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The effect of extracellular pH on matrix turnover by cells of the bovine nucleus pulposus

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Abstract It has long been known that very acidic conditions can be found in degenerate discs. The effect of these acid conditions on matrix turnover are, however, unknown. This study aimed to examine the effect of acidity on production of matrix components and on agents which break down the matrix in order to gain insight into the effect of pathological values of pH on matrix turnover. Cells were isolated from the nucleus of bovine discs and from bovine articular cartilage, embedded in alginate beads and cultured at pH levels maintained within the ranges seen in normal and pathological discs: pH 7.4–pH 6.3 for 48 h. Rates of sulphated glycosaminoglycan (GAG) and protein synthesis were measured, as well as rates of production of some agents involved in matrix breakdown, i.e. total and activated matrix metalloproteinases (MMPs) and their inhibitors (TIMPs). The results showed that acid conditions had a profound effect on cell matrix turnover; at pH 6.4, total production of most species measured was inhibited by more than 50% compared to production at pH 7.2; production of sulphated GAGs and of TIMP-1 fell by >90%. However production of active metalloproteinases by disc cells was relatively insensitive to pH, with activity at pH 6.3 not statistically different from that at pH 7.2. These findings indicate that exposure to acid conditions appears particularly deleterious for the disc matrix, as it inhibits the disc cells from synthesising functionally important molecules such as the sulphated GAGs but does not prevent the production of agents able to degrade matrix components. The low values of pH seen in some degenerate discs are thus likely to be involved in breakdown of the disc matrix.

Keywords Extracellular pH · Sulphate incorporation · Metalloproteinases · Articular chondrocytes · Lactic acid

Introduction

Nucleus pulposus cells are solely responsible for the production and maintenance of the extracellular matrix of the nucleus throughout life [31]. The cells have no direct contact with each other and rely on cell-matrix interactions and signals from the environment to maintain the function of the tissue. The cells synthesize all the matrix components [10, 37, 38, 50] and also produce degradative enzymes including metalloproteinases (MMPs) and their inhibitors (tissue inhibitors of metalloproteinases or TIMPs), involved in matrix turnover [7, 45, 53]. The fine balance between synthesis of components and the breakdown of tissue determines the composition of the matrix; tissue integrity is thus dependent on the activity of the nucleus pulposus cells [53]. In vivo [20, 43] and in vitro [14] environmental factors such as mechanical stress [30, 34] and nutrient levels have been found to affect matrix composition, presumably by affecting macromolecule biosynthe-

sis and production of proteases. Matrix acidity is another important factor governing matrix production; a fall in matrix pH has been shown to modify protein and proteoglycan synthesis by intervertebral disc explants [39] as in other cartilages [12]. How such factors alter the production and activity of degradative enzymes is, however, relatively unexplored.

Nucleus pulposus cells in situ, like those of other cartilaginous tissues, exist in an extracellular environment where the pH of the interstitial fluid is more acidic than that of most other tissues. Several factors contribute to the acidic pH. Firstly, the tissues carry a high fixed negative charge due to the presence of many COO^- and SO_4^{2-} groups on the constituent glycosaminoglycan (GAG) chains of the proteoglycans. The fixed negative charge attracts high levels of free cations including Na^+, K^+, and H^+ ions, in accordance with the Gibbs-Donnan equilibrium [32]. The H^+ ion concentration within the cartilaginous matrix is thus always greater than in its surroundings, and consequently pH is on average 0.5 pH units lower than that of surrounding serum or synovial fluid [12, 32]. In addition, the disc is exposed to large and varying loads; 20–25% of fluid from the disc is expressed and regained during a normal diurnal cycle [4]. During fluid expression, proteoglycans are concentrated; the consequent rise in fixed charge density and H^+ concentration increases tissue acidity. Secondly, both articular cartilage and intervertebral disc are avascular and therefore steep gradients of metabolites develop within these tissues [48]. Since metabolism of the cells is largely by anaerobic glycolysis (up to 95% of all glucose metabolism), large amounts of lactic acid are produced [17, 20]; lactic acid diffusion across a large thickness of dense matrix (up to 8 mm for adult lumbar discs) is slow, resulting in elevated acidity within the matrix and around the cells, particularly in the centre of the disc [16, 47]. Thirdly, and to a lesser extent, acid extrusion mechanisms will acidify the pericellular matrix further. The maintenance of a constant intracellular pH is of paramount importance for cell viability and function; therefore, the disc cells possess acid extruding proteins of which the $Na^+ \times H^+$ exchanger is the most prominent [44]. These mechanisms are all thought to maintain the extracellular environment in disc and cartilage at pH 7.2–pH 6.9 [12]. Disturbances in lactic acid transport or lactic acid metabolism can, however, increase lactic acid concentrations substantially [2, 15] and influence extracellular acidity; values of pH below pH 6.5 have been measured in degenerate discs [9, 25].

