth of the synovium called pannus invades the cartilage from the joint margins and is considered to be responsible for the erosive changes. Contrasting with this view of immune driven chronic inflammation is the finding that synovial fluid from patients with rheumatoid arthritis often contains large numbers of acute inflammatory cells, polymorphonuclear leucocytes (PMNs).¹ The persistence of these cells at a site of chronic inflammation may in part be due to chemotactic properties of cartilage breakdown products,² but the question arises as to what part, if any, they play in the destructive processes. Polymorphonuclear leucocytes have been described at the cartilage pannus junction³ and can occasionally be the dominant cell type.⁴ As PMNs are short lived cells their presence suggests that they could play an important part in cartilage erosion, possibly affecting the early stages of the disease and disease exacerbations.⁵ Cartilage integrity may also be compromised by a generalised loss of proteoglycan that is not confined to areas of erosion. Synovial fluid PMNs could contribute to this process.

Immune complexes, which have been demonstrated in the superficial layer of rheumatoid cartilage,⁶ can provide an anchorage and a trigger for PMN activation.⁷ As a consequence, reactive oxygen species and proteolytic enzymes will be released directly onto the surface of the cartilage, a process enhanced by frustrated

phagocytosis.⁸ In cell free systems in vitro, reactive oxygen species have been shown to degrade a variety of macromolecules including proteoglycan, a cartilage matrix component.⁹ Degradation of intact cartilage matrix has also been described.^{10 11} Polymorphonuclear leucocyte elastase, a proteolytic enzyme, has been extracted from arthritic cartilage and has been shown to be capable of degrading cartilage in vitro.¹²

Study of the direct actions of PMNs on cartilage matrix has been hampered because existing assays of cartilage breakdown require time periods well in excess of the lifespan of the cells. In this study, the problem has been overcome by developing a novel investigational method which involves layering PMNs onto cryostat sections of cartilage. This allows proteoglycan loss to be assessed histochemically over a period of one hour. This model has been used to determine if PMNs can cause cartilage damage in vitro and to assess the relative importance of reactive oxygen species and proteolytic enzymes in the effects of PMNs on cartilage.

Materials and methods

ISOLATION OF POLYMORPHONUCLEAR LEUCOCYTES

Male Wistar rats (mean (SD) weight 250 (10) g; allowed free access to food and water) were injected intraperitoneally with 10 ml 5% oyster glycogen in Hanks's balanced salt solution (HBSS). After 16 hours the rats were killed by cervical dislocation and the peritoneal cavity of each rat lavaged with 10 ml HBSS containing 0.01 M trisodium citrate. The lavage fluid was recovered and layered directly onto Histopaque 1077 and centrifuged at 400 *g* for 30 minutes at room temperature. Buffy coat cells were discarded with the rest of the supernatant. The cell pellet was resuspended in HBSS before being centrifuged at 300 *g* for 10 minutes. Cells were resuspended at the desired concentration in HBSS.

CARTILAGE CULTURE

Bovine nasal cartilage was obtained from the local abattoir. A 3 mm diameter cork borer was used to prepare cylinders of cartilage which were cut into 1 cm lengths. These were chilled by precipitate immersion in *n*-hexane at -70°C . Cross sections of cartilage were cut at 2 or 3 μ m on a Brights cryostat with a cabinet temperature of -25°C and with the knife cooled with cardice. Three sections were picked up onto

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each microscope slide. Perspex rings (1.3 cm diameter and 0.3 cm deep) were secured around each cartilage section using a solution of polyvinylalcohol (Cryoembed, Brights) to form an open chamber of total volume 0.25 ml. Cartilage sections were incubated with PMNs at 1×10^7 cells/ml in HBSS for periods up to 60 minutes at 37°C in a humidified chamber. Sections were then fixed in buffered formalin for 10 minutes and the Perspex rings removed.

