

High mobility group box-1 (HMGB-1) in osteoarthritic cartilage

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Abstract

Objective

Nucleosomal high mobility group box-1 (HMGB-1) is translocated and released from necrotic and activated cells as an endogenous danger signal (alarmin) and cytokine. It was hypothesised that it plays a role in osteoarthritis (OA) characterised by cellular activation, inflammation and enchondral bone formation.

Methods

Bovine knee joint samples, collected from culled animals, were scored using histologic/histochemical grading to intact looking, mild, moderate or severe and immunohistochemically stained for HMGB-1. Chondrocyte pellets, produced from human bone marrow-derived mesenchymal stem cells and stimulated with tumour necrosis factor- α (TNF- α), were similarly stained.

Results

In healthy looking OA cartilage chondrocyte nuclei were usually HMGB-1 negative and in mild OA staining was restricted to nuclei. In moderate OA lesions HMGB-1 was also seen in the cytoplasm and occasionally pericellular matrix and in severe OA lesions often also in intra- and inter-territorial matrix. The tidemark in healthy cartilage did not contain HMGB-1, which however was seen at this interface as linear deposits even in intact-looking and mild OA lesions, as multiple wave-like deposits in moderate and as heavy granular deposits in severe lesions. TNF- α stimulation of chondrocytes caused translocation of HMGB-1 from the nucleus to the cytoplasm.

Conclusions

In resting chondrocytes tight nucleosomal HMGB-1 binding might cause steric hindrance of immunostaining. TNF- α or OA-mediated activation leads to nuclear staining and cytoplasmic translocation. Advancing OA leads to increasingly intense extra-/pericellular deposition of HMGB-1 alarmin, indicating local chondrocyte activation and/or necrosis. In particular, HMGB-1 at the tidemark might play a role in the pathological thickening of subchondral bone plate/osteophyte formation.

Key words

Osteoarthritis, cartilage, chondrocyte, bone formation, HMGB-1 protein

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Introduction

Osteoarthritis (OA) is a common degenerative joint disease characterised by a painful aseptic inflammation and often limited range of motion of the joint. OA leads to destruction of the joint surface, thickening of the subchondral bone plate and formation of osteophytes at the joint margins. Cartilage surface irregularities develop and increase friction. It is not known what molecules mediate development of subchondral sclerosis and marginal osteophytes.

High mobility group box-1 (HMGB-1) is a highly conserved non-histone nuclear protein expressed by all eukaryotic cells. It binds to and bends DNA and participates in the transcription of multiple genes (1). It has been recently discovered that HMGB-1 is released to extracellular space (2), either upon stimulation of the cells (2-3) or passively from cells undergoing necrosis (3). Hypoxia is one of the stimuli able to lead to mobilisation and release of HMGB-1 (4). HMGB-1 induces production of tumour necrosis factor- α (TNF- α) and interleukin-1 β (5). Cytoplasmic and/or extracellular HMGB-1 has been described in experimental arthritis and synovial fluid and tissues in rheumatoid arthritis (6-7). HMGB-1 has been studied in OA, but only in synovial fluid (4). The aim of the present study was to analyse HMGB-1 in cartilage in OA.

HMGB-1 is chemotactic to endothelial cells, monocyte/macrophages and osteoblasts (6, 8). It triggers differentiation of mesenchymal stromal (stem) cells (MSC) into osteoblasts (7). It was hypothesised that necrotic and/or activated chondrocytes release HMGB-1 into extracellular cartilage matrix, where it might play potent roles as a cytokine and particularly as a stimulus for endochondral bone formation.

Materials and methods

Animals

Bovine knee joints were collected from a performance testing station from six 30–61 month old animals culled because of low total merit index. Based on a thorough clinical examination (locomotion scoring, haematological and biochemical laboratory tests) all animals were considered to be healthy

enough to be slaughtered in a commercial slaughterhouse. They all suffer at this stage from OA of the large weight bearing joints. The bones forming the knee joint were removed at the abattoir. Healthy articular cartilage samples obtained from knee joints of six dairy bulls, younger than 20 months of age, were used as control material.

