

*OsteoArthritis and Cartilage* (2004) 12, 485–496

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doi:10.1016/j.joca.2004.02.012

# Osteoarthritis and Cartilage

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## Increased stromelysin-1 (MMP-3), proteoglycan degradation (3B3- and 7D4) and collagen damage in cyclically load-injured articular cartilage

Peggy M. Lin, Chih-Tung Christopher Chen\* and Peter A. Torzilli

*Laboratory for Soft Tissue Research, Hospital for Special Surgery, New York, USA*

### Summary

**Objective:** To determine whether load-induced injury causes alterations in proteoglycan (PG), stromelysin-1 (MMP-3) and collagen in articular cartilage.

**Methods:** Mature bovine cartilage was cyclically loaded at 0.5 Hz with 1 and 5 MPa for 1, 6 and 24 h. Immediately after loading explants were evaluated for cell viability. Alterations in matrix integrity were determined by measuring PG content, PG degradation using 7D4 and 3B3(-) antibodies, broken collagen using COL2-3/4m antibody, and stromelysin-1 content using a MMP-3 antibody.

**Results:** Mechanical load caused cell death and PG loss starting from the articular surface and increasing in depth with loading time. There was a decrease in the 7D4 epitope (native chondroitin sulfate) in the superficial zone of cartilage loaded for longer than 1 h, but an increase around chondrocytes in the deep zone. The 3B3(-) staining for degraded/abnormal chondroitin-4-sulfate neopeptide appeared only in cartilage loaded under the most severe condition (5 MPa, 24 h). The elevation of stromelysin-1 was co-localized with broken collagen (COL2-3/4m) at the articular surface in explants loaded with 1 and 5 MPa for 24 h.

**Conclusions:** Cell death and PG loss occurred within 6 h of cyclic loading. The elevation of MMP-3 following cell death was consistently found in the superficial zone of loaded cartilage. Since MMP-3 can degrade PG and super-activate procollagenase, the increase of MMP-3 can therefore induce matrix degradation and PG depletion in mechanically injured articular cartilage, both of which are important to the development of osteoarthritis.

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**Key words:** Load-induced injury, Proteoglycan degradation, Stromelysin-1, Cell death, Cartilage.

### Introduction

The normal function of articular cartilage relies on the structural integrity and biochemical composition of the extracellular matrix (ECM), mainly collagen and proteoglycan (PG). The balance and organization of these macromolecules in the cartilage matrix are, somehow, disrupted due to trauma injury and degenerative joint diseases (DJD) such as osteoarthritis (OA). In the early stages of OA, the degradation and loss of proteoglycans and collagen can significantly change cartilage material properties owing to the presence and activation of degradative enzymes<sup>1,2,10,13,16,40</sup>.

Among of the earliest changes in OA cartilage are the loss of PG (aggrecan) and reduction of aggrecan size. These change are due mainly to the proteolytic cleavage of the core protein within the interglobular domain (IGD) by two distinct enzymes, matrix metalloproteinases (MMP-1, -2, -3, -7, -8, -9, and -13) and aggrecanases (ADAMTS-4 and ADAMTS-5)<sup>1,11,16</sup>. In addition to modifications in the core protein, there are many alterations to the GAG side chains of degenerative cartilage including shortening of the chondroitin sulfate (CS), increased ratio of

chondroitin-4-sulfation to total CS, and decreased KS/CS ratio<sup>6,10,24,28,34</sup>. Increased levels of the 'native' chondroitin-4-sulfation neopeptide recognized by the 7D4 antibody and of GlcA1,3GalNAc6S- neopeptide (consisting of non-reducing terminal saturated glucuronic acid residue adjacent to N-acetylgalactosamine-6-sulfate) recognized by 3B3(-) are found in human OA cartilage and synovial fluid of diseased joints<sup>4,31,39</sup>. The increased activity of both epitopes is also seen in several animal models of OA<sup>4,31,40</sup>. This indicates a change in the differentiated state of the chondrocytes, since the 3B3(-) epitope is found only in fetal cartilage and postnatal growth plate but not in normal adult articular cartilage<sup>3</sup>. The breakdown of type II collagen recognized by the COL2-3/4m and several other antibodies, is profound at late stages of OA and significantly increased with the arthritic severity, and thought to follow the degradation of PG<sup>12</sup>.

Although cartilage injury is a known risk factor that leads to the development of OA, the role of proteolytic enzymes in matrix degeneration from acute load injury is unclear<sup>13</sup>. The structural damage and morphological changes due to trauma injury, such as clefts, PG loss and collagen breakdown, have been well documented<sup>7,9,15,17,21,30,35,37</sup>. However, several recent studies have provided indirect but very interesting links between MMPs and matrix degradation<sup>18,19</sup>. Within hours of an inter-articular injury, the concentration of MMP-3 in the synovial fluid increases by as much as 40-fold and persists above normal levels for up to 17 years<sup>18</sup>. Increased amounts of PG fragments in the synovial fluid are seen together with the upregulation of

\*Address correspondence and reprint requests to: Dr Chris Chen, Laboratory for Soft Tissue Research, The Hospital for Special Surgery, 535 East 70th Street, New York, NY 10021-4010, USA. Tel: +1 212-606-1068; Fax: +1 212-249-2373; E-mail: [chench@hss.edu](mailto:chench@hss.edu)

Received 17 January 2003; revision accepted 24 February 2004.

MMP-3<sup>19</sup>. While these studies did not determine the ratio between MMP-3 and its natural inhibitor, TIMP-1, an index of MMP activation, others have reported that in OA the levels of TIMP-1 are markedly increased above physiological levels but not as high as MMP-3<sup>18</sup>. Of further interest is that increased levels of MMP-3 are also seen in the synovial fluid and cartilage of over-exercised and animal models of OA<sup>11,25,27,38</sup>. Since PG degradation and loss occurs in the early stages of OA and are important to cartilage functional properties, the aim of our study was to determine the *in situ* changes of cell viability, matrix integrity, and MMP-3 content due to load injury using histochemical and immunohistochemical methods. We hypothesized that cell death, PG loss, and collagen damage would correlate with the changes in CS and MMP-3 in load-injured cartilage.

## Materials and methods

### CARTILAGE EXPLANTS AND MECHANICAL LOADING

Mature bovine (adult 18–24 months) shoulder joints were obtained from a local abattoir within 4 h post-mortem. Full-thickness cartilage explants (7 mm in diameter) were excised from the weight-bearing region of the humeral head using a biopsy punch and surgical scalpel as previously described<sup>6</sup>. Each cartilage explant was then sliced through its deep zone, using a custom-fabricated cutting template and razor blade, to produce a uniform thickness of  $1.00 \pm 0.05$  mm ( $n=11$ ) with an intact articular surface. The explants were incubated in serum-free Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL, Grand Island, NY) with 1% antibiotic/antimycotic, 10 mM HEPES buffer (Gibco) and 50 µg/ml ascorbic acid at 37°C, 5% CO<sub>2</sub> and 95% atmosphere in 100% humidity. Culture media was changed three times a week with the addition of fresh ascorbic acid. Four week-old bovine growth plate cartilage, obtained from a local abattoir, and human osteoarthritic cartilage, from an 85-year-old patient who had a total hip replacement performed at the Hospital for Special Surgery with the approval of IRB, were used as positive controls for the histology and immunolocalization, as described below.

