Influence of interleukin-1β and hyaluronan on proteoglycan release from equine navicular hyaline cartilage and fibrocartilage

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Proteoglycan (PG) release, in response to recombinant human interleukin-1β (rh-IL-1β), was measured in cartilage explants obtained from the equine distal sesamoid bone (navicular bone). Fibrocartilage from the surface of the navicular bone apposing the deep digital flexor tendon and hyaline cartilage from the surface of the navicular bone articulating with the middle phalanx were labelled with $^{35}SO_4$. Hyaline cartilage from the distal metacarpus was used as a control tissue. Following radiolabel incorporation, the three cartilage types were treated with rh-IL-1β (100 U/mL) in the presence of hyaluronan (0.2, 2, 20, 200 and 2000 μg/mL). rh-IL-1β-Induced PG release was measured by scintillation assay of PG-bound radiolabel. Increases in PG release of 94% ($P < 0.01$), 101% ($P < 0.05$) and 122% ($P < 0.05$), in response to rh-IL-1β, were noted in fibrocartilage, navicular hyaline cartilage and metacarpal hyaline cartilage, respectively. Hyaluronan (0.2 μg/mL) significantly reduced rh-IL-1β-induced PG release in metacarpal hyaline cartilage ($P < 0.01$). In fibrocartilage and navicular hyaline cartilage, hyaluronan did not reduce PG release and at some concentrations appeared to increase PG release, although this was not statistically significant. These experiments show that (i) fibrocartilage and hyaline cartilage of the navicular bone release PGs in response to rh-IL-1β, and (ii) hyaluronan does not prevent rh-IL-1β-induced breakdown of navicular bone cartilage.

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INTRODUCTION

Navicular disease is an important cause of forelimb lameness in the horse. This is a chronic condition that affects the distal sesamoid bone (navicular bone), podotrochlear bursa (navicular bursa) and deep digital flexor tendon (Pleasant & Crisman, 1997). Several theories of the pathogenesis of navicular disease have been proposed, suggesting vasculopathic (Colles, 1979) or biomechanical (Ostblom *et al.*, 1982) origins leading to a degenerative condition of navicular fibrocartilage comparable to that of osteoarthritis of diarthrodial joints of the distal limb of the horse (Doige & Hoffer, 1983; Svalastoga *et al.*, 1983).

The navicular bone consists of a core of cancellous bone that is surfaced with cartilage. The articular surface, which articulates with the head of the middle phalanx, is surfaced with hyaline cartilage as is found covering the articular surfaces of diarthrodial joints, whilst the flexor surface, over which the deep digital flexor tendon runs, is covered by fibrocartilage (Getty, 1975). The navicular bone is an integral functional component of the distal interphalangeal joint (Getty, 1975; Pleasant & Crisman, 1997). Distinction between hyaline cartilage and fibrocartilage is drawn, primarily, on preponderance of collagen fibre subtype; hyaline cartilage contains primarily Type II collagen, whilst fibrocartilage is composed, in major part, by type I collagen (Girdler, 1998; Mueller *et al.*, 1999; PerezCastro & Vogel, 1999). These two cartilage types further differ in constituent proteoglycan (PG) subtypes (Ehlers & Vogel, 1998).

Navicular disease is characterised by degeneration of the fibrocartilage of the flexor surface of navicular bone apposing the deep digital flexor tendon (Doige & Hoffer, 1983; Svalastoga *et al.*, 1983; Pool *et al.*, 1989; Wright & Douglas, 1993). The fibrocartilage demonstrates thinning, fibrillation, loss of PG staining, death of superficial chondrocytes and chondrone formation by adjacent viable cells. These histologic observations are similar to those made in hyaline cartilage in cases of osteoarthritis (Doige & Hoffer, 1983; Svalastoga *et al.*, 1983). However, the hyaline cartilage of the articular surface of the navicular bone rarely exhibits such signs of degeneration in cases of navicular disease (Pool *et al.*, 1989).