In this study we examined the effect of acidity on production of matrix components and on agents that break down the matrix, in order to gain insight into the effect of pathological values of pH on matrix turnover. We compared the behaviour of nucleus pulposus cells to that of articular chondrocytes as, although in many respects the cellular environment of these two cell types is similar, the disc cells are routinely exposed to more acidic values of extracellular pH in vivo [13, 24]. We used isolated cells rather than the explants used in previous studies [12, 39], because in explant cultures, extracellular pH is modified by matrix properties, varies throughout the explant and is difficult to control [5]. The effects of pH were assessed on some species involved in matrix turnover; proteoglycan and protein synthesis were determined using radiotracer incorporation, enzymes involved in matrix degradation, (MMPs) and their inhibitors (TIMPs) were assessed using fluorescent substrate cleavage, MMP-2 and -9 were measured by gelatin zymography, TIMP-1 and -2 activities were detected using an immunoassay kit and ELISA system respectively.

Materials and methods

Cell isolation

For these tests, we used bovine tail intervertebral discs and bovine metacarpal phalangeal articular cartilage from 18- to 24-month steers. These discs were chosen as a model for human discs as they are easily available, carry mechanical load [21] and have been shown to be similar to human discs in composition, tissue properties and matrix biosynthesis [40] and in cell type [18]. Twenty-four tails and 18 metacarpal-phalangeal joints were used for the tests reported here.

All dissections were carried out aseptically. For each experiment, the upper five intervertebral discs from each tail were carefully exposed and a square removed in the central gelatinous section of the disc, the nucleus pulposus. Great care was taken not to cut into the 'inner annulus', which is arbitrarily defined as the tissue containing ill-organised lamellae, between the gelatinous nucleus and the fibrous organised rings of the outer annulus. The dissected nucleus was then placed in a 25 ml tissue culture flask containing Dulbecco's Modified Eagles Medium (DMEM) (1 g l^{-1} glucose, 25 mM HEPES, no $NaHCO_3$; Life Technologies, Paisley, UK), supplemented with antibiotics/antimycotics (500 units ml^{-1} penicillin G, 500µg ml^{-1} streptomycin sulphate and 25µg ml^{-1} amphotericin B; Life Technologies, Paisley, UK). Articular cartilage was shaved from the opened joint as described previously [44] and similarly placed in DMEM supplemented with antibiotics in 25 ml tissue culture flasks. The dissected tissues were then enzymatically digested as previously described [22] at 37°C in DMEM containing 1% (vol/vol) antibiotic/antimycotic solutions with 1 mg ml^{-1} collagenase (collagenase 1, Sigma Chemical Co., Poole, Dorset, UK) under 95% air/5% CO_2 for 18–20 h. After incubation, the digested tissue suspension was filtered to isolate the cells, which were then washed three times by repeated centrifugation (1000 g for 5 min) and resuspended in DMEM. The cell suspension was assessed microscopically for cell viability using trypan blue exclusion and cell number. Only cell preparations with cell viabilities >95% were then used.

Alginate bead culture

Isolated cells were encapsulated in alginate beads (sodium alginate; Fluka Chemicals, Poole, UK) at a density of 4.10^6 cells ml^{-1} as described previously [18] and cultured in 24-well culture plates (five beads per well) [31]. The wells contained 2 ml of DMEM held at controlled pH, 3% Insulin Transferase Supplement (ITS+3; Sigma Chemical Co., Poole, UK) [6], antibiotics/antimycotics (0.5% antibiotic/antimycotic, 0.5% gentamycin) and 50µg ml^{-1} ascorbate (Sigma Chemical Company, Poole, UK). The pH of the medium

was altered in the range pH 6.3–pH 7.5 by adding $NaHCO_3$ and HCl as required. The multi-well plates were then incubated for 48–96 h at 37°C in a humidified atmosphere of 95% air/5% CO_2. The medium was changed every 2 days. For each value of pH, beads from one well (five beads) were resuspended in 0.5 ml of citrate buffer (0.15 M NaCl, 55 mM Na citrate, pH 7.4) to depolymerise the alginate and the cells counted using a haemocytometer; viability was assessed using trypan blue exclusion. The remaining wells were used for biochemical assessment, with each assay carried out in triplicate in each experiment.