Mechanical injury was induced using a mechanical explant test system (METS) as previously described<sup>8,36</sup>. Each explant was placed in a loading chamber and immersed in culture media with its articular surface facing a porous (35 µm) load-platen. The cartilage explants were cyclically loaded in confined compression with 1 or 5 MPa at 0.5 Hz for 1, 6 and 24 h (normal joint loading for bovine was estimated to be 1800 cycles per day at 1 MPa). In each loading condition, five cartilage disks were loaded ( $n=5$ ) and five disks served as non-loaded controls, the controls positioned in the loading chambers without the load-platen. In all test conditions, explants were kept in DMEM at 37°C in 100% humidity with 5% CO<sub>2</sub> and 95% air.

### ANALYSES OF CELL VIABILITY

Cell viability in each explant was evaluated immediately after loading using a combination of two fluorescent probes: (1) propidium iodide (PI), a cell impermeable dye to stain dead cells red, and (2) fluorescein diacetate (FDA), a cell metabolic dye to stain viable cells green<sup>8,37</sup>. After loading, loaded and control explants were placed in phosphate buffered saline (PBS, 0.1 M phosphate-sodium, 0.15 M NaCl, pH=7.2) for 20 min to gain their original

volume. A slice of ~300 µm thickness was cut transversely through the explant and stained with 30 µM PI (Sigma) and 5 µM FDA (Sigma) in PBS for 5 min. Sections were then washed in PBS for 5 min to remove residual dyes from the tissue matrix and viewed using a fluorescent microscope (NIKON Optiphot-2, Melville, NY, USA) with a dual green and red barrier filter (wavelength ranges of 505–530 and 580–620 nm, respectively). The fluorescent images were captured using a cooled color CCD camera (Optronics, Goleta, CA).

### HISTOLOGY AND IMMUNOHISTOCHEMISTRY

After slicing the cartilage explant for cell viability, the remaining specimen was embedded and frozen in OCT (Tissue-Tek, Sakura, Torrance, CA) for histological and immunohistochemical assays. Non-loaded controls were frozen next to loaded explants within the same block to facilitate comparison. Six µm thick sections were cut in a cryostat and adhered to pre-coated slides (Fisher). Slides were air-dried overnight and then dried in acetone before being stored at -80°C until use. Prior to staining, slides were thawed and fixed in 4% paraformaldehyde for 10 min. After rehydrating in PBS, the slides were washed three times in PBS before proceeding with Safranin-O staining, or incubating in primary antibodies.

### SAFRANIN-O

Cartilage specimens were stained with Safranin-O histochemical stain to analyze proteoglycan distribution. Slides were first placed in Mayer's Hematoxylin for 2 min and rinsed in water. These slides were then placed in Fast Green FCF Yellowish for 10 min and rinsed in acetic acid (1%) until the glass was free of stain. Next, the slides were placed in Safranin-O for 30 min, and rinsed in water and 95% EtOH until the glass was free of stain. After air drying, the slides were cover-slipped.

### 3B3(-) AND 7D4

The non-specific binding in slides was blocked with 10% goat serum and 1% bovine serum albumin (BSA). After rinsing three times with PBS and 1% BSA, the slides were incubated with the monoclonal CS antibodies, 3B3(-) antibody (Seikagaku, Falmouth, MA) and 7D4 (generously provided by Dr Bruce Caterson). The slides were incubated with primary antibodies for 1 h at room temperature and followed by three washes with PBS and 1% BSA<sup>6</sup>. The slides were then incubated with the goat-anti-mouse secondary antibody conjugated to FITC (Sigma) in the dark for 1 h. Slides were kept in the dark for 1 h at room temperature. Subsequent washing was also done in the dark to prevent photo-bleaching. The sections were then counter-stained with 0.5 µM PI for cell nuclei and embedded in the mounting medium containing anti-fade agent, gelvatol (Sigma).

### MMP-3

Slides were treated with a 0.1% Triton X-100 solution for 10 min for permeabilization. After washing three times with PBS, non-specific binding was blocked with 10% goat serum containing 1% BSA. The slides were then rinsed three times with PBS and 1% BSA. The slides were then incubated with MMP-3 monoclonal antibody (Chemicon, Temecula, CA) for 1 h at room temperature<sup>11</sup>.

The remainder of the protocol was identical to that followed for the CS antibodies.

#### BROKEN COLLAGEN

Localization of denatured type II collagen was determined using COL2-3/4m antibody (kindly provided by Dr A. R. Poole) as previously described, except using a FITC-conjugated secondary antibody<sup>7,12</sup>. Briefly, the non-specific binding in cartilage sections was blocked with 10% goat serum and 1% bovine serum albumin (BSA). Sections were treated with 1 mg/ml testicular hyaluronidase (Behring Diagnostics, La Jolla, CA) at room temperature for 30 min to enhance antigens retrieval before incubated with COL2-3/4m primary antibody at 37°C for 30 min. Sections were then incubated with the goat-anti-rabbit secondary antibody conjugated to FITC (Sigma) in the dark at room temperature for 1 h. Cell nuclei were counter-stained with propidium iodide. The sections were embedded in anti-fade mounting medium as described above.

#### ANALYSIS

The histological preparations were viewed with a light microscope while the immunohistochemical preparations were viewed using a fluorescent microscope as mentioned above. Images were captured using a color CCD camera (DXM1200, Nikon, Melville, NY) and commercially available software (ACT1, Nikon; SigmaScan, SPSS, Chicago, IL).

#### SEMI-QUANTITATIVE ANALYSIS AND STATISTICAL ANALYSIS

The loss of safranin-O, MMP-3 staining and broken collagen was always found to start at the articular surface and to be uniform across the explant's width. Thus, the changes of staining from the articular surface were measured using SigmaScan Pro imaging software (SPSS Science, Chicago, IL) as previously described<sup>8</sup>. The cells with positively 3B3- staining in each section were also counted manually. The differences between groups were analyzed using the unpaired Student's (two-tailed) *t*-tests (Excel, Microsoft, Redmond, WA). All data are expressed as mean±standard error of the mean (SEM). Statistical significance level ( $\alpha$ ) was set at 0.05.

## Results

#### CELL DEATH IN THE SUPERFICIAL ZONE DUE TO LOADING

No dead cells were found in the non-loaded controls and cartilage loaded at 1 MPa for 1 h (Fig. 1A). A progressive increase in the depth of dead cells from the articular surface, proportional to the loading time and load magnitude, was seen in the loaded cartilage (Fig. 1B–F). This was consistent with our previous findings<sup>8</sup>. In the explants loaded at 1 MPa, dead cells were localized only in the superficial zone (SZ) (Fig. 1C–E). In the most severe loading condition, 5 MPa for 24 h, cell death was seen in the SZ and middle zone (MZ) (Fig. 1F). In the deep zone no cell death was seen except on the cut edges, which was attributed to the cutting process (Fig. 1B–F).