Interleukin-1 (IL-1) is a cytokine that has been implicated as a mediator of articular cartilage damage in joint disease. Inhibition

of proteoglycan synthesis by IL-1 has been reported in humans (Arner & Pratta, 1989) and horses (May, 1989; MacDonald *et al.*, 1992; Morris & Treadwell, 1994; Platt & Bayliss, 1994). Furthermore, IL-1 causes breakdown of articular cartilage through induction of metalloproteases (MMPs), although it is uncertain whether this cytokine-induced MMP induction occurs in equine adult cartilage (Platt & Bayliss, 1994). Investigations of potential cytokine-induced changes in PG metabolism of the two cartilage types of the distal sesamoid bone (navicular bone) of the equine limb have not been reported.

Hyaluronan (sodium hyaluronate) is used in equine medicine as an antiarthritic drug and is licensed for both intravenous and intra-articular administration (Veterinary Data Sheet Compendium, 1999–2000). It is indicated for the treatment of acute sterile synovitis and degenerative joint disease (DJD) (McIlwraith, 1989; Kawcak *et al.*, 1997). Hyaluronan possesses a diverse range of anti-inflammatory effects; it has been shown to inhibit PGE_2 synthesis in equine synoviocyte cultures (Frean & Lees, 2000) and to lower synovial fluid PGE_2 concentrations in horses with experimentally induced joint disease (Kawcak *et al.*, 1997). Further, chondroprotective qualities have been ascribed to hyaluronan because of its effects on cartilage proteoglycan (PG) synthesis (Frean *et al.*, 1999) and breakdown. Hyaluronan reduces PG release induced by a variety of mediators (Homandberg *et al.*, 1994; Iwata, 1994; Homandberg *et al.*, 1998), including IL-1. Bovine cultured cartilage explants treated with IL-1 showed accelerated PG release that was abrogated by hyaluronan (Morris *et al.*, 1992).

Few studies of the normal and pathological metabolism of navicular bone cartilage have been undertaken; equine rheumatology has been more focused on the articular cartilage of diarthrodial joints. In view of the suggestion that navicular disease might be a degenerative disease of navicular fibrocartilage (Doige & Hoffer, 1983; Svalastoga *et al.*, 1983), it was considered pertinent to investigate IL-1-induced PG release from navicular hyaline cartilage and fibrocartilage, as well as from articular hyaline cartilage from a diarthrodial (metacarpophalangeal) joint frequently affected by DJD.

Similarly, investigations of hyaluronan have focused on its effects on the metabolism of articular, rather than navicular, cartilage despite hyaluronan having been proposed as a potential treatment for navicular disease (Turner, 1989; Trotter, 1991). We investigated the effects of hyaluronan on IL-1-induced PG release from navicular hyaline cartilage, fibrocartilage and articular hyaline cartilage.

MATERIALS AND METHODS

Selection of material

This experiment was repeated once; each experiment used tissue from the metacarpophalangeal joints and forelimb navicular bones of one animal. Tissue was obtained within 2 h of death from adult horses less than 7 years old (based on examination of dentition). Cartilage was only used if, on examination of the distal limb joints, ligaments and tendons, no macroscopic signs of musculoskeletal disease, either acute or chronic, were evident. Criteria for selection were as follows: articular and navicular cartilage did not show erosion or wear lines; synovial membrane did not exhibit signs of acute or chronic inflammation; periarticular osteophytes were absent; synovial fluid of the metacarpophalangeal and distal intercarpal joints was normal in quantity, viscosity and colour, i.e. no blood was present; and there was no evidence of intra- or periarticular fractures.