QUANTITATIVE HISTOCHEMISTRY

Sections were rinsed with distilled water and transferred to a staining bath consisting of 0.05% alcian blue 8GX in 0.025 M acetate buffer with magnesium chloride at 0.5 mol/l. After 24 hours sections were treated with three 10 minute changes of distilled water before being dehydrated with alcohol, cleared with xylene, and mounted with DPX. The intensity of staining in each section was quantified with a Vickers M85 scanning and integrating microdensitometer¹³ using a $\times 40$ objective, a wavelength of 550 nm, a band width of 20 nm, a mask diameter of 15 μ m, and a scanning spot size of 0.2 μ m. For each section, clear field readings were taken (background) and clear field readings with an imposed neutral density of 1.0 (ND1). Ten integrated readings were taken from areas of cartilage matrix free of lacunae. The extinction of each section was < 0.7 . From these values the mean integrated extinction (MIE) was calculated as (mean integrated absorbance-background)/ND1. Results were expressed as $MIE \times 100$.

CHEMILUMINESCENCE

Chemiluminescence was measured on an LKB 1251 luminometer set to a temperature of 37°C. Measuring cuvettes contained PMNs at 5×10^6 cells/ml with 5 μ M luminol in a total volume of 1 ml HBSS.

ELASTASE ASSAY

Release of an enzyme with elastase activity was measured from PMNs at 1×10^7 cells/ml HBSS incubated at 37°C for periods up to one hour. At each time point the tubes were centrifuged at 200 g for 10 minutes at 1°C. Supernatants were then assayed for elastase activity by spectrophotometric assay.¹⁴ In brief, the substrate was MeO-Suc-Ala-Ala-Pro-Val-p-NA used at a final concentration of 1 mmol/l, in 100 mM TRIS-HCl buffer (pH 8.0) containing 200 mM NaCl and 0.001% Brij. A volume of 100 μ l supernatant was added to 900 μ l enzyme substrate. Incubation was at 37°C for 30 minutes and the reaction was stopped with 100 μ l 50% (v/v) aqueous acetic acid. Absorbance was measured at 405 nm. Elastase activity was quoted as unit/ml (one unit releases 1 μ M p-NA/minute at 37°C).

CHEMICAL TREATMENT

Polymorphonuclear leucocytes were treated with one or a combination of the following: phorbol myristate acetate (PMA) at 3.2 μ mol/l,

superoxide dismutase at 60 U/ml, catalase at 1000 U/ml, taurine at 2.5 mmol/l, diphenylene iodonium at 5 and 10 μ mol/l, Foipan at 100 μ mol/l and ONO-5046 at 100 nmol/l to 100 μ mol/l.

SOURCE OF CHEMICALS

Most chemicals were obtained from Sigma Chemical Company. Foipan and ONO-5046 were from ONO Pharmaceuticals, Japan.

STATISTICAL ANALYSIS

Results were analysed using the unpaired Student's *t* test.

Results

Over 60 minutes HBSS caused some loss of glycosaminoglycans from 2 μ m cryostat sections of bovine nasal cartilage (fig 1). At times of 20–60 minutes, the glycosaminoglycan loss was significantly increased by the presence of PMNs at 1×10^7 cells/ml ($p < 0.001$; comparison with HBSS treated cartilages). When the cells were stimulated with PMA at 3.2 μ mol/l, a further significant loss of glycosaminoglycans was seen at the same time points ($p < 0.001$; comparison with non-stimulated PMN treated cartilages). By 60 minutes, a comparison with non-treated control cartilages showed that almost 90% of

Figure 1 Effects of polymorphonuclear leucocytes (PMNs) on glycosaminoglycan content of 2 μ m cryostat sections of bovine nasal cartilage. Sections were stained with alcian blue and the dye content was measured by microdensitometry. Values are mean (SEM) ($n=6$). HBSS=Hank's balanced salt solution; PMNs=PMNs at 1×10^7 cells/ml; and PMA=phorbol myristate acetate at 3.2 μ mol/l.