Macroscopic grading

All joint surfaces from OA knee joints (7 joints) from 6 animals (from one animal samples were collected from both knee joints) were evaluated macroscopically. The appearance of macroscopically visible pathological changes in different sampling areas of individual joints were recorded before harvesting of the samples (more than one from each joint) as follows: Grade 1 samples (number of samples collected = 7), macroscopically no visible lesions; Grade 2 samples (number of samples = 3), minor lesions, joint surface roughening and irregularities of the articular cartilage, which penetrate less than 2 mm into the cartilage; Grade 3 samples (number of samples = 2), moderate lesions, joint surface roughening and more than 2 mm penetration; grade 4 samples (number of samples = 2), full thickness, osteochondral lesions. The depth of the lesions was recorded using a caliper ruler. In addition to the above mentioned OA joint samples, samples were also collected from six animals from six healthy knee joints (no OA) and thus without any macroscopically visible lesions in the joint surfaces at all (number of samples = 6); these samples from healthy areas of healthy joints are referred to grade 0 samples.

Histological scoring

Bone/articular cartilage samples were fixed in 4% neutral phosphate-buffered formaldehyde for 2 weeks and decalcified for 5 weeks in 10% EDTA solution prior to embedding in paraffin. For additional softening of the cartilage samples, open surfaces were treated with 10% nitric acid prior cutting with the microtome. 3–4 μ m sections were stained with haematoxylin and eosin or safranin O. Samples were scored using the histologic/histochemical grading

system (HHGS) (9), which is based on the structure of the cartilage (0–6), number of cells (0–3), Safranin O staining (0–4) and tidemark integrity (0–1) and ranges from 0 to 14, zero representing histologically healthy-looking samples. 1–3 tissue sections were analysed for each of the HHGS values. For analytical purposes the samples, based on their HHGS values, were divided into four categories, no apparent cartilage pathology (HHGS =0) and mild (HHGS <3), moderate (HHGS 4–8) or severe (HHGS >9) OA changes. HHGS score does not take into account the area of the cartilage change (10, 11).

Chondrogenesis and TNF- α stimulation

Incomplete chondrogenic medium (Cambrex, Charles City, IA, USA) with transforming growth factor- β 3 (R&D Systems, Minneapolis, MN, USA) added to final concentration of 10 ng/ml, was pipetted to test tubes, each containing 2.5×10^5 cells, which had been centrifuged (pelleted) at $150 \times g$ for 5 minutes. Non-adherent pellets formed in polypropylene tubes were kept in Complete chondrogenic medium at $+37^\circ\text{C}$ in a humidified 5% CO_2 -in-air. The medium was replaced every 3 days. Samples were differentiated for 21 days and then cultured without or with 10 ng/ml TNF- α for one more day. After this samples were fixed in 10% formalin and embedded in agarose, followed by dehydration in ethanol series, clearing in xylene and embedding in paraffin. 4 μm paraffin sections were cut on objective slides. Chondrogenic differentiation was confirmed using proteoglycan and collagen type II staining (data not shown).

Immunohistological staining

Tissue sections were deparaffinised in xylene and rehydrated in a graded ethanol series. After deparaffinisation of the bone-cartilage sections, antigens were retrieved for 30 minutes at $+37^\circ\text{C}$ using 4 mg/ml pepsin dissolved in 0.01 N HCl, whereas cultured cell pellet sections were covered with 10 mM sodium citrate buffer, pH 6, and subjected to AR 98°C-S30M program for 24 minutes using MicroMed T/T Mega Labo-

Table I. The histological/histochemical grading score or HHGS value¹ compared to the High-mobility group box-1 (HMGB-1) staining² in bovine osteoarthritis (OA) and in healthy control cartilage samples compared to

HHGS ²	HMGB-1 staining			Tidemark staining
	nuclear	cytoplasmic	extracellular ³	
Intact	– ⁴	–	–	faint, linear
Mild	+	–	–	faint, linear
Moderate	+	++	++	parallel lines/waves
Severe	+	++	+++	scattered, granular
Control joints ⁵	+ ⁶	–	–	–

¹HHGS value: intact OA cartilage, HHGS = 0 (n=7); mild changes ($\neq 0$) in OA cartilage, HHGS ≤ 3 (n=2); moderate changes in OA cartilage, HHGS = 4–8 (n=3); severe changes in OA cartilage = HHGS ≥ 9 (n=2); control joints = healthy joints (no OA), with no macroscopic pathology (n=6).

²Immunohistochemical HMGB-1 staining was scored using the following system: – = no visible staining, + = low intensity staining, ++ = moderate intensity staining, +++ = high intensity staining.

³Except cartilage-bone tidemark labelling, which is provided separately in the last column of the table.

⁴Max. 10% of the nuclei were HMGB-1 immunoreactive in intact looking cartilage areas in OA cartilage samples.

⁵Samples from healthy joints, with no osteoarthritis and with HHGS value = 0.

⁶Up to 90% of the nuclei were HMGB-1 immunoreactive.

ratory Microwave Systems (Milestone, Sorisole, Italy).