Cartilage harvesting

Metacarpophalangeal joint: using sterile instruments, the metacarpophalangeal joint was opened through a lateromedial incision of the collateral ligaments, overlying extensor tendons and dorsal joint capsule. Cartilage was harvested from the articular surface of the distal metacarpus; a skin biopsy punch (4 mm diameter) was used to define cartilage discs which were then removed from the underlying bone using a scalpel and forceps. *Navicular bone*: an incision was made through the extensor tendons and the dorsal aspect of the distal interphalangeal joint. This incision was continued through the lateral and medial collateral ligaments and palmar joint capsule to remove the second phalanx and expose the navicular bone. The lateral and medial collateral sesamoidean ligaments and the unpaired distal sesamoidean ligament were then sectioned to free the navicular bone from the first and third phalanges, respectively. The entire navicular bone was transferred to culture medium and discs of cartilage harvested as described above; hyaline cartilage was obtained from the dorsal surface of the navicular bone, whilst fibrocartilage was harvested from the flexor (palmar) surface. Explants were weighed and placed in 48-well culture plates (one explant per well) in 1 mL Dulbecco's Modified Eagle Medium (DMEM) supplemented with foetal calf serum (FCS) (10% v:v) and penicillin/streptomycin (1% v:v) and kept in standard culture conditions for 24 h.

Pulse-chase protocol

Twenty-four hours after harvesting, a medium change was made with addition of $Na_2{}^{35}SO_4$ (5 μCi/well). Explants were incubated in FCS/$^{35}SO_4$-supplemented medium for 48 h, following which they were washed twice in fresh medium to remove unincorporated radioisotope and placed in DMEM supplemented with penicillin/streptomycin but not FCS.

Treatment with rh-IL-1β in presence of hyaluronan

Prelabelled articular cartilage, navicular hyaline and navicular fibrocartilage explants were treated with rh-IL-1β in the absence or presence of a hyaluronan preparation (Hyonate®, Bayer Animal Health, Bury St. Edmunds, UK) licensed for therapeutic use in horses in the UK. Treatment groups were as follows: untreated, rh-IL-1β (100 U/mL), and rh-IL-1β (100 U/mL) plus hyaluronan (0.2, 2, 20, 200 and 2000 μg/mL). Hyaluronan dilutions were prepared in DMEM. Explants were incubated under treatment conditions for 48 h, following which culture medium was

harvested and frozen at −20 °C until assayed for ^{35}sulphated PGs released in response to rh-IL-1β.

Assay of PG release

Culture medium was eluted over Sephadex G-25 M gel (PD-10 columns; Pharmacia) using guanidine-HCl/Na-acetate as eluent. Eluate was collected in 0.5 mL fractions in scintillation vials for β-activity assay following addition of scintillation fluid. PG samples were found to pass through PD-10 columns completely in a total eluent volume of 12 mL; thus 30 0.5 mL eluate fractions were collected.

Correction for DNA content

PG release was corrected for explant cellularity, measured as explant DNA content using the bisbenzimidazole fluorescent dye (Hoechst 33258) technique (Kim *et al*., 1988). Following guanidine HCl/Na-acetate extraction, cartilage explants were digested with papain to release chondrocyte DNA. Explants were placed in 1 mL Eppendorf tubes in 1 mL of sodium acetate buffer (0.1 M; pH 5.8) containing EDTA (2.4 mM) and L-cysteine (5 mM). Papain was added to an activity of 10 U/mL. Samples were incubated at 60 °C for 6 h on a heating block. Following papain digestion, 100 μL aliquots of digest solution were mixed with 2 mL of dye solution. Fluorescence of samples was determined using a fluorimeter with excitation and emission wavelengths set at 365 nm and 458 nm, respectively. Absolute DNA concentrations of samples were determined by comparison with known DNA standards.

Statistical analysis

In each experiment, four cartilage explants comprised each treatment group; data from both experiments were combined, hence $n = 8$. Statistical analysis was conducted using a proprietary statistical software package. Data were analysed by one-way analysis of variance, followed by a pairwise comparison using Fisher's multiple range test. *P*-values of 5% and less were interpreted as statistically significant.

RESULTS

Articular hyaline cartilage, navicular hyaline cartilage, and navicular fibrocartilage showed basal levels of release of PG-bound $^{35}SO_4$. Compared to unstimulated controls, all three cartilage types demonstrated enhanced release of PG-bound $^{35}SO_4$ when treated with rh-IL-1β.