Figure 2 Effects of phorbol myristate acetate (PMA) at 3.2 μ mol/l on the chemiluminescence of polymorphonuclear leucocytes (PMNs) at 5×10^6 cells/ml in the presence of luminol at 5 μ M. Values are mean ($n=3$).

glycosaminoglycans had been depleted from sections treated with stimulated PMNs. Sections treated with PMA alone showed a similar loss of glycosaminoglycan to the HBSS treated sections.

Figure 3 Elastase release into medium from polymorphonuclear leucocytes (PMNs) in the presence or absence of phorbol myristate acetate (PMA) at $3.2 \mu\text{mol/l}$. Values are mean (SEM) ($n=3$).

Figure 4 Microdensitometric values of alcian blue stained $2 \mu\text{m}$ cartilage sections showing glycosaminoglycan content after a one hour treatment. The effects of adding scavengers of reactive oxygen species or an inhibitor of their production to phorbol myristate acetate (PMA) stimulated polymorphonuclear leucocytes (PMNs) are shown. Values are mean (SEM) ($n=6$). HBSS=Hanks's balanced salt solution; PMNs=PMNs at 1×10^7 cells/ml; PMA=PMA at $3.2 \mu\text{mol/l}$; SOD=superoxide dismutase at 60 U/ml ; Cat=catalase at 1000 U/ml ; taurine was used at 2.5 mmol/l ; and DPI=diphenylene iodonium.

Figure 5 Microdensitometric values of alcian blue stained $3 \mu\text{m}$ cartilage sections showing glycosaminoglycan content after a one hour treatment. The effects of adding serine protease inhibitors are shown. ONO-5046 has activity against elastase whereas Foipain is a broad spectrum serine protease inhibitor but lacks activity against elastase. Values are mean (SEM) ($n=6$). NTC=non-treated control; HBSS=Hanks's balanced salt solution; PMNs=polymorphonuclear leucocytes at 1×10^7 cells/ml; and PMA=phorbol myristate acetate $3.2 \mu\text{mol/l}$.

A consequence of PMA stimulation of PMNs is the production of reactive oxygen species. Figure 2 shows luminol enhanced chemiluminescence of PMNs at 5×10^6 cells/ml in the presence or absence of PMA. Chemiluminescence of non-stimulated cells was close to background readings from HBSS (1.3 mV at the peak). Stimulation of cells with PMA resulted in a rapid increase in the chemiluminescent signal, which peaked at four minutes (474 mV) and then rapidly decreased. By 20 minutes the signal was 10% of the peak value.

Figure 3 shows the release of elastase from PMNs. Non-stimulated cells released only low levels of elastase during the 60 minute time course. When these cells were stimulated with PMA, there was a significant increase in enzyme release at 10 minutes ($p < 0.001$; comparison with non-stimulated cells), which became greater at subsequent time points. By 60 minutes, measured enzyme release from stimulated cells was almost 30 times greater than that from non-stimulated cells (0.33 mU/ml/10^7 cells compared with 8.92 mU/ml/10^7 cells).

Figure 4 shows the effect of adding scavengers of reactive oxygen species, or an inhibitor of the metabolic burst, to cartilage sections being treated with stimulated PMNs for 60 minutes. Superoxide dismutase at 60 U/ml , catalase at 1000 U/ml , taurine at 2.5 mmol/l , and diphenylene iodonium at 5 and $10 \mu\text{mol/l}$ did not affect glycosaminoglycan loss from sections treated with stimulated PMNs. When these agents were added alone or in combination with PMA onto sections of cartilage, they resulted in glycosaminoglycan loss similar to HBSS treated cartilages. In addition, at the stated doses, each of these agents quenched or prevented a chemiluminescent response.