Matrix synthesis rates

^{35}S-sulphate incorporation

Synthesis of sulphated glycosaminoglycans (GAG) was determined from ^{35}S-sulphate incorporation [33] determined as described previously [18]. Briefly, after 48 h at controlled pH, beads were resuspended in 0.5 ml of 25 mM HEPES-buffered DMEM held at their incubation pH, supplemented with 5μCi ml^{-1} of ^{35}S-sulphate (Amersham Pharmacia Biotech, Little Chalfont, UK) and incubated for a further 4 h at 37°C and 5% CO_2. Radiolabelling was stopped by removing the beads from the medium and washing them twice in ice-cold PBS supplemented with 2 mM $CaCl_2$, 5 mM Na_2SO_4 at 4°C. The beads and incubation medium were frozen at –20°C until processed. The bound and free ^{35}S-sulphate radiolabels were separated by dialysis after disaggregating the beads in a citrate buffer [18]. When the dialysate was at background levels, the tube contents and the tubing were placed into a scintillation vial with 4 ml of scintillant, and the activity present in the GAGs synthesised by the cells and in the culture medium was measured on a beta scintillation counter.

Rates of proteoglycan synthesis were calculated assuming that the specific activity of ^{35}S-sulphate was the same in the medium and incorporated proteoglycans, and could be expressed by:

$$R = \frac{\frac{P}{M} \times \frac{S}{T}}{C}\left(\frac{\text{nmol sulphate}}{\text{million cells} \times \text{hour}}\right)$$

Where:
- P = the activity (counts per minute) of ^{35}S-sulphate incorporated into proteoglycans by the cell
- M = activity of total ^{35}S-sulphate in culture medium
- S = concentration of non-radioactive sulphate in culture medium (nM)
- T = labelling period in hours
- C = number of cells (million) in sample

^{3}H-leucine incorporation

Leucine is an essential amino acid and can be used as a general marker of protein synthesis. Measurement of its rate of incorporation was carried out in a similar manner to that described for ^{35}S-sulphate. Briefly, after 48 h at controlled pH, beads were resuspended in 0.5 ml of 25 mM HEPES-buffered DMEM held at their incubation pH, supplemented with 10μCi ml^{-1} of ^{3}H-leucine. Radiolabelling was stopped by washing the beads twice in ice-cold PBS supplemented with 2 mM $CaCl_2$, 5 mM Na_2SO_4 at 4°C. The beads and medium were frozen at –20°C until dialysed as above to separate the bound and free ^{3}H-leucine radiolabel, and their activity determined as described above. The leucine incorporation rate was calculated from the above equation, except here activities referred to ^{3}H-leucine and S refers to the concentration of non-radioactive leucine in the culture medium.

Measurements of matrix metalloproteinase activity

Coumarin-labelled peptide

MMP proteolytic activity in conditioned medium obtained from culturing cells for 48 h was determined using a fluorimetric method [27]. Briefly, 150 μl of conditioned medium was incubated for 2 h at 37°C with 425 μl assay buffer (200 mM Tris HCl, 20 mM $CaCl_2$, pH 7.8) and substrate (coumarin-labelled peptide, Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2, Calbiochem-Novabiochem, Nottingham, UK) added to a final concentration of 6.4 μM. The assay mixture (i.e. buffer plus medium sample but no substrate) was used as a blank. Total MMP activity was determined by adding 1 mM p-aminophenyl mercuric acetate (APMA; Sigma Chemical Co., Poole, UK) [3] to the assay mixture and incubating 1 h at 37°C prior to addition of the MMP substrate. The assay mixture plus 1 mM APMA stock without substrate was used as a blank.

After incubation with MMP substrate, the reaction was stopped by addition of 600 μl 3% acetic acid. The fluorescence intensity of samples was then measured immediately on a fluorimeter (Perkin-Elmer LS30, excitation wavelength 320 nm, emission wavelength 405 nm). A standard curve using the free fluorescent product (Mca-Pro-Leu-OH; Calbiochem-Novabiochem, Nottingham, UK) was used to calibrate the readings by preparing serial dilutions of the control compound in assay buffer and 3% acetic acid over the concentration range of 3.125–3200 nM.