Endogenous peroxidase was quenched in 0.3 % H_2O_2 in methanol for 25 minutes. Sections were incubated in 10% normal goat serum diluted in 0.1% bovine serum albumin (BSA) in 10 mM phosphate buffered 140 mM saline, pH 7.4 (PBS) for 1 hour prior to incubation for 12 hours at $+4^\circ\text{C}$ in 0.5 $\mu\text{g}/\text{ml}$ (in 0.1 % BSA-PBS) affinity purified rabbit anti-human HMGB-1 IgG cross-reactive with the highly conserved HMGB-1 of bovine origin (Abcam ab 18256, Cambridge, UK). Similarly diluted normal rabbit IgG (Jackson ImmunoResearch 011-000-003, Suffolk, UK) was used as a negative staining control.

Colour was developed using the Vectastain Elite ABC kit (Vector laboratories, Burlingame, CA) as described in detail elsewhere (12). If not stated otherwise, all incubations were performed at $+22^\circ\text{C}$ and the slides were washed 3x5 minutes in PBS between the steps.

Also another antibody, affinity purified monoclonal mouse anti-human HMGB-1 IgG_{2a} (Assay Designs, Inc. CSA-614, Ann Arbor, Michigan, USA) was used at 6 $\mu\text{g}/\text{ml}$ in 0.1% BSA-PBS, with an appropriate Vectastain Elite ABC kit. Similarly diluted monoclonal mouse IgG_{2a} (DAKO X0944, Dako, Glostrup, Denmark) against glucose oxidase of

Aspergillus niger was used as a negative staining control.

Statistical analyses

Fisher's exact test was used to explore differences in HMGB-1 expression data in bovine OA in different histological/histochemical grading system (HHGS) groups and in healthy control cartilage samples. *P*-values of less than 0.05 were considered statistically significant.

Results

General characterisation

Samples from healthy joints (n=6) did not contain any macroscopic or microscopic pathology (HHGS=0). All sampling areas of the OA joints were graded to four grades based on their macroscopic appearance and further scored into four categories based on microscopic findings (see Table I for the HHGS scores). As an example, Figure 1A shows an area with a macroscopic grade 3 change indicated with an arrow. Figure 1B shows a microphotograph of a deep surface cleft, a mix of chondrocyte clusters and hypocellular areas and a broken and partly duplicated tidemark. Also the proteoglycan content of the cartilage lesion affects the HHGS value and this was evaluated from Safranin O stained slides; Figure 1C shows some hypochromatic, proteoglycan poor (blue) areas. The area of the grade 3 lesion shown in Figure