#### PROTEOGLYCAN LOSS INCREASED WITH LOADING TIME

Uniform Safranin-O staining was seen throughout all zones in the non-loaded controls, except for a lighter

staining near the articular surface where the PG content is known to be lower (Fig. 2A). In the explants loaded at 1 MPa, a progressive loss of PG staining was found to increase with loading time, starting at the articular surface (Fig. 2C) and progressing to greater than 100  $\mu$ m (Table I) in the cartilage after 24 h of loading (Fig. 2E). The same trend of PG loss was seen in the explants loaded at 5 MPa with the greatest PG loss in the 24-h loaded explants (Table I, Fig. 2D and F). In the growth plate, the Safranin-O staining was uniformly distributed throughout the intercellular regions with a stronger staining in the proliferative zone than in the calcified region (Fig. 2B).

#### LOSS OF 7D4 STAINING WITH LOADING

In the non-loaded controls, there was an intense staining of 7D4 epitope throughout the full thickness of the cartilage, with the strongest staining in the SZ at the articular surface (Fig. 3A). Within the SZ, there was an uneven staining with the strongest staining in the pericellular and territorial regions around the chondrocytes. This staining was consistent with the localization of PG concentration from the histological preparation and from other studies in normal cartilage<sup>6</sup>. In the growth plate, the 7D4 epitope was seen in the lower proliferative region and throughout the calcified zone. Within the calcified zone, the staining was localized only around the chondrocytes. In the proliferative zone, there was strong staining in chondrocytes and the regions adjacent to them (Fig. 3B).

In explants loaded for 1 h at 1 MPa, a weak 7D4 staining in the SZ and MZ was consistently seen (Fig. 3C). There was no discernible difference in 7D4 staining in the interterritorial regions between the loaded and the control explants. A stronger staining in the pericellular and territorial regions was also seen. In explants loaded for 6 and 24 h, a greater loss of 7D4 staining was seen (Fig. 3D). The staining at the surface and in the SZ was faint and discontinuous, while in the middle and deep zones the sections did not stain at all. In the explants that did stain, a weaker staining in the interterritorial matrix of the deep zone was found, even though the immunostaining in the pericellular and territorial regions was still strong (Fig. 3D). This loss of 7D4 staining was attributed, in part, to the loss of PG in this zone. The lack of 7D4 staining was also seen in the explants loaded at 5 MPa. In explants loaded for 24 h, the loss of staining was seen in all sections and included the SZ and MZ of the explants (not shown).

#### 3B3(-) STAINING IN LOADED CARTILAGE

No staining against 3B3(-) epitope was found in the non-loaded controls (Fig. 4A), similar to previously reported in normal human cartilage<sup>23,40</sup>. Neither was there 3B3(-) staining within any of the explants loaded at 1 MPa, nor explants loaded at 1 MPa for 24 h (Fig. 4C). However, positive 3B3(-) staining was repeatedly seen in the explants loaded at 5 MPa for 24 h (4 of 5, Fig. 4D). In all these explants, an increased staining (Table I) around and within the chondrocytes was seen in the deep zones (Fig. 4D). The most intense staining for the epitope appeared to be concentrated within the pericellular matrix of the chondrocytes. No staining was seen in the territorial and interterritorial regions of loaded explants (Fig. 4A and C). In the growth plate, 3B3(-) staining was seen in the proliferative zone with the most intense staining in the extracellular matrix (ECM) just outside the chondrocytes (Fig. 4B).

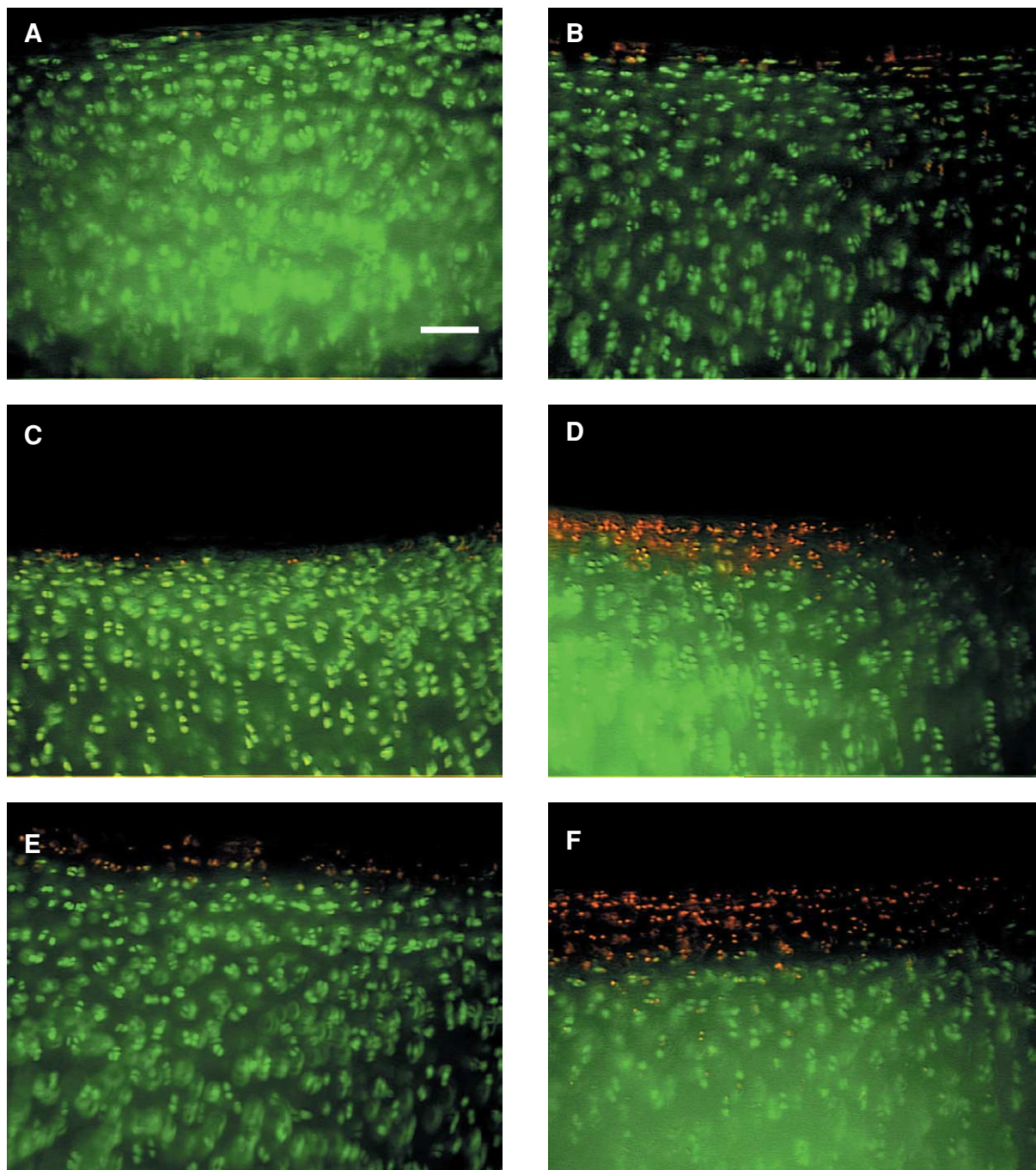


Fig. 1. Cell viability in mechanically loaded articular cartilage. Red fluorescence indicates dead cells and green fluorescence indicates viable cells. Cartilage explants were loaded at 1 MPa (A, C, E) and 5 MPa (B, D, F) for 1 h (A, B), 6 h (C, D) and 24 h (E, F). There were negligible dead cells at the articular surface in cartilage loaded with 1 MPa for 1 h. The depth of dead cells from the articular surface increased with loading magnitude (1–5 MPa) and loading time (1–24 h). Bar=200  $\mu$ m.

