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Chondrocyte Differentiation in Human Osteoarthritis: Expression of Osteocalcin in Normal and Osteoarthritic Cartilage and Bone

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Abstract. Osteocalcin (OC), which is a marker of the mature osteoblasts, can also be found in posthypertrophic chondrocytes of the epiphyseal growth plate, but not in chondrocytes of the resting zone or in adult cartilage. In human osteoarthritis (OA), chondrocytes can differentiate to a hypertrophic phenotype characterized by type X collagen. The protein- and mRNA-expression pattern of OC was systematically analyzed in decalcified cartilage and bone sections and nondecalcified cartilage sections of human osteoarthritic knee joints with different stages of OA to investigate the differentiation of chondrocytes in OA. In severe OA, we found an enhanced expression of the OC mRNA in the subchondral bone plate, demonstrating an increased osteoblast activity. Interestingly, the OC protein and OC mRNA were also detected in osteoarthritic chondrocytes, whereas in chondrocytes of normal adult cartilage, both the protein staining and the specific mRNA signal were negative. The OC mRNA signal increased with the severity of OA and chondrocytes from the deep cartilage layer, and proliferating chondrocytes from clusters showed the strongest signal for OC mRNA. In this late stage of OA, chondrocytes also stained for alkaline phosphatase and type X collagen. Our results clearly show that the expression of OC in chondrocytes correlates with chondrocyte hypertrophy in OA. Although the factors including this phenotypic shift in OA are still unknown, it can be assumed that the altered microenvironment around osteoarthritic chondrocytes and systemic mediators could be potential inducers of this differentiation.

Key words: Osteocalcin — Cartilage — Osteoarthritis — Chondrocytes — Differentiation.

Osteocalcin (OC) is the major noncollagenous protein in bone. It has a molecular weight of 5.8 kD and contains three γ-carboxyglutamic (Gla) acid residues [1]. The formation of the Gla acid residues occurs posttranslationally and is a vitamin K-dependent process, which is stimulated by vitamin D3. Although the function of OC *in vivo* is unclear, however, the binding of calcium and hydroxyapatite via the Gla acid residues indicates a role in the mineralization process. Hauschka et al. [2] have hypothesized that OC might have regulatory functions to prevent excessive bone mineralization. Ducy et al. [3] showed recently that bone development is normal in OC-deficient mouse mutants until birth. Six months after birth, however, these mutants had an increased bone mass. Therefore, OC is proposed to regulate bone matrix formation.

Numerous studies have investigated the role of OC in cartilage and bone development. The postnatal formation of the bone matrix is accompanied by the differentiation of pluripotent, mesenchymal stem cells into chondrocytes. These chondrocytes may hypertrophy and form a cartilaginous matrix, which later serves as a scaffold for the mineralization. The differentiation of chondrocytes and the mineralization of the extracellular matrix are strictly regulated and occur in a sequential manner [4–6]. During endochondral bone formation, chondrocytes of the epiphyseal growth plate are arranged in zones according to morphological and phenotypical criteria. Chondrocytes of the hypertrophic cartilage, located near the mineralized bone matrix, have a large cell volume and a high metabolic activity [7]. These hypertrophic chondrocytes express not only matrix molecules, which are typical for chondrocytes of the resting zone or adult articular cartilage such as cartilage collagen types II or IX [8, 9], but also type X collagen. In adult articular cartilage, type X collagen is restricted to the deep cartilage zone and the adjacent calcified cartilage [10, 11].

In vitro, fetal chicken chondrocytes from the hypertrophic zone were found to differentiate in cell culture and express proteins like alkaline phosphatase (ALP), OC, and osteopontin [12–15], which characterize a posthypertrophic phenotype. In addition, expression of ALP, osteopontin, and osteonectin was identified in the growth plate chondrocytes from other species such as mouse or human [16, 17]. When analyzing the ossification process during fracture healing using animal models, cells expressing markers of a chondrocyte and osteoblast phenotype in the fracture callus were identified [18–21]. Therefore, it has been hypothesized that chondrocytes may undergo a further differentiation from the hypertrophic stage into a posthypertrophic-like phenotype.