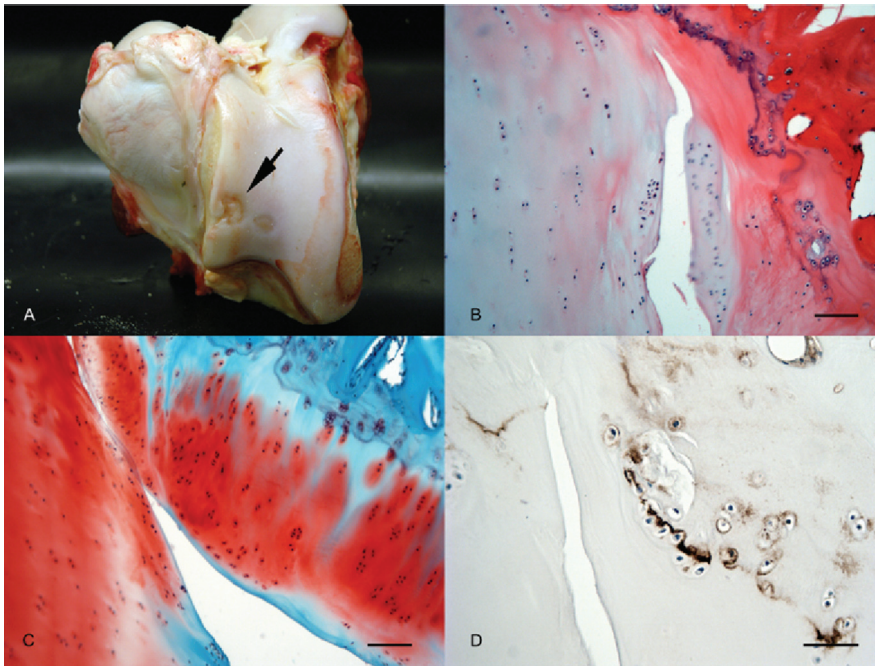


Fig. 1. **A.** Macroscopic grade 3 osteoarthritic joint lesion (arrow) in the lateral femoral condyle, which has a roughened articular surface. Histological/histochemical grading scale value for this sample was 8. **B.** Haematoxylin-eosin staining shows a surface cleft and chondrocyte cloning (clusters) intermingled with hypocellular areas. **C.** A consecutive section in Safranin O staining showing areas of hypochromasia. **D.** A consecutive section in immunohistochemical high mobility group box-1 (HMGB-1) staining. This figure series shows that the strong extracellular HMGB-1 deposits co-localise exactly at the cartilage-bone interface to those areas where also the most advanced OA changes are localised. In addition, cell associated HMGB-1 can be seen in chondrocytes and their extracellular matrix. Scale bar in B and C 100 μ m and in D 50 μ m.

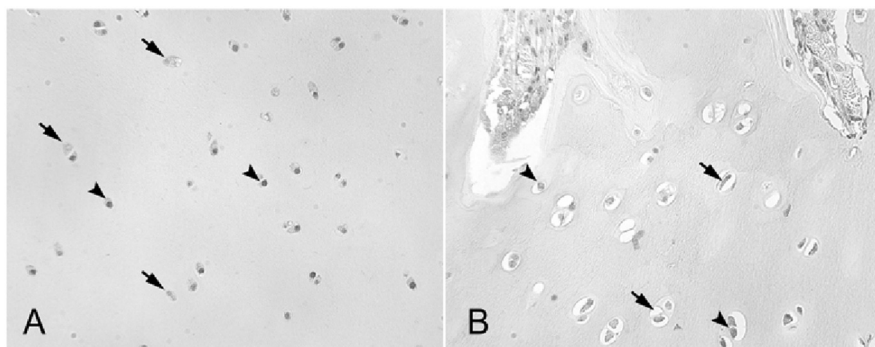


Fig. 2. **A.** High mobility group box-1 (HMGB-1) staining of healthy articular cartilage obtained from a healthy control joint not affected by OA and without any macroscopically visible pathology and with a histological and histopathological grading score (HHGS) value = 0. Approximately 90% of the chondrocyte nuclei are HMGB-1 immunoreactive (arrow heads). Some of the HMGB-1 negative cells are marked with arrows. **B.** HMGB-1 staining of the tidemark of a healthy control joint. Notice that no HMGB-1 immunoreactivity can be seen at the interface between the non-calcified and calcified articular cartilage.

1A has according to the haematoxylin-eosin (Fig. 1B) and Safranin O (Fig. 1C) staining a HHGS value 8. These HHGS values were compared with the immunohistochemical staining of HMGB-1, which in this same area formed parallel lines or waves, or was already almost granular (Fig. 1D). Analysis of the local

severity of the OA (HHGS values) and HMGB-1 staining showed a clear correlation between these two (Table I).

HMGB-1 staining of the healthy control samples

Both HMGB-1 antibodies (polyclonal and monoclonal) gave similar staining

patterns in all samples, for example, lack of nuclear staining in some cells was not antibody dependent. In the healthy control samples (no OA and no macroscopically visible pathology, HHGS value = 0) 90% of the chondrocyte nuclei were HMGB-1 positive (Fig. 2A). Deeper down in the cartilage the proportion of HMGB-1 positive cells was smaller and chondrocyte nuclei stained more weakly (Fig. 2B). In these healthy control samples there was no HMGB-1 in the cytoplasm or outside the chondrocytes in the pericellular or territorial cartilage matrix. In the healthy control samples there was no HMGB-1 staining in the tidemark, the area at the interface between the hyaline non-calcified articular cartilage and calcified cartilage (Fig. 2B, compared to Fig. 3 below).

Correlation between the HHGS value and HMGB-1 staining of the chondrocytes and their surrounding

Samples from OA joints were taken from macroscopically graded areas, but scored according the severity of the OA lesions using the HHGS value. These scores divided between different grades as follows: all samples from macroscopically healthy-looking "grade 1" OA cartilage (n=7) had a HHGS value = 0; two of the "grade 2" OA samples (n=3, with joint surface roughening and irregularities of the articular cartilage, which penetrated less than 2 mm into the cartilage) had a HHGS value in the 1-3 category and one had a HHGS value in the 4-8 category; two "grade 3" samples with joint surface roughening penetrating more than 2 mm had a HHGS value in the 4-8 category; and two "grade 4" samples taken from areas with full thickness, osteochondral lesions had a HHGS value in the ≥ 9 category.