Strong 3B3(-) staining was also found in the human OA cartilage (not shown).

#### INCREASED MMP-3 STAINING IN THE SUPERFICIAL ZONE

In the control explants, there was faint or no staining for MMP-3 at the articular surface (not shown). This was compared to a strong staining in the human OA cartilage

(Fig. 5A) and bovine growth plate (Fig. 5B) where staining for MMP-3 was uniformly distributed throughout the ECM. There was little or no MMP-3 staining at the articular surface or within the ECM in the explants loaded at 1 or 5 MPa for up to 6 h (Table I, Fig. 5C, D). However, in explants loaded at 1 MPa for 24 h, an increase in the intensity of MMP-3 staining at the articular surface and within the SZ was found (Table I, Fig. 5E). A similar

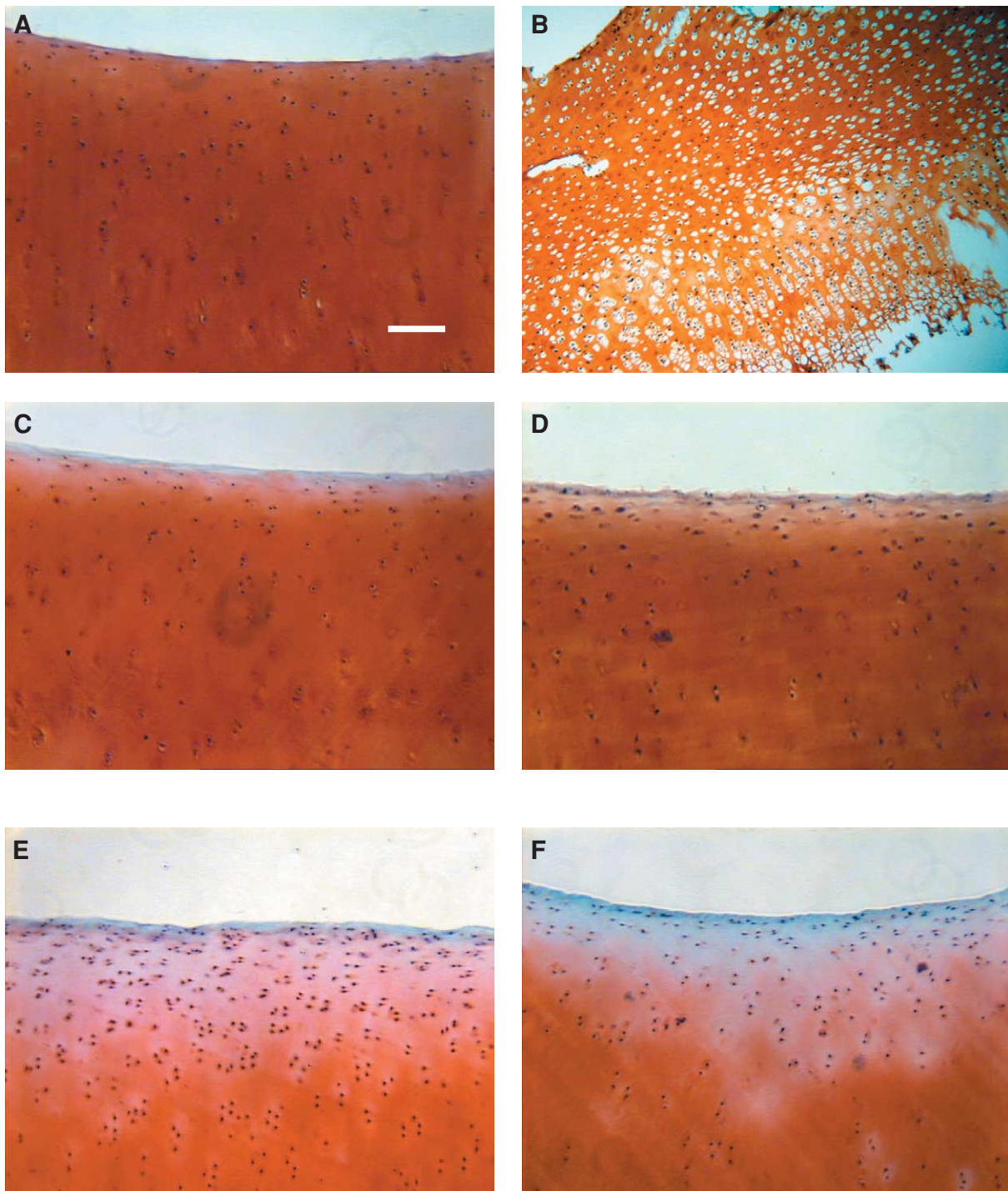


Fig. 2. Histological staining of proteoglycan (safranin-O) in bovine articular cartilage and growth plate. The intensity of red (safranin-O) staining indicates the concentration of PG. A uniform red staining was seen in all regions of normal adult articular cartilage (A) and immature bovine growth plate (B). A slightly loss of red staining was seen at the articular surface of cartilage explants loaded with 1 MPa (C) and 5 MPa (D) for 1 h. An increased loss of red staining was seen in the superficial and middle zones of explants loaded with 1 MPa (E) and 5 MPa (F) for 24 h. Bar=200  $\mu$ m.

increase in MMP-3 staining was also found in the explants loaded at 5 MPa for 24 h (Table I, Fig. 5F). Unlike non-loaded controls, the MMP-3 staining was uniformly distributed within the SZ (Fig. 5E and F). Of interest was that the region of increased MMP-3 staining overlapped with the region where there was chondrocyte death.