Gelatin zymography

Matrix metalloproteinase activity was also measured by gelatin zymography (which allows measurement of gelatinase activity; MMP-2 and MMP-9) and can detect both the active and pro-forms of the enzymes at picogram quantities [26]. Gelatin zymography was carried out on conditioned medium obtained from 48 h incubation of cells in alginate beads, concentrated by centrifugation at 4°C using Microcon-10 ultrafiltration units (Microcon, Millipore Corp., Bedford, USA). Gelatinase activity was examined using 7.5% polyacrylamide gels containing 0.05% gelatin. Gel electrophoresis of samples was carried out under denaturing conditions [28] in a Mini-Protean II vertical electrophoresis apparatus at 150 V and 4°C for 40–60 min. MMP-2 and MMP-9 standards (Calbiochem-Novabiochem, Nottingham, UK) were run alongside samples, as molecular markers to calibrate the gels. The gels were then washed and incubated at 37°C and shaken gently overnight in a buffer (50 mM Tris-HCl, 200 mM NaCl, 5 mM $CaCl_2$ and 0.02% Brij-35; pH 7.8). The gels were then stained for 30 min in 0.1% (w/v) Coomassie brilliant blue in 45% (w/v) methanol, 10% (w/v) acetic acid, followed by destaining for 2 h in 10% (w/v) methanol, 7.5% (w/v) acetic acid. The gels were then dried overnight and scanned.

TIMP immunoassays

TIMP-1 levels were measured using a TIMP-1 immunoassay kit (Chemicon International, Chandlers Ford, Hampshire, UK; catalogue no. ECM496) for the measurements of human and bovine TIMP-1 levels with a sensitivity of 1.2 ng ml^{-1}. The assay was carried out as directed by the manufacturers' instructions on conditioned media obtained from culturing cells embedded in alginate for 48 h and concentrated by centrifugation.

TIMP-2 levels were measured using a TIMP-2, human ELISA system (Amersham Pharmacia Biotech, Amersham, UK; catalogue no. RPN 2618) for the measurements of human and bovine TIMP-2 levels with a sensitivity of 3 ng ml^{-1}. The assay was carried out as directed by the manufacturers instructions on conditioned media obtained from culturing cells embedded in alginate for 48 h and concentrated by centrifugation.

Statistical analysis

Unless otherwise stated, data are presented as the mean ± the standard error of the mean (SEM) of at least three separate experiments ($n \geq 3$). In each experiment, each condition tested was carried out in triplicate. Significant differences were determined using Student's unpaired *t*-test.

Results

Effect of extracellular pH on rates of tracer incorporation

Table 1 shows the rates of ^{35}S-sulphate incorporation by articular chondrocytes and intervertebral disc cells cultured in alginate beads. The rate of sulphated GAG production by nucleus pulposus cells was circa four times greater than that of articular chondrocytes.

Table 1 ^{35}S-sulphate incorporation rates for freshly isolated articular chondrocytes and nucleus pulposus intervertebral disc cells cultured in alginate bead culture for 48 h at pH_o 7.4. Results are given as mean±SEM for *n* separate experiments (**P<0.01, nucleus cells statistically different from articular chondrocytes)

Cell type	^{35}S-sulphate incorporation rate [pM (1×10^6 cells)$^{-1}$ h^{-1}]
Articular chondrocytes	83.9±15.5 (n=6)
Nucleus pulposus cells	308.2±35.7 (n=9)**

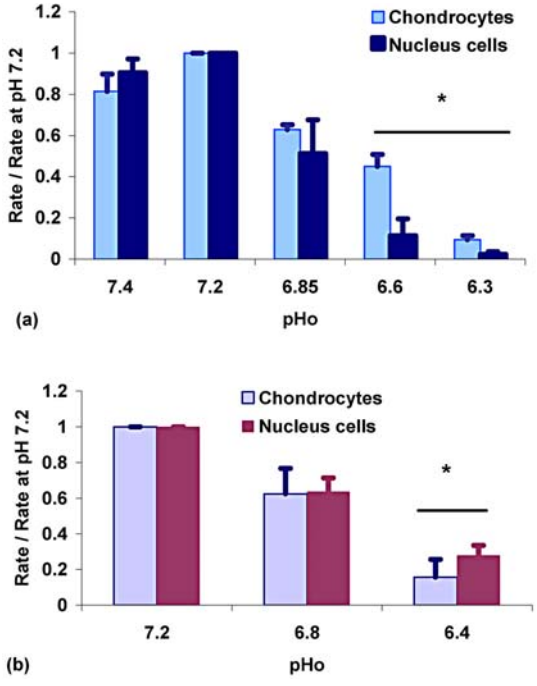

Fig. 1 Rates of incorporation, normalised to rates at pH 7.2, are shown for **a** ^{35}S-sulphate incorporation into sulphated GAGs and **b** ^{3}H-leucine incorporation into proteins. Chondrocytes and nucleus cells were cultured in alginate beads for 48 h in medium at varying pHs prior to radiolabelling for 4 h. Results are given as mean± SEM (n=6) (*P<0.05; statistically different from articular chondrocytes)