Metacarpal hyaline cartilage

In control explants there was a basal level of release of PG-bound $^{35}SO_4$. A concentration of rh-IL-1β of 100 U/mL increased PG release by 122% ($P < 0.05$) (Table 1). Cytokine-stimulated explants treated with hyaluronan (0.2, 2, 20 and 200 μg/mL) exhibited decreases (between 14% and 59%) in PG release compared to explants treated with rh-IL-1β alone, although statistical significance was only shown at a hyaluronan concentration of 0.2 μg/mL ($P < 0.01$). The highest concentration of hyaluronan (2000 μg/mL) reduced PG release to below the basal level of PG release of control explants. (Fig. 1).

Table 1. Effect of rh-IL-1β on PG release by equine cartilage explants

Cartilage Type	PG release (CPM/μg DNA)		% increase in PG release vs Control
	Control	rh-IL-1β (100U/mL)	
Metacarpal Hyaline	2223±143	4955±1127	122*
Navicular Fibrocartilage	1544±144	3088±252	101*
Navicular Hyaline	977±106	1898±249	94**

*$P<0.5$, **$P<0.1$; Fisher's multiple range test. Each treatment group represent 8 replicate wells±SEM.

Navicular fibrocartilage

Unstimulated explants showed a basal level of release of PG-bound $^{35}SO_4$ that was significantly enhanced by rh-IL-1β; a 101% increase in PG release was obtained ($P < 0.05$) (Table 1). Hyaluronan demonstrated no consistent effect in altering cytokine-induced PG release; compared to rh-IL-β alone, hyaluronan (0.2 and 2000 μg/mL) with rh-IL-1β yielded respective decreases of 6% and 15% in PG release, whilst hyaluronan (2, 20 and 200 μg/mL) with rh-IL-1β increased PG release by 21, 68 and 28%, respectively. The concentration-response relationship for hyaluronan appeared to be bell-shaped (Fig. 2).

Navicular hyaline cartilage

Compared to unstimulated explants, rh-IL-1β (100 U/mL) significantly increased PG-bound $^{35}SO_4$ release by 94% ($P < 0.01$)

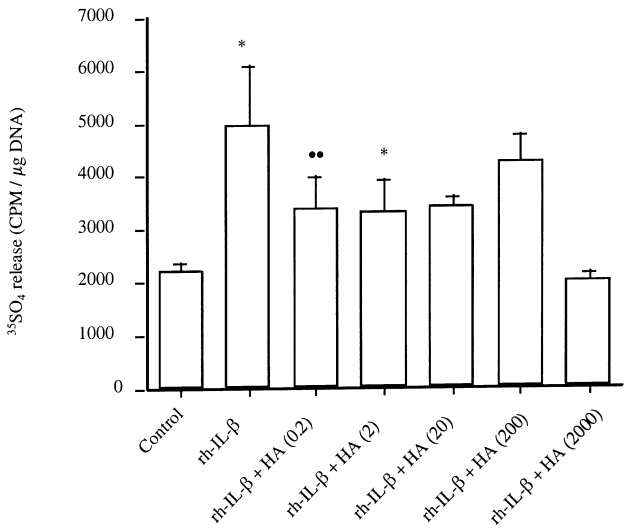

Fig. 1. Effect of hyaluronan on PG release by metacarpal hyaline cartilage treated with rh-IL-1β. Each treatment group represents mean of 8 replicate wells ± SEM. Hyaluronan (HA) concentrations are expressed in μg/mL, rh-IL-1β concentration is 100 U/mL throughout. *$P < 0.05$ vs. control; **$P < 0.01$ vs. rh-IL-1β; Fisher's multiple range test.