#### INCREASED BROKEN COLLAGEN IN THE SUPERFICIAL ZONE

There was a strong staining of broken collagen (COL2-3/4m) in the human OA cartilage (Fig. 6A) and bovine growth plate (Fig. 6B) which was compared to no staining in the non-loaded cartilage (Table I, Fig. 6C). A weak of

Table I

Sample group	Non-loaded control	Growth plate	1 MPa 1 h	5 MPa 1 h	1 MPa 24 h	5 MPa 24 h
Loss of safranin-O staining ( $\mu\text{m}$ )	7 $\pm$ 4	NA	16 $\pm$ 8	22 $\pm$ 13	123 $\pm$ 73*	104 $\pm$ 49*
3B3- stained cells/section	0 $\pm$ 0	197 $\pm$ 27*	0 $\pm$ 0	0 $\pm$ 0	0.2 $\pm$ 0.2	3.2 $\pm$ 0.4*
Depth of MMP-3 staining ( $\mu\text{m}$ )	0.3 $\pm$ 0.2	NA	0.2 $\pm$ 0.1	0.7 $\pm$ 1.0	15.0 $\pm$ 4.6*	18.9 $\pm$ 11.8*
Depth of COL2-3/4m staining ( $\mu\text{m}$ )	3.7 $\pm$ 3.3	NA	4.4 $\pm$ 2.7	2.6 $\pm$ 1.5	28.6 $\pm$ 11.0*	58.3 $\pm$ 16.9*

\*Indicates  $P < 0.05$  as compared to non-loaded control. NA indicates 'not available'. Semi-quantitative analysis of the sections of bovine articular cartilage and growth plate ( $n=5$ , in each group) staining with safranin-O, and antibodies recognizing 3B3 (-) neopeptide, MMP-3, and denatured collagen (COL2-3/4m). Data are presented as means $\pm$ standard errors of the mean.

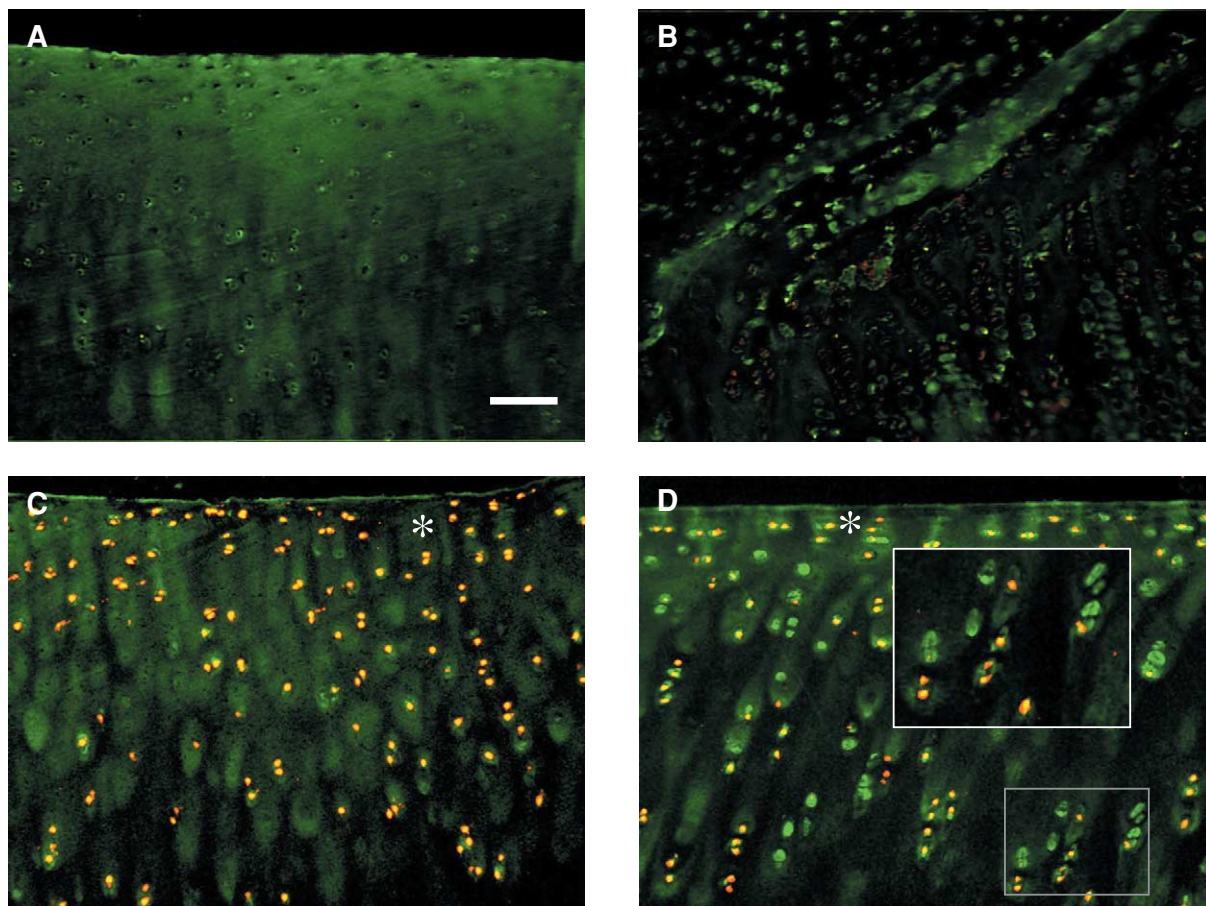


Fig. 3. The 7D4 (green) staining of native chondroitin sulfate in bovine cartilage. All cell nuclei were counterstained with propidium iodide (red fluorescence). A strong (green) staining was seen in the territorial and pericellular regions of normal (non-loaded) adult articular cartilage (A) and immature bovine growth plate (B). A loss of 7D4 staining was seen in the superficial zone of articular cartilage loaded for 1 h at 1 MPa (C). An increased loss of 7D4 stain in the superficial and middle zones was seen (asterisk) after loaded for 24 h at 1 MPa (D). In the explant loaded for 24 h, an increased 7D4 staining was seen (arrows) in the peri-cellular regions (inserts). Bar=200  $\mu\text{m}$ .

staining was found in the explants loaded at 5 MPa for 1 h (Table I, Fig. 6D). In explants loaded at 1 and 5 MPa for 24 h, a strong staining for broken collagen (COL2-3/4m) was found within the SZ (Table I, Fig. 6E, F). This change was co-localized with the increase of MMP-3 and cell death, and consistent with our previous finding<sup>7,8</sup>.

### Summary and discussion

In this study, we load-injured cartilage explants and analyzed where specific PG modification or loss occurred as

well as the progressive changes occurring in the tissue matrix as a function of loading time. The findings of this study are summarized in Fig. 7. Cell death and PG loss in load-injured cartilage progressively increased from the articular surface to the deeper zones with loading time and load magnitude due in part to the heterogeneous property of articular cartilage<sup>33</sup>. An increased of chondroitin sulfate synthesis (3B3-) was found in the deep-zone chondrocytes after load-induced injury. The increased levels of MMP-3 were co-localized with dead chondrocytes and broken collagen. Accordingly, cyclic loading greater than 1 MPa for more than 6 h was responsible for the increased levels of MMP-3.