Figure 1a illustrates the effect of acidifying the extracellular medium on the rate of ^{35}S-sulphate incorporation. Results are shown for freshly isolated articular chondrocytes and nucleus pulposus cells cultured in alginate beads for 48 h in medium whose extracellular pH was maintained in the range pH 7.4–pH 6.3. Rates were normalised to control values at pH 7.2 for each experiment, as there were large animal-animal differences in rates, as seen previously [29]. The rate of synthesis showed a biphasic response to extracellular pH for both disc cells and chondrocytes. The maximum rate was seen at pH 7.2, where the rate was 10–20% higher than at pH 7.4. With fall in pH, rates of synthesis decreased for both cell types; however, acidification inhibited GAG production by nucleus pulposus cells more than that by articular chondrocytes. For instance, at pH 6.6, synthesis decreased by 80% and 50% relative to the maximum (pH 7.2) for nucleus pulposus and articular chondrocytes respectively.

Acidifying the extracellular environment, as shown in Fig. 1b, also significantly inhibited protein synthesis for both articular chondrocytes and nucleus pulposus cells, as shown by its effect on the rates of ^{3}H-leucine incorporation. However, the inhibitory effect of pH on ^{3}H-leucine incorporation was smaller than that on ^{35}S-sulphate incorporation; at pH 6.8 for instance, rates for nucleus cells fell by circa 38% and 50% respectively (Fig. 1a,b).

Effect of extracellular pH on MMP activity

Table 2 shows the MMP activities in the conditioned medium obtained from the culture of nucleus pulposus cells and articular chondrocytes in alginate beads at pH 7.2 for 24 h. The activities per million cells were very similar for both cell types.

Figure 2 illustrates MMP activity in conditioned medium for both nucleus pulposus disc cells and articular chondrocytes pre-incubated for 48 h in alginate beads over a range of extracellular pH levels. The medium was changed after 24 h and conditioned media collected for the final 24 h. The results were normalised to measured activity at pH 7.2 for each individual experiment. MMP activity for both cell types was maximum at pH 7.2; a bi-

Table 2 Matrix metalloproteinase (MMP) activity in conditioned medium from articular chondrocytes and nucleus pulposus intervertebral disc cells cultured in alginate beads for 48 h, measured using a coumarin dye assay [28]. Results are given as mean±SEM ($n \geq 4$)

Cell type	MMP activity [nM (1×10^6 cells)$^{-1}$ h^{-1}]
Nucleus pulposus cells	1.068±0.160 (n=6)
Articular chondrocytes	1.041±0.075 (n=4)

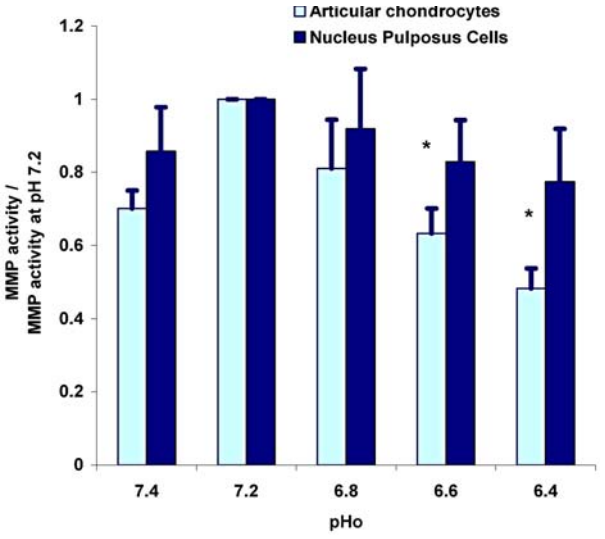

Fig. 2 Effect of extracellular pH on matrix metalloproteinase (MMP) activity in conditioned medium from bovine articular chondrocytes and nucleus pulposus cells cultured in alginate beads for 24 h at controlled pH levels. MMP activity was assayed using a fluorescent coumarin dye [27] and activities normalised to activities at pH 7.2. Results are given as mean±SEM (n>3.0) (*P<0.05; statistically significantly different from pH 7.2)