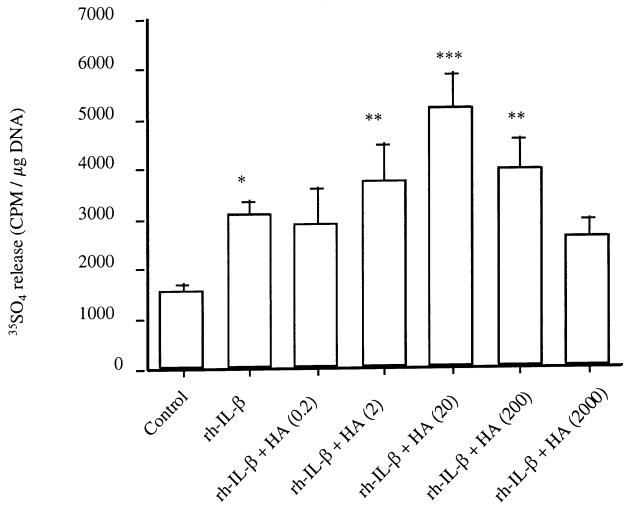

Fig. 2. Effect of hyaluronan on PG release by navicular fibrocartilage treated with rh-IL-1β. Each treatment group represents mean of 8 replicate wells ± SEM. Hyaluronan (HA) concentrations are expressed in μg/mL, rh-IL-1β concentration is 100 U/mL throughout. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control; Fisher's multiple range test.

(Table 1). Explants treated with cytokine and hyaluronan also demonstrated significantly enhanced PG release, although this was not related to hyaluronan concentration; compared to unstimulated controls, increases in PG release of between 5 and 105% were obtained (Fig. 3).

DISCUSSION

The consistent effect of increased PG-bound $^{35}SO_4$ release from explants treated with rh-IL-1β, obtained with the three types of equine cartilage used in this study, suggests that this cytokine exerts a catabolic effect on equine cartilage, consistent with its degradative effects on cartilage of other species. The exact mechanism of this degradative effect on PG was not investigated in this work, but much experimental evidence in other species suggest that the effects of IL-1 are attributable largely to enhanced expression of metalloproteinases by chondrocytes (Dayer *et al.*, 1986; Lefebvre *et al.*, 1991).

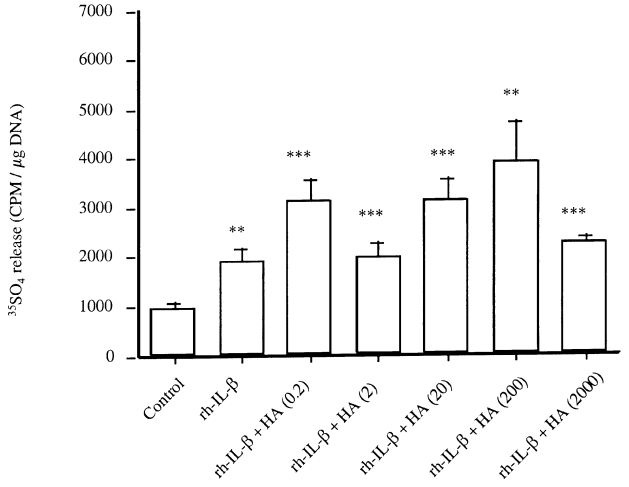

Fig. 3. Effect of hyaluronan on PG release by navicular hyaline cartilage treated with rh-IL-1β. Each treatment group represents mean of 8 replicate wells ± SEM. Hyaluronan (HA) concentrations are expressed in μg/mL, rh-IL-1β concentration is 100 U/mL throughout. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control; Fisher's multiple range test.

The considerable variation, between replicate explants, in PG release that was noted in all three equine cartilage explant types treated with IL-1 is similar to that occurring in bovine articular cartilage explants; mean increases in release of PG, in response to Il-1β, of 163% with a range between 59% and 422% have been reported (Morris *et al.*, 1992). In each of our experiments replicate explants were chosen randomly from pooled cartilage harvested from the metacarpophalangeal joints and forelimb navicular bones of the forelimbs of a single animal. The possibility exists that this variation reflects interlimb differences in cartilage matrix metabolism; however, this is unlikely as variation in basal release of $^{35}SO_4$ from unstimulated replicates was narrow. Variation might also be ascribed to varying sensitivity of chondrocytes, from different zones of the joint or navicular bone, to the action of rh-IL-1β. Differences in the rate of matrix synthesis and breakdown have been demonstrated in cartilage obtained from different areas of the same joint (Murray *et al.*, 1996). Variation in metabolic activity has also been described in chondrocytes from different depths of articular cartilage (Mitrivic & Darmon, 1994; Hayashi & Jasin, 1995).