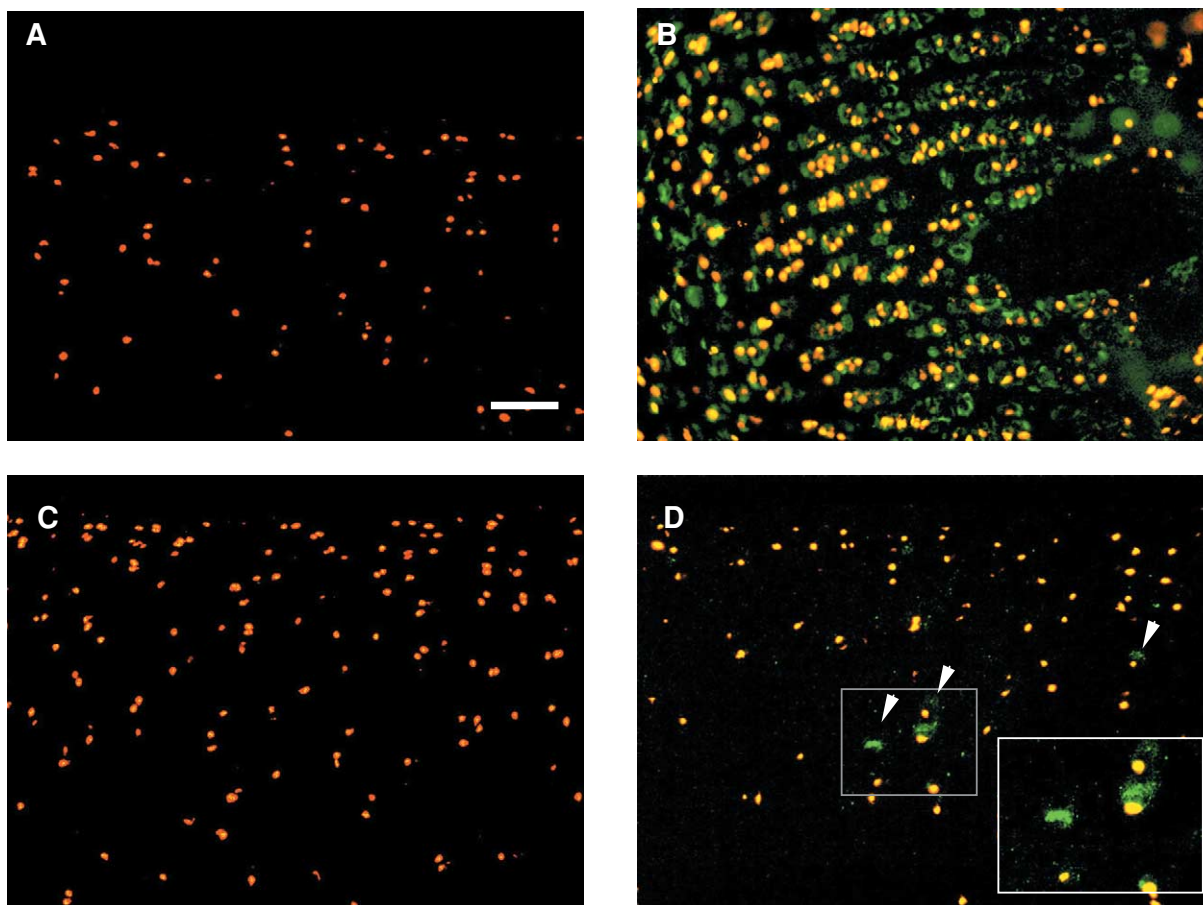


Fig. 4. The 3B3(-) (green) staining of abnormal/degraded chondroitin sulfate in bovine cartilage. All cell nuclei were counterstained with propidium iodide (red fluorescence). No 3B3(-) staining was seen in the non-loaded adult articular cartilage (A). This was compared to a strong staining in the immature growth plate (B). No 3B3(-) staining was either seen in cartilage explants loaded with 1 MPa for 24 h (C). However, 3B3(-) staining around and within the chondrocytes was seen (arrows) in the deep zone of explants loaded with 5 MPa for 24 h (D). Bar=200  $\mu$ m.

*In situ* PG loss is usually due to (1) an imbalance of metabolism (catabolism>anabolism), (2) degradation and loss of the aggrecan molecules, and (3) convective fluid flow during mechanical loading due to the use of porous indenter. Since our loading times were much shorter than the half-life of aggrecan (>3 years)<sup>29</sup> and newly synthesized GAG (>12 days)<sup>29</sup>, the loss of PG in the loaded cartilage was most likely caused by PG degradation and convective fluid flow during mechanical loading. In our study, the loss of PG began at the articular surface after 1 h of loading. By 24 h, PG loss extended into the MZ to a depth of approximately one-third of the full thickness. Proteoglycan loss due to mechanical loading has also been reported in several other studies<sup>15,24,37</sup>. These studies found that more than 4% of the newly synthesized PGs of non-loaded explant are lost daily and that the PG loss increases when the cartilage is cyclically loaded<sup>36</sup>. Our findings agree with these studies. In addition, we found that the loss of PG was located within the SZ and MZ where the convective fluid flow is greatest. Although the increase and activation of MMP-3 would also result in aggrecan damage and sequential PG loss, we found that it did not take place until a longer time period of loading had occurred (>6 h).

A loss of 7D4 staining was also found in the SZ and MZ after loading and the loss progressively increased with

loading time. In control explants, the 7D4 epitope was concentrated within the SZ and MZ. Since *in situ* CS and 7D4 epitopes in normal cartilage are proportional to PG concentration, the loss of PG in the uppermost layers of loaded cartilage is closely related to the loss of 7D4 staining. We found an increase in 7D4 staining in the middle and deep zones around the chondrocytes of the explants loaded at 1 and 5 MPa for 1 to 24 h. The increased 7D4 staining was especially strong within the pericellular matrix of chondrocytes. Of interest was that the increased 3B3(-) staining was also seen in the same region. Unlike 7D4 staining, the 3B3(-) staining appears limited to the DZ of cartilage and only in the explants loaded at 5 MPa for 24 h. These changes in CS side chain metabolism are similar to that found in early human OA and a number of animal models of OA<sup>5,6,34,40</sup>.

Chondroitin sulfate epitopes such as 3B3(-) and 7D4 have been used as markers for osteoarthritis because they can be measured in the synovial fluid of joints and in the tissue itself. Previous findings in patients with OA and rheumatoid arthritis (RA) have shown an increase in CS epitopes as well<sup>4,5,34</sup>. Roberts *et al.*<sup>32</sup> found a greater immunoreactivity in specimens of a higher degenerative grade for 3B3(-) and 7D4. Although we found that mechanically loading cartilage caused damage at the articular