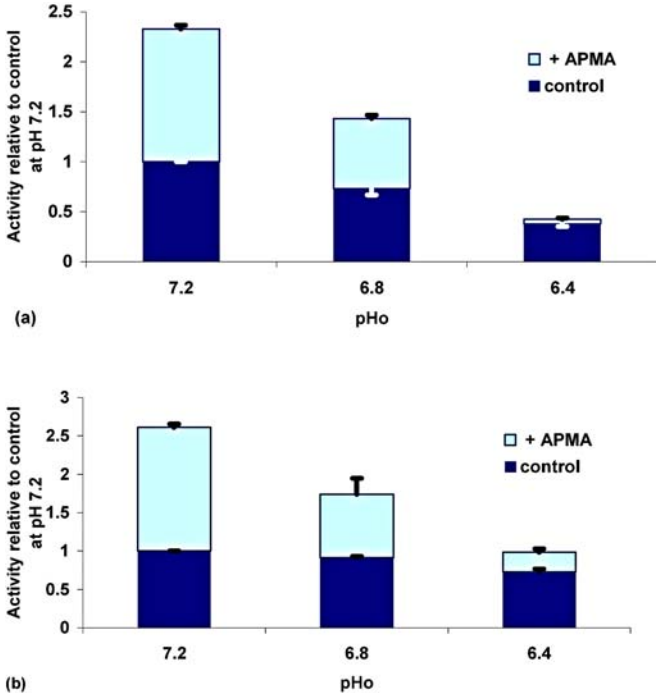

Fig. 3 The effect of extracellular pH on MMPs produced by **a** articular chondrocytes and **b** nucleus pulposus cells. MMP activity at different pH levels is shown for conditioned media (control) and after addition of 1mM p-aminophenyl mercuric acetate (APMA) activated all the MMPs produced. The rates were normalised to rates at pH 7.2. Results are given as mean±SEM (n>3.0)

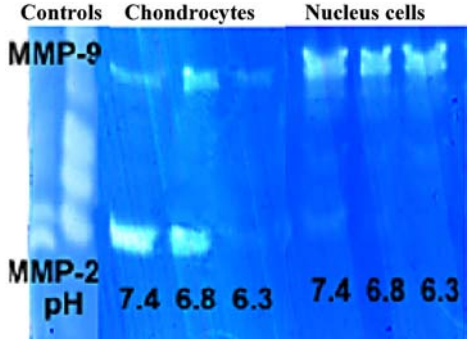

Fig. 4 Typical zymogram showing the effect of pH on MMP activity for bovine articular chondrocytes and nucleus pulposus invertebral cells cultured for 48 h in alginate beads. MMP activity was assayed by gelatin zymography for activites of the gelatinases MMP-2 and -9

modal relationship between MMP activity and extracellular pH was observed for both cell types. However, MMP activity for articular chondrocytes was significantly depressed at acidic pH; the MMP activity at pH 6.4 was 50% of that at pH 7.2. In contrast, MMP activity for nucleus pulposus cells was only marginally affected by acidity; the MMP activity at pH 6.4 was 80% that seen at pH 7.2, and the difference in activity was not significant.

MMPs are secreted as latent proforms and are activated by the cleavage of a short peptide [7]. The coumarin dye assay only measures the activated MMPs, and therefore to measure total MMP produced, all the MMPs were activated by addition of 1 mM APMA (p-aminophenyl mercuric acetate) [3]. Figure 3 illustrates the effect of extracellular pH on the activity of the total MMPs produced after activation by APMA compared to the actual MMP activity measured in the conditioned medium. Total MMP activity and hence the total MMPs produced by nucleus pulposus and articular chondrocytes were significantly reduced in acid conditions. Total MMP production was inhibited to a greater extent by acidification than MMP activity, particularly for nucleus pulposus cells. For nucleus cells, relative to values at pH 7.2, the MMP activity in the conditioned medium was inhibited by only 20% at pH 6.4; in contrast, total MMP production fell by 60%. At pH 6.4, most of the MMPs produced by articular chondrocytes and nucleus pulposus cells were activated; in contrast, at pH 7.2, less than 50% of the MMPs were in active form.