In contrast to healthy cartilage samples, in intact-looking OA cartilage samples (HHGS = 0) most nuclei were HMGB-1 negative (Fig. 3A). This was confirmed by staining the nuclei of the same sample slides with haematoxylin which clearly showed that most of the chondrocyte nuclei indeed lacked HMGB-1 immunoreactivity (Fig. 3B). In mild OA lesions HMGB-1 staining was seen, mostly restricted to cell nu-

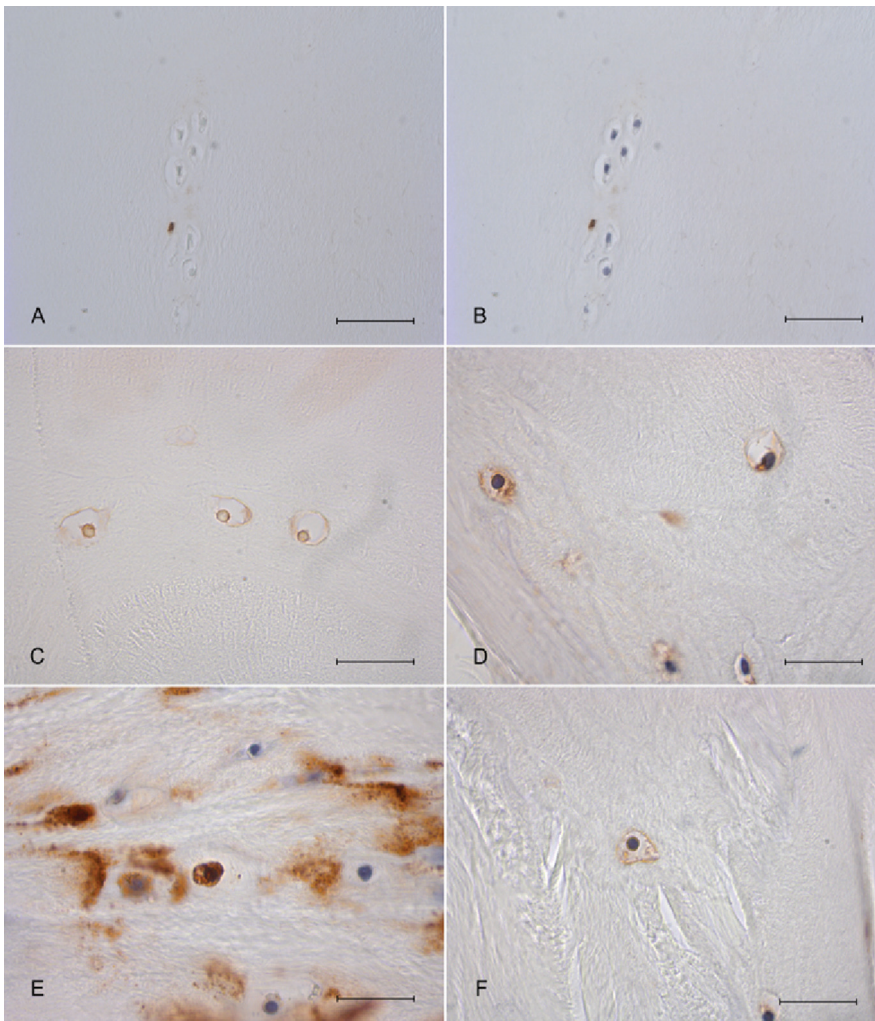


Fig. 3. High mobility group box-1 (HMGB-1) in bovine osteoarthritic cartilage.
A. In healthy-looking cartilage areas from an osteoarthritic joint only max. 10 % of chondrocyte nuclei were HMGB-1 positive, this view showing one such HMGB-1 immunoreactive cell nucleus.
B. The section used for the previous microphotograph shown in panel A was demounted, counterstained with haematoxylin and mounted again to clearly demonstrate that many of the chondrocyte nuclei are not immunoreactive for HMGB-1.
C. In mild osteoarthritis (OA) lesions HMGB-1 immunoreactive cells are more numerous and most of the HMGB-1 immunoreactivity is restricted to chondrocyte nuclei.
D. In moderate OA lesions HMGB-1 is more clearly seen translocated to the cell cytoplasm and in this field also occasionally secreted to the pericellular matrix intimately surrounding the chondrocyte located in its cartilage lacuna.
E. In severe OA HMGB-1 is more strongly expressed and clearly also deposited more widely in the extracellular, intra- or even interterritorial matrix surrounding the chondrocyte.
F. HMGB-1 formed occasionally ring-like structures suggesting membrane-bound staining. Avidin-biotin-peroxidase complex staining, no counterstain in A and C, haematoxylin counterstain in B, D, E and F. Scale bar length 20 μ m.

clei (Fig. 3C). In moderate OA lesions HMGB-1 was already seen clearly translocated to the cell cytoplasm (Fig. 3D) and occasionally to the pericellular matrix (Fig. 3D). In severe OA lesions HMGB-1 was also found in pericellular and intra- and inter-territorial matrix (Fig. 3E). In addition, some immunoreactive cells HMGB-1 were seen mainly as a membrane-type staining (e.g. Fig. 3F).