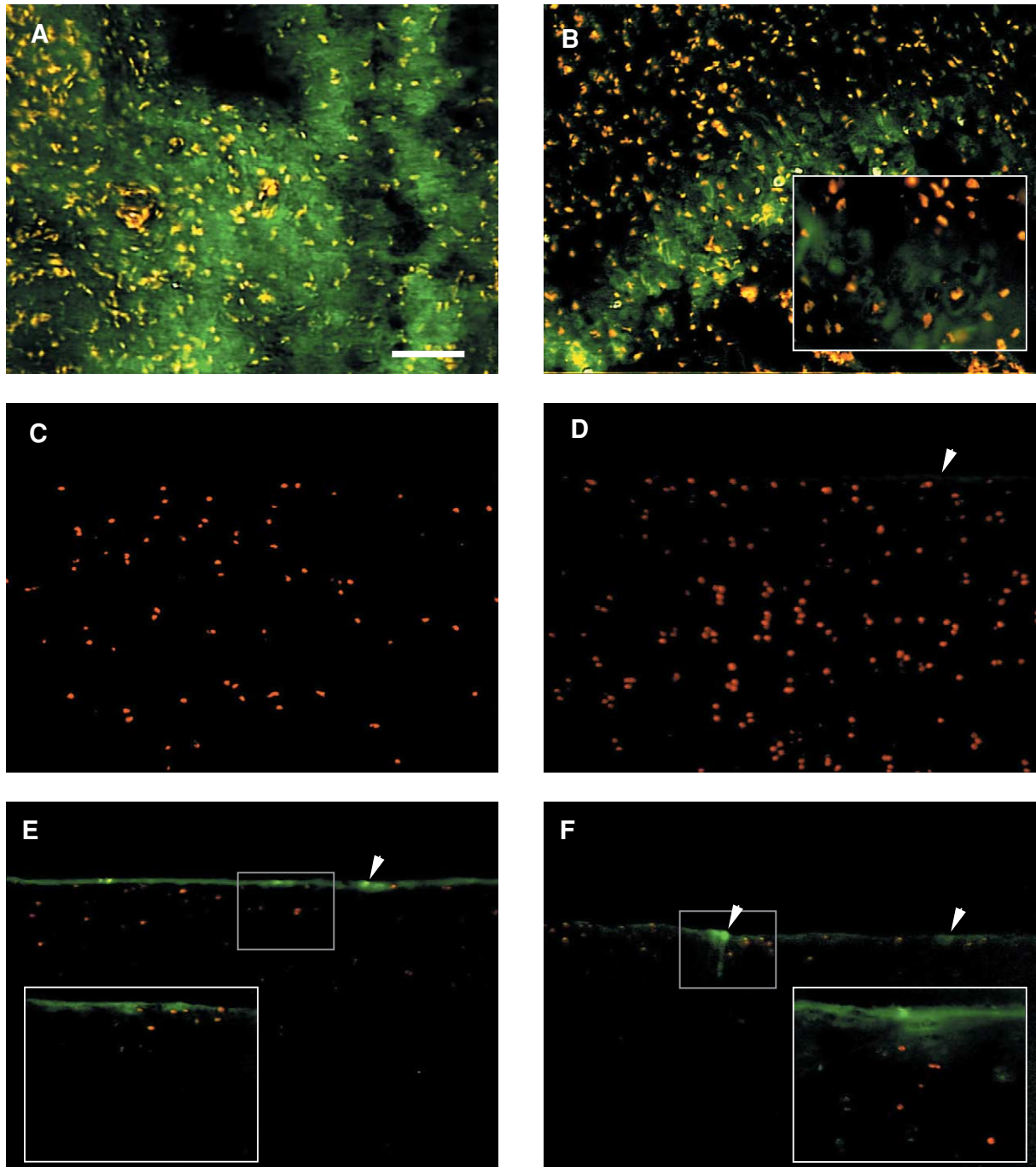


Fig. 5. Immunolocalization of MMP-3 (green) in human and bovine cartilage. A strong staining of MMP-3 was seen in human OA cartilage (A) and bovine growth plate (B). A weak or no staining was seen in the explants loaded for 1 h at 1 MPa (C). A weak staining was seen (arrow) in the explant loaded for 1 h at 5 MPa (D). An increase of MMP-3 staining was seen (arrows) in the superficial zone of explants loaded with 1 MPa (E) and 5 MPa (F) for 24 h. Bar=200  $\mu$ m.

surface, 3B3(-) staining was seen only in the deep zone of explants loaded under the most severe condition (5 MPa for 24 h). This suggests that new CS synthesis occurs only after loading at high stresses for at least 24 h. Roberts *et al.* also reported seeing pericellular staining for CS epitopes within cartilage<sup>32</sup>. This pattern may be explained by the fact that newly synthesized proteoglycans are secreted from chondrocytes but take time to diffuse throughout the

ECM<sup>29,36</sup>. Quinn *et al.*<sup>29</sup> suggested that the highest turnover of proteoglycans occurs in the pericellular matrix of chondrocytes, and that chondrocytes contribute to the remodeling and repair of the ECM in order to maintain biomechanical functionality after the tissue is damaged.

The presence of 3B3(-) and increased levels of 7D4 epitopes are normally found in the developmental stages of cartilage growth, such as growth plate and fetal cartilage,