Figure 4 illustrates the typical effect of extracellular pH on gelatin zymograms from conditioned media obtained from articular chondrocytes and nucleus pulposus disc cells cultured in alginate beads as described above. Five zymograms were run; all gels produced a similar pattern to that seen in Fig. 4. Both cell types produced MMP-2 and MMP-9, but in different proportions. Articular chondrocytes produced mainly MMP-2, and little MMP-9 was evident. Nucleus cells, however, produced virtually only MMP-9 at pH 7.4. For articular chondrocytes, both MMP-2 and MMP-9 activities in conditioned media were sensitive to pH and decreased steeply below pH 6.8. For nu-

Table 3 TIMP-1 and -2 levels produced by articular chondrocytes and nucleus pulposus intervertebral disc cells cultured in alginate beads for 48 h at pH 7.4. Levels were measured using a human TIMP-1 immunoassay and human TIMP-2 ELISA system. Results are given as mean±SEM, (n>3) (*P< 0.05, TIMP-2 levels produced by nucleus cells are significantly different from TIMP-1 levels produced by nucleus cells and from TIMP-2 levels produced by articular cartilage)

Cell type	TIMP-1 levels [ng $(1\times10^6$ cells$)^{-1}$ h^{-1} ml^{-1}]	TIMP-2 levels [ng $(1\times10^6$ cells$)^{-1}$ h^{-1} ml^{-1})]
Nucleus pulposus cells	1.85±0.349 (n=4)	0.572±0.136 (n=3)*
Articular chondrocytes	1.28±0.356 (n=4)	1.236±0.235 (n=3)

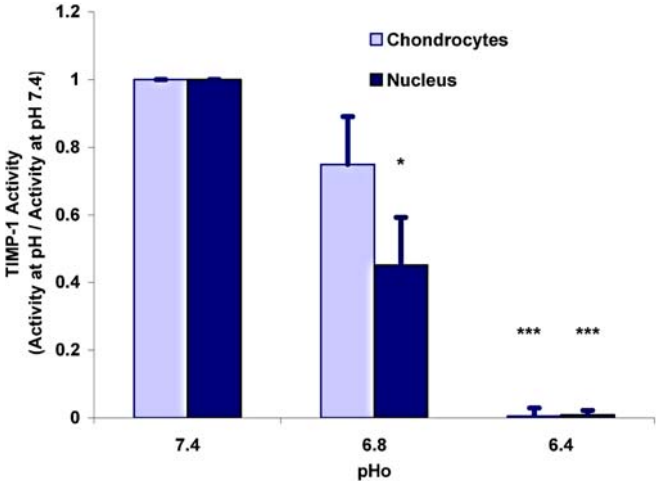

Fig. 5 The effect of pH on TIMP-1 production by bovine articular chondrocytes and nucleus pulposus intervertebral disc. The cells were cultured in alginate beads for 48 h in medium. The levels ere normalised to levels at pH 7.4. Results are given as mean±SEM (n>3.0) (*P<0.05, ***P<0.001)

cleus pulposus disc cells, however, while MMP-2 activity was sensitive to pH, falling in acid pH, MMP-9 activity was not affected by acidification.

Effect of pH on TIMP production

A human TIMP-1 immunoassay and a human TIMP-2 ELISA system were used to measure TIMP levels in conditioned media obtained by culture nucleus cells and articular chondrocytes encapsulated in alginate beads for 48 h in medium at different pH levels. Table 3 illustrates the levels of TIMP produced by nucleus pulposus disc cells and articular chondrocytes at pH 7.4. TIMP-1 and -2 levels were similar in articular chondrocytes. For the nucleus, however, while TIMP-1 levels were higher than those in articular chondrocytes, TIMP-2 levels were lower than those in articular chondrocytes, and were approximately only one-third of the TIMP-1 levels produced by nucleus pulposus cells.

Figure 5 illustrates the effect of pH on TIMP-1 levels for both nucleus pulposus disc cells and articular chondrocytes cultured for 48 h in alginate beads. Results are shown normalised to pH 7.4. TIMP-1 levels for both cell types were maximum at pH 7.4. Acidity greatly reduced the levels of TIMP-1 in the medium; at pH 6.4 TIMP-1 levels were <3% of those seen at pH 7.4 for both cell types. TIMP-2 activity showed similar responses to pH (not shown).

Discussion

The results of this study indicate that, in the intervertebral disc nucleus and articular cartilage, extracellular pH has a strong influence on both the production of matrix components and the production of agents involved in the breakdown of the matrix. For both disc and cartilage cells cultured for 48 h in alginate beads, the production of matrix components showed a biphasic response to pH (Fig. 1), as seen previously after short exposures of disc explants and cartilage cells in suspension culture to changes in extracellular pH [39, 54]. Rates were highest at around pH 7.0–7.2 (the estimated pH in normal discs [19]), where they were 10–20% greater than at pH 7.4 (the extracellular pH of most body fluids). In acid conditions, rates fell steeply. At pH 6.6, the rates of sulphated GAG production by disc cells were only 11% those at measured at pH 7.2 (Fig. 1a). Protein synthesis appeared less sensitive to pH, as observed in similar systems [39, 54], but was still strongly inhibited under acid conditions (Fig. 1b).