HMGB-1 at the tidemark and cartilage-bone interface

In contrast to the healthy control cartilage samples, in the OA joint samples HMGB-1 deposits were always seen in apparently intact as well as in mildly affected OA lesions as a continuous and faintly staining line at the interface between the articular cartilage and the underlying calcified cartilage and bone (Fig. 4A). In moderate OA lesions

HMGB-1 deposits were more intense and formed often several tidal waves at the tidemark (Fig. 4B). Finally, in severe OA lesions these often duplicated HMGB-1 deposits were even more intense and formed in many places irregular granular deposits at the tidemark and at the cartilage-bone interface (Fig. 4C). Staining controls confirmed the specificity of the staining (staining in Fig. 4D and staining control of the consecutive section in the insert to Fig. 4D).

TNF- α stimulation of chondrocyte pellets

TNF- α stimulation of chondrocytes lead to translocation of HMGB-1 from the nucleus to the cytoplasm. Prior to stimulation (Fig. 5A) only 10% of the cells were HMGB-1 positive and HMGB-1 was visible only in the nuclei of these cells. After TNF- α stimulation the number of immunoreactive cells rose from 10% to 20% and HMGB-1 was translocated from the nuclei to the cytoplasm (Fig. 5B) and some specific HMGB-1 staining was also seen in the extracellular matrix. Staining control confirmed the specificity of the staining (Fig. 5C).

Comparison of HMGB-1 grade with the HHGS values and healthy controls

HMGB-1 grades differed significantly in different HHGS groups, i.e. in healthy-looking, mild, moderate and severe OA (and in healthy control cartilage) as to nuclear, cytoplasmic and extracellular HMGB-1 staining and as to the HMGB-1 staining of the tidemark.

Discussion

In a single joint the HHGS value, i.e. the degree of local OA changes, varied according to the sampling region, probably reflecting the biomechanical loading pattern, joint incongruities and injuries and varying inherent disease susceptibility of different articular regions. Comparison of the severity of the OA lesions and HMGB-1 staining patterns disclosed an interesting association between these two. First, most but not all chondrocyte nuclei in healthy control samples were HMGB-1 positive