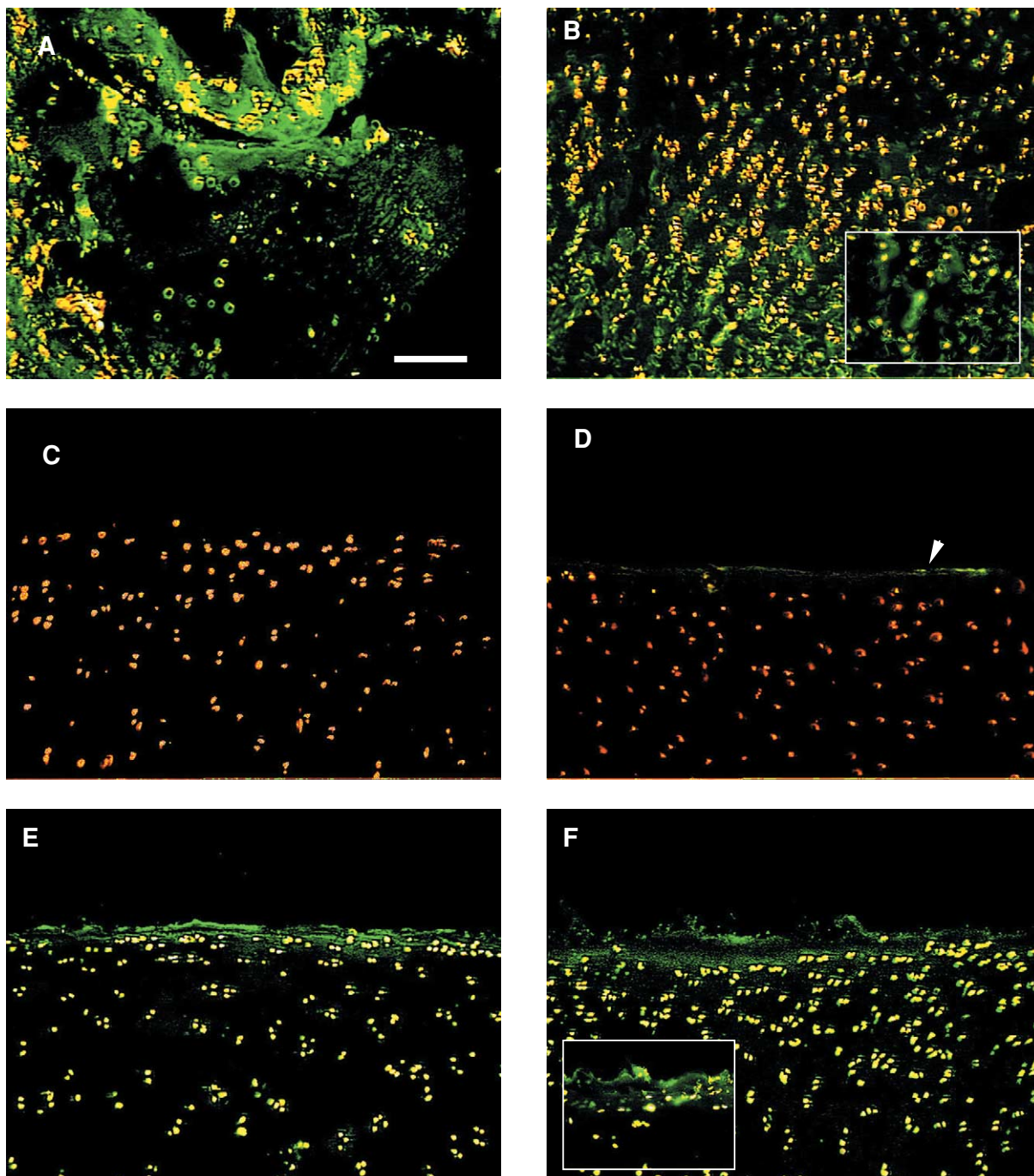


Fig. 6. Immunolocalization of broken collagen with COL2-3/4m (green) antibody in bovine cartilage. A strong staining of COL2-3/4m was seen in human OA cartilage (A) and bovine growth plate (B), which were compared to no staining in non-loaded control (C), and a weak staining at the articular surface (arrow) of cartilage loaded at 5 MPa for 1 h (D). A strong COL2-3/4m staining was seen in the superficial zone of explants loaded with 1 MPa (E) and 5 MPa (F) for 24 h. Bar=200  $\mu$ m.

and in OA tissue. In our study, positive 3B3(-) staining was found throughout the bovine growth plate, consistent with studies in dog and rabbit<sup>3,6</sup>. A strong 7D4 staining was also found in the proliferative region and within the pericellular matrix of the hypertrophic chondrocytes, again consistent with studies in dog and opossum growth plate<sup>4</sup>. The presence of 3B3(-) and increased levels of 7D4 epitopes in OA cartilage is thought to be due to a change in the

differentiated state of the chondrocytes<sup>22</sup>. Our finding of increased 3B3(-) and 7D4 epitopes in loaded cartilage suggests that an alteration in chondrocyte metabolism had occurred and was in response to the mechanical load (injury). This finding is similar to OA tissue and may be due to the chondrocyte's attempt to repair the injured tissue. Another possibility is that loading caused the activation of other enzymes or caused previously 'hidden' epitopes to

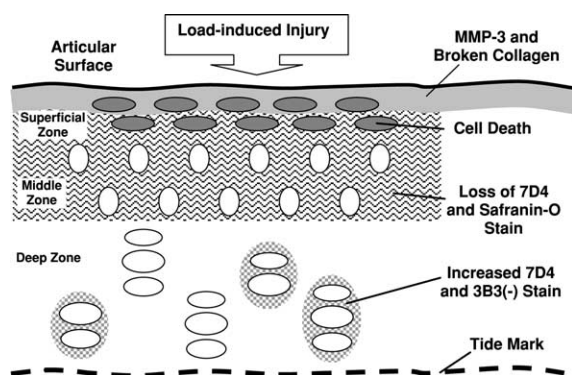


Fig. 7. Summary of *in situ* changes due to load-induced injury. The loss of 7D4 and safranin-O is located in the superficial and middle zones. Increased levels of MMP-3 are co-localized with cell death and broken collagen. The increased 3B3(-) and 7D4 staining in the deep zone indicates an alteration of GAG.

become revealed and recognized by the antibodies<sup>10</sup>. Since we found no loss of Safranin-O (PG) staining in the region where the increased 7D4 and 3B3(-) staining was seen, the enzymatic induction of 7D4 and 3B3(-) epitopes is likely to be a minor mechanism.

Our finding of increased MMP-3 in loaded cartilage agrees with *in vitro* and *in vivo* findings from other groups<sup>18–20,26</sup>. Patwari *et al.* measured the mRNA expressions in 2- to 3-week-old bovine cartilage loaded with peak stresses of 11.5 MPa. They found a 10-fold increase of MMP-3 mRNA in the loaded cartilage over control 1–24 h after load removal<sup>26</sup>. In addition, Martin *et al.* showed an increase in MMP-3 proenzyme expression in normal human cartilage after 20% cyclical compression at 0.5 Hz for 2 h<sup>20</sup>. Our result is also consistent with the levels of MMP-3 seen in the synovial fluid of patients soon after a traumatic knee injury<sup>18,19</sup>.

However, it should be noted that the MMP-3 antibody used in our study could not distinguish between the different forms of MMP-3. In articular cartilage, there exist three forms of MMP-3: proenzyme, conjugated to TIMP, and active. In the ECM of normal cartilage, MMP-3 is conjugated to TIMP and inactive before dissociation from TIMP. Therefore, the existence of MMP-3 does not always produce aggrecan breakdown and cartilage matrix degradation. Despite the uncertainty of activation of MMP-3, two previous studies may shed some light on the functional roles of MMP-3 in injured cartilage. Lark *et al.* correlated the presence of MMP-3 with MMP-degraded aggrecan (by using an antibody against VDIPEN<sup>341</sup> neopeptide) in the cartilage from patients with OA and RA<sup>16</sup>. In murine antigen-induced arthritis (AIA), van Muers *et al.* showed the co-localization of MMP-degraded aggrecan (VDIPEN<sup>341</sup> antibody) with collagenase-cleaved collagen, all in the superficial zone<sup>38</sup>. In the same study, cartilage from MMP-3 knockout mice showed no PG and collagen degradation during AIA, suggesting that MMP-3 (stromelysin-1) played a pivotal role in cartilage degradation<sup>38</sup>. Our finding of co-localization of MMP-3 enzyme and broken collagen in cartilage after mechanical loading agrees with these findings<sup>38</sup>. Together, these data suggest that the expression, presence, and activation of MMP-3 plays a key role in the development of OA as well as in acutely load-injured cartilage.

One of the most interesting findings in this study was that the increased MMP-3 was co-localized with the occurrence of cell death in the SZ. It would be interesting to know how and why the MMP-3 is located in this region. Since the increased MMP-3 takes place at least 10 h after the occurrence of cell death, the increase in MMP-3 is more likely from adjacent viable cells rather than a release of proenzyme from the dead cells. It is known that MMP-3 can super-activate procollagenase by changing collagenase from the proenzyme (inactive) to the active form<sup>14</sup>. The co-localization of MMP-3 and cell death in the SZ of loaded cartilage may be a response to remove the dead cells and damaged matrix (collagen and PG) in order to facilitate cartilage repair and the migration of viable cells<sup>13,14</sup>. If this is true, then the role of MMP-3 in load-injured cartilage may be constructive rather than destructive. Further studies are needed to elucidate the functional roles of MMP-3 as well as the relationship between cell death and MMP-3 localization in acutely load-injured cartilage.

### Acknowledgements

The authors thank Dr Bruce Caterson for providing the 7D4 antibody and Dr A. Robin Poole, for providing COL2-3/4m antibody, and Dr Madhu Bhargava for assistance in immunohistochemical analyses. This study was supported by the National Institutes of Health Grant AR45748 (PAT).

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