These experiments indicate a possible chondroprotective effect of hyaluronan on equine diarthrodial hyaline cartilage through inhibition of IL-1-induced PG breakdown. However navicular hyaline cartilage and fibrocartilage failed to respond to hyaluronan in the same manner. These differences might be explained by interjoint and intrajoint regional differences in chondrocytes' response to osteoarthritic mediators and matrix synthetic capacity (Mitrivic & Darmon, 1994; Little *et al.*, 1996; VanOsch *et al.*, 1998; Arokoski *et al.*, 1999). It is possible that such differences might extend to the tissues' response to hyaluronan.

These results are similar to those of experiments investigating the effects of hyaluronan (Larsen *et al.*, 1992; Morris *et al.*, 1992) and Hylan (a cross-linked hyaluronan derivative) (Larsen *et al.*, 1992) on IL-1-induced PG release in bovine articular cartilage explants. Morris *et al.* (1992) reported a reduction in proteoglycan release from bovine articular cartilage explants, in response to hyaluronan, in both unstimulated controls and cytokine-stimulated explants. Reductions in cytokine-induced PG release were noted at hyaluronan concentrations of 0.5, 1 and 1.5 mg/mL, that is, at concentrations in the same range as those used in this study (Morris *et al.*, 1992). Larsen *et al.* (1992) similarly observed decreased release of $^{35}SO_4$ from IL-1-stimulated bovine cartilage explants treated with hyaluronan (2 mg/mL) and Hylan (2 mg/mL). Hylan was also noted to reduce $^{35}SO_4$ release from explants exposed to oxygen-derived radicals.

These experiments are, to our knowledge, the first investigations of PG metabolism of either navicular fibrocartilage or navicular hyaline cartilage. Navicular cartilage, both hyaline and fibrocartilage, responded in a manner similar to that of diarthrodial joint hyaline cartilage with regard to rh-IL-1β-induced

PG release. In the context of the suggestion that navicular disease is an osteoarthritis-like condition, these results raise several points of discussion. A proposed initiating factor of diarthrodial joint osteoarthritis is inflammation of the synovial membrane. It is possible that a similar synovial involvement might explain or contribute to the genesis of navicular disease, as the flexor surface of the navicular bone lies adjacent to an enclosed synovial structure, the navicular bursa. Indeed, inflammation of the navicular bursa has been described in cases of navicular disease. Demonstration of IL-1 activity in fluid obtained from the navicular bursa of affected horses might confirm the *in vitro* observations of this hypothesis that navicular fibrocartilage breakdown is, at least in part, IL-1-dependent.

The hyaline cartilage of the articular surface of the navicular bone also lies within a synovial joint, the distal interphalangeal (coffin) joint. Our results demonstrate that navicular hyaline cartilage responds in the same way as diarthrodial hyaline cartilage to IL-1. Indeed in cases of osteoarthritis of the distal interphalangeal joint, degenerative changes to the phalangeal articular cartilage are often mirrored in damage to navicular bone hyaline cartilage.

Despite the close proximity to each other of the navicular bone synovial spaces and cartilage surfaces, damage to the hyaline cartilage is rarely seen in navicular disease. Similarly osteoarthritis of the distal interphalangeal joint is not usually associated with navicular disease (although the two syndromes can occur concurrently). If it is indeed assumed that navicular disease is an osteoarthritis-like condition, initiated by synovitis, it would appear that the factors initiating inflammation of the navicular bursa are different to those initiating synovitis of diarthrodial joints.

It is apparent from these results that navicular fibrocartilage, like articular cartilage of diarthrodial joints, can be induced to release PG in response to a mediator of osteoarthritis, i.e. IL-1. These biochemical characteristics lend support to the histological evidence of others that this disease has osteoarthritic properties.

This work suggests that hyaluronan does not prevent cytokine-induced PG release from navicular cartilage, unlike its effect on articular cartilage of a diarthrodial joint. Based on this evidence, hyaluronan would appear not to be an appropriate therapeutic for navicular disease.

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