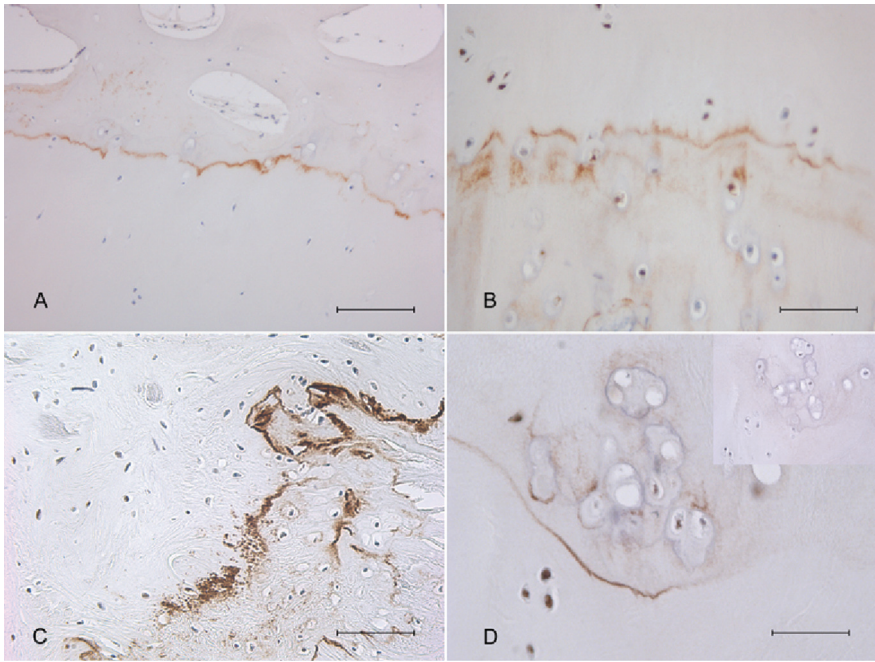


Fig. 4. High mobility group box-1 (HMGB-1) in the tidemark between the articular cartilage and bone. **A.** In apparently healthy-looking or mildly affected articular cartilage areas from osteoarthritic joints HMGB-1 forms smooth and intact linear deposits at the tidemark between the cartilage and bone. **B.** In moderate osteoarthritis (OA) lesions HMGB-1 deposits are increased and occur at intervals producing a wave-like pattern. **C.** In a severe OA lesion, with a cleft protruding down to the subchondral bone, HMGB-1 deposits are even more intense forming granular deposits and an interrupted line at the tidemark. **D.** This panel shows linear HMGB-1 deposits at the tidemark and the insert to its upper right shows the negative control staining of the consecutive section of exactly the same area, which confirms specificity of the HMGB-1 immunolabelling. Scale bar length in A and C 100 μ m and in B and D 50 μ m.

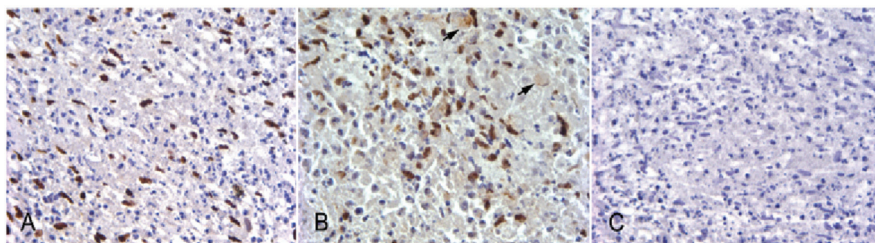


Fig. 5. HMGB-1 translocation from the nucleus to the cytoplasm in tumour necrosis factor alpha (TNF- α) stimulated stem cell derived chondrocytes.

A. Prior to the TNF- α stimulation, approximately 10% of chondrocytes are high mobility group box-1 (HMGB-1) positive. Positive reaction is seen mainly in the nuclei of the cells as a well restricted, intense colour reaction.

B. After TNF- α stimulation the number of immunoreactive cells arises. HMGB-1 is translocated from the nuclei also to the cytoplasm of these cells (arrows). Some HMGB-1 immunoreactivity is also seen in the extracellular matrix as a faint specific background staining.

C. A negative staining control confirms the specificity of the HMGB-1 immunostaining. Avidin-biotin-peroxidase complex staining counterstained with haematoxylin.

and, surprisingly, most of the chondrocyte nuclei located in healthy-looking OA cartilage were HMGB-1 negative. Due to the universal nucleosomal presence of HMGB-1 and its essential role in DNA packaging in eukaryotic cells (1), all nuclei must naturally contain HMGB-1. As a matter of fact, such lack of staining of the cartilage cells

and normal muscle cells can be seen in publication microphotographs in some earlier publications (13, 14). In one of these papers, this issue was even raised and addressed with lack of sensitivity of the staining method (13). Although this is in principle possible, it is unlikely for two reasons. First, HMGB1 is a highly abundant nuclear protein with approxi-

mately 10^6 molecules per cell (15). Second, its amount varies quite little between different types of eukaryotic cells (16) although HMGB-1 amount doubles in dividing cells (17) and increased 1.5–2.5-fold upon oestrogen stimulation of oestrogen-dependent breast carcinoma cells (18). DNA is tightly packed into nucleoprotein complexes, which are composed of nucleosomal repeating units. HMGB-1 is an abundant non-histone protein, which also binds to and bends DNA and participates in the transcription of multiple genes (1). Native HMGB-1 inhibits DNA replication, but after post-translational modification by acetylation this effect is diminished (19). This suggests that post-translational modifications allow at least partial dissociation of HMGB-1 from its molecular binding partners seen as nuclear HMGB-1 staining. It could be speculated that in resting chondrocytes HMGB-1 is so tightly bound to its target molecules that, in spite of its presence, its immunoreactive sites are blocked by a steric hindrance or conformational changes and can therefore not be visualised; such a phenomenon in antibody binding has been earlier reported by Romani and co-workers and was explained by steric hindrance or conformational changes (20). Apparently, chondrocytes in healthy cartilage participate more actively in cartilage remodeling than chondrocytes in mild OA; alternatively, this difference in nuclear HMGB-1 staining in healthy and mild OA cartilage sections might reflect the younger age of the healthy controls compared to the OA animals. The above-mentioned activation-induced acetylation alters the charge of HMGB-1, inhibits its binding to DNA and enables its translocation from the cell nucleus to the cell cytoplasm (21). Indeed, upon increasing severity of OA, HMGB-1 became progressively translocated from the cell nuclei to the cell cytoplasm and was further secreted (2–3) and/or passively released (3) into the extracellular space surrounding the chondrocyte lacunae. It is concluded that HMGB-1 could be used as a topological marker for the severity of the involvement of the cartilage in OA. Non-calcified cartilage-calcified carti-

Table II. Statistical comparison of high-mobility group box-1 (HMGB-1) staining in bovine osteoarthritis in different histological/histochemical grading system (HHGS) groups and in healthy controls.