Extracellular pH also affected MMP production. MMPs are secreted by cells as inactive pro-enzymes and require activation by cleavage of a short pro-peptide before they are able to degrade matrix macromolecules [7]. They are generally activated by proteolysis in vivo, but in vitro can be activated by mercurial compounds. Total MMPs can thus be assayed by addition of APMA, which activates all MMPs [3]. Total MMP production, like that of other proteins (Fig. 1b) fell steeply with fall in pH (Fig. 3) for both articular chondrocytes and disc cells. Production of active MMPs was less sensitive to pH, but for articular chondrocytes, showed a biphasic response, with the production maximal at pH 7.2 and falling by >50% at pH 6.4 (Fig. 2). For disc cells, however, pH had relatively minor effects on production of activated MMPs (Fig. 2, Fig. 3), with production falling only by a nominal 20% at pH 6.4. The differential sensitivity may arise in part because bovine disc cells (in contrast to reports on ovine cells) produced mainly MMP-9, while chondrocytes produced mainly MMP-2, and production and activation of these enzymes may be regulated differently by extracellular pH (Fig. 4). It should be noted, however, that here we only assayed freely diffusible MMPs, and thus might have underestimated total MMP-2 production; MMP-2 has been reported to localise at the cell surface of ovine disc cells cultured in alginate beads [42].

MMPs appear to play an important role in disc degeneration [8, 11, 45, 53]. Regulation of their production and activity in the disc is not well understood. Several possible protease pathways appear involved in MMP activation in the disc [11, 42], but even if activated, the presence of inhibitors can prevent tissue degradation. Tissue inhibitors of metalloproteinases (TIMPs) bind to MMPs with 1:1 stoichiometry [7], with the resulting complex unable to cleave macromolecules. We found that TIMP production is also strongly downregulated by pH, with TIMP-1 production rates at pH 6.4 less than 2% of those at pH 7.2 (Fig. 5). As TIMP-1 production fell much more rapidly than production of total MMPs in acid conditions (Fig.3, Fig. 5), the resulting imbalance possibly explains the increase in proportion of active MMPs at low pH (from less than 50% at pH 7.2 to 80–98% at pH 6.4 for disc and cartilage respectively). Extracellular pH thus appears to play an important regulatory role both in the secretion of pro-MMPs and TIMPs (Fig. 3).

Although the environment of disc cells is likely to be more acid than that of cartilage cells, nucleus cells do not seem well adapted to a low pH environment. In cartilage at low pH, matrix turnover appears to slow markedly, with both production of active MMPs and biosynthesis of matrix components inhibited (Fig. 1, Fig. 3, Fig. 4). In contrast, exposure to acid conditions would appear particularly deleterious for the disc matrix, as it inhibits the disc cells from synthesising functionally important molecules such as the sulphated GAGs responsible for maintaining swelling pressure [52], but does not prevent the production of agents able to degrade matrix components. The acid conditions seen in degenerate discs [9, 25] are thus likely to be involved in breakdown and degeneration of the disc matrix.

It is not known how the low values of pH measured in degeneration [9, 25] arise. Lactic acid concentrations have been shown to rise rapidly after blood supply to the disc is reduced [15]; thus, loss of nutrient transport routes is one possible route to low pH. Another possible route is the presence of pro-inflammatory cytokines such as IL-1 or TNFα, thought to be involved in disc and cartilage degeneration and in sciatica [23, 41, 51]. These increase the rate of lactic acid production markedly [49], and hence tend to acidify the matrix, particularly in an avascular tissue such as the disc [47]. Thus, in addition to stimulating protease production [51], pro-inflammatory cytokines may be responsible for the low pH values seen in degenerate discs [9], adding further to the processes leading to disc degradation.

In this study we only examined representative markers of both biosynthesis and degradation. We did not attempt to determine whether different proteoglycan species found in the disc such as aggrecan or decorin [46], or whether other major disc components such as the different collagen types [10], are differentially regulated by pH. We also only examined the behaviour of some classes of MMPs and inhibitors. Other proteases such as aggrecanases [45] and the serine proteinases [1, 35], which can both degrade matrix components and activate MMPs, and protease inhibitors [36] are also found in the disc and are involved in matrix turnover and degradation. The results found here do, however, demonstrate that the complex balance between synthesis and protease production and inhibition, which regulate matrix turnover and hence ultimately matrix integrity and composition, can be strongly influenced by pH. Thus a full understanding of the regulation of turnover and degradation of disc matrix will not be achieved unless the effect of extracellular pH is also considered.

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