Figure 5 shows the effect of two serine protease inhibitors on glycosaminoglycan loss from cartilage sections induced by PMA stimulated PMNs. Foipain is active against a range of serine proteases but lacks activity against elastase. At $100 \mu\text{mol/l}$ there was no protection of the cartilage sections from glycosaminoglycan loss. In contrast, ONO-5046, which has activity against elastase, showed a significant dose-response protection of cartilage sections from 100 nmol/l to $100 \mu\text{mol/l}$.

Figure 6 shows that elastase at 10 mU/ml in HBSS supplemented with Brij can cause glycosaminoglycan loss from cartilage sections.

Figure 6 Microdensitometric values of alcian blue stained $3 \mu\text{m}$ cartilage sections showing glycosaminoglycan content after a one hour treatment. The effect of elastase at 10 mU/ml (in Hanks's balanced salt solution (HBSS) containing 0.001% Brij) is shown. Values are mean (SEM) ($n=6$).

A 40% loss of glycosaminoglycans was seen at 20 minutes ($p < 0.001$), which became greater with time, reaching 63% loss at 60 minutes. In contrast, HBSS/Brij caused only a 9% loss of glycosaminoglycans at 60 minutes.

Discussion

We have devised a novel in vitro method for assessing the direct action of inflammatory cells on cartilage matrix over times which can range from minutes to hours. There are many potential advantages of this system over cartilage coculture experiments which may last for days or weeks. These include a reduction in the risk of in vitro changes to cell function, the use of a defined incubation medium free from serum, the ability to use short lived cells, and the ability to investigate the effects of transient cell processes. This model would also lend itself to a rapid screen of agents that could protect cartilage from exogenous cellular destruction and would require only small amounts of perhaps valuable compound.

This study has shown that when PMNs on the surface of cartilage are activated with PMA in vitro they are able to cause substantial breakdown of cartilage proteoglycan (measured as loss of sulphated glycosaminoglycans). Two consequences of the activation process could give rise to cartilage degradation. The first is the respiratory burst responsible for the production of reactive oxygen species and the second is the release of proteolytic enzymes.

The first event in the respiratory burst is the production of superoxide from a substrate of molecular oxygen and a membrane bound enzyme NADPH oxidase. Superoxide in turn is acted on by the enzyme superoxide dismutase to form hydrogen peroxide. This acts as a substrate for the enzyme myeloperoxidase which produces hypochlorous acid.¹⁵ The hydroxyl radical can be produced by the reaction of superoxide with hydrogen peroxide in the presence of an iron catalyst (Fenton reaction).¹⁶ This species is particularly destructive to biological molecules,¹⁷ but is not thought to be produced in significant amounts by stimulated PMNs.¹⁷ The rate constants for the reactions superoxide to hydrogen peroxide and hydrogen peroxide to hypochlorous acid are such that the main oxidant of PMNs appears to be hypochlorous acid.¹⁸ In addition, lactoferrin, a protein found in the secondary granules of PMNs, is able to chelate free iron in a form which is unable to participate in hydroxyl radical formation.¹⁹

Chemiluminescent studies showed that the production of reactive oxygen species by PMNs stimulated with PMA peaked at four minutes after activation and then decreased rapidly. A number of scavengers of reactive oxygen species, either alone or in combination, were added to PMA stimulated PMNs. These were taurine, catalase, and superoxide dismutase, showing activity against hypochlorous acid, hydrogen peroxide, and superoxide respectively. All these agents were effective in quenching the chemiluminescent measurement. When these scavengers were added to stimulated PMNs in the presence of cartilage, however, proteoglycan

loss occurred to the same extent as in their absence. These data are in agreement with the negative effects of superoxide dismutase and catalase in other models of PMN induced cartilage breakdown,^{19, 20} but in these previous experiments claims could be made that the scavengers were ineffective because they lost activity or that they were excluded from the cartilage/PMN microenvironment. The latter is given credence by the finding that degradation of a fibronectin substrate by PMNs in vitro could occur in the presence of inhibitors when the cells were bound to the substrate.²¹ Schalkwijk *et al* showed that the serine protease inhibitor, α_1 protease inhibitor, afforded no protection of murine patellar cartilage against PMN attack, but that a low molecular mass elastase inhibitor was effective.²⁰ These results were taken to show an exclusion of α_1 protease inhibitor from the cartilage/PMN interface, though inactivation by reactive oxygen species could provide an alternative explanation.²²