HHGS	Nuclear		Cytoplasmic	Extracellular		Tidemark			
	<10%	>10%		Moderate	Strong	No visible reaction	Faint, linear	Parallel lines/waves	Scattered, granular
Intact (n=7)	7	0	0	0	0	0	7	0	0
Mild (n=2)	0	2	0	0	0	0	2	0	0
Moderate (n=3)	0	3	3	3	0	0	0	3	0
Severe (n=2)	0	2	2	0	2	0	0	0	2
Controls (n=6)	0	6	0	0	0	6	0	0	0
Fisher's exact test	p<0.001		p<0.001	p<0.001		p<0.001			
p<0.001 according to most extensive staining									

lage tidemark interface in the healthy control cartilage samples was HMGB-1 negative, while HMGB-1 was detected at this interface in all of the OA samples, even in the areas where no lesions apparent to the human eye in HHGS scoring were evident. It has to be concluded that in OA joints HMGB-1 was translocated from the nuclei to the cytoplasm and then secreted extracellularly to be deposited at the tidemark.

The HMGB-1 release has been generally considered a feature of necrosis but not apoptosis. HMGB-1 can also be released by activated cells although this has not been studied before in chondrocytes. Hypoxia is one of those stimuli able to induce translocation of HMGB-1 from the nucleus to the cell cytoplasm and further via a non-conventional lysosomal secretory route to the extracellular space. We show that stimulation with TNF- α mobilised HMGB-1 so that it could be more often visualised in nuclei and some of it had been translocated to cytoplasm and even extracellularly. It remains unclear, what is the mechanism of deposition of HMGB-1 in OA around chondrocytes and in particular at the tidemark. However, in immunohistopathological analysis extracellular HMGB-1 was always seen at this location in OA. In the growth plate and articular cartilage the calcified cartilage is resorbed by chondroclasts and replaced by blood vessels, osteoblasts and bone in enchondral bone formation. HMGB-1 is chemotactic to monocyte/macrophages, vascular endothelial cells and osteoblasts (6, 8). In addition, HMGB-1 participates in the recruitment and differentiation of

MSCs along the osteoblastic lineage (7). These earlier observations and the current finding on the linear deposition of HMGB-1 at the tidemark, even in apparently healthy-looking or mildly affected OA lesions, suggests an involvement in the mineralisation and enchondral bone formation at the cartilage-bone interface area, perhaps as a result of hypoxia-induced HMGB-1 release from the hypertrophic chondrocytes in the deep cartilage zone.

In advancing OA lesions HMGB-1 deposits at the cartilage-bone interface increased progressively. In moderate lesions HMGB-1 often already formed multiple parallel wave-like lines suggesting pulse-like release at intervals. This correlates well with the duplication and often even multiplication of the tidemark upon increasing age (22) and in OA (23). Furthermore, in severe OA lesions HMGB-1 staining of the tidemark was intense and granular. Calcified cartilage fastens the cartilage to subchondral bone and provides a calcified scaffold for the vascular in-growth and enchondral bone formation. Such increasing HMGB-1 deposition at the tidemark upon advancing OA is compatible with its role in enchondral bone remodelling in mildly affected OA and subchondral bone sclerosis and osteophyte formation in more advanced OA. Cell surface staining was seen in some cells in the moderate and severe OA lesions. Receptors for advanced glycation end products (RAGE) are up-regulated in OA chondrocytes (24) and may take part in the degradation of cartilage (25). Chondrocyte toll-like receptors 2 (TLR2) and 4 (TLR4) also bind HMGB-

1. Cell membrane HMGB-1 staining indicates that not only is HMGB-1 secreted and/or passively released (26), but it also binds to its cellular targets compatible with auto- and paracrine mechanism of action. HMGB-1 and its receptors RAGE, TLR2 and TLR4 deserve more attention in OA.

Finally, the present work discloses an amazing microheterogeneity in the HHGS score and HMGB-1 staining pattern. The association between the severity of histological OA involvement and HMGB-1 staining pattern indicates that this is a truly significant (patho)physiological phenomenon and suggests that OA even in a single joint proceeds at different pace in topologically distinct areas of the joint.

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