To investigate further the role of reactive oxygen species in cartilage degradation, an NADPH oxidase inhibitor, diphenylene iodonium,²³ was used. By blocking the first step in the respiratory burst, this compound effectively prevents the formation of reactive oxygen species. This was confirmed by chemiluminescent measurement. When this compound was used in the cartilage model, cartilage degradation by stimulated PMNs still occurred. This also showed that the degradation of cartilage matrix by stimulated PMNs was due primarily to the release of proteolytic enzymes rather than through the release of reactive oxygen species. Reactive oxygen species may still be implicated in some aspects of cartilage damage in vivo, however, and we are currently investigating possible indirect effects in our laboratory.

Previous experiments using cell free systems for generating reactive oxygen species have shown proteoglycan and cartilage to be susceptible to breakdown.⁹⁻¹¹ The difference between these and the current results suggests that cell free systems for producing reactive oxygen species are not mimicking cellular production in terms of the mixture and concentration of reactive oxygen species produced, their location and control, and are therefore too artificial to allow assessment of the consequences of cellular oxygen metabolites. In the light of this discrepancy other biological conclusions based on the effects of reactive oxygen species in cell free systems should be interpreted with caution.

In this study we have used two serine protease inhibitors in an attempt to identify the enzyme(s) responsible for PMN induced cartilage glycosaminoglycan loss. Foipan is a broad spectrum serine protease inhibitor, but lacks activity against elastase.²⁴ ONO-5046, on the other hand, has selective action against elastase.¹⁴ ONO-5046 but not Foipan afforded significant protection of the cartilage from degradation by PMNs. This is strong evidence that the degradation seen in this model is elastase dependent. Inhibitors of other protease classes have not been included either because of the sensitivity of the assay to vehicle solutions or

due to fears of non-specific reactions, particularly with the metal ion chelators.

Supernatants from stimulated PMNs were unable to cause significant glycosaminoglycan loss from cartilage sections over a one hour incubation (data not shown). This suggests that only in the microenvironment created by the close proximity of PMNs to cartilage can the concentration of protease build up to a level that will result in cartilage glycosaminoglycan loss. Elastase alone at concentrations measured in supernatants of stimulated cells caused cartilage glycosaminoglycan loss, but only after the addition of detergent, which greatly enhances its activity. Nonetheless this shows that elastase is capable of degrading cartilage. Whether elastase has an additional role in activating proenzymes to contribute to cartilage breakdown is not clear from these experiments.

A role for PMNs destroying cartilage matrix *in vivo* can be questioned on the basis that PMN depletion experiments increased the severity of an animal model of arthritis²⁵ and arthritis can be induced in Beige mice which are deficient in PMN elastase and cathepsin G.²⁶ These models show an acute influx of PMNs and fluid exudate, suggesting that the destructive capability of these cells may be countered by protease inhibitors in the exudate. A cell free acute inflammatory exudate will completely inhibit PMN induced cartilage breakdown *in vitro*.²⁷ In rheumatoid arthritis, however, there is an influx of PMNs into synovial fluid in the chronic phase of the disease where there is evidence for oxidative and proteolytic damage to protease inhibitors.²⁸ This presumably accounts for the ability to detect active elastase in some synovial fluids from patients with rheumatoid arthritis.²⁹ Thus PMN elastase may well contribute to cartilage glycosaminoglycan loss in rheumatoid arthritis making it a rational target for therapeutic intervention.

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