In the mature adult articular cartilage, chondrocytes have a stable phenotype with a low metabolic activity [22]. However, during the disease process of OA, phenotypic changes occur in the synthesis of extracellular matrix components. Expression of collagens such as type II and VI are increased [23–25] and the synthesis of noncollagenous matrix molecules, such as proteoglycans, fibronectin, and tenascin are also activated [26, 27]. The increased expression of type X

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collagen in the calcified cartilage zone of osteoarthritic cartilage samples suggests that adult osteoarthritic chondrocytes may differentiate into a hypertrophic phenotype [11], as described for epiphyseal growth plate cartilage.

Due to the lack of data on the differentiation potential of adult mammalian articular chondrocytes *in vivo*, we focused on the expression of OC, ALP, and type X collagen in human OA. In this study, we have demonstrated that in osteoarthritic human cartilage chondrocyte hypertrophy is accompanied also with the expression of OC.

Materials and Methods

Cartilage Samples

A total of 33 osteoarthritic cartilage/bone samples were obtained from 17 patients with primary OA (54–75-year-old donors) undergoing total knee replacement. Clinical data were carefully reviewed to exclude any forms of secondary OA and inflammatory joint diseases such as rheumatoid arthritis. Normal cartilage was collected from five human knees at the time of autopsy within 18 hours after death (34–56-year-old donors). In these five knees, the femorotibial and patellafemoral compartments were totally free from any signs of OA. The localizations, where samples were taken from, were carefully monitored by video printouts. Osteoarthritic changes were classified histomorphologically, using the grading system of Otte [28] briefly explained below. Normal cartilage (grade 0) has a smooth surface and a regular zonal distribution of the chondrocytes. Mildly affected cartilage (grade I) has a fibrillated surface and a superficial loss of proteoglycans (safranin-O staining); the zonal structure is intact. Moderately affected cartilage (grade II) has deep clefts reaching down to the middle cartilage zone; clusters of proliferating chondrocytes are present. In severely affected cartilage (grade III), clefts are reaching down to the deep zone; the tangential zone is lost and chondrocyte clusters are present. In grade IV, the cartilage tissue was fully destroyed. Five normal samples, 10 samples with slight, 10 samples with moderate, 10 samples with severe osteoarthritic lesions, and 3 samples with a complete loss of the cartilage layer were included in this study.

Preparation of Tissue Samples

Cartilage/bone samples (1.0 cm thick) with a cartilage surface of about 2.0 cm × 0.5 cm were incubated in freshly prepared paraformaldehyde, 4% (w/v), buffered with 0.01 M sodium phosphate, pH 7.4, containing 0.14 M NaCl (PBS) for 12 hours at 4°C. The tissue samples were decalcified in diethyl-pyrocarbonate (DEPC)-treated 0.2 M EDTA, pH 8.0 for several weeks at 4°C. The buffer was changed twice a week. Tissue specimens were then dehydrated in grading concentration of ethanol and xylene and finally embedded in paraffin. In addition, adjacent cartilage sections were taken without subchondral bone. These samples were fixed and embedded without undergoing the long decalcification procedure. Cartilage/bone and cartilage sections (5 μm) were mounted on glass slides precoated with 1% (v/v) 3-aminopropyl triethoxysilane and heated for 90 minutes at 65°C.

Preparation of Osteocalcin RNA Probe

Total RNA was extracted from human knee cartilage. Cartilage (0.5 g) was frozen in liquid nitrogen and pulverized using a stainless steel mortar and pestle. Extraction was performed using acid guanidinium isocyanate as described by Chomczynski et al. [29]. Total RNA (1 μg) was transcribed into single-stranded cDNA using AMV reverse transcriptase (Boehringer Mannheim, Mannheim, Germany) and Oligo-p(dT) 15 as a primer according to the manufacturer's protocol (Boehringer Mannheim). Double-stranded cDNA was generated by polymerase chain reaction (PCR) using modified primers containing a new HindIII site in the upstream primer (5′-ctc aag ctt ctc gcc cta ttg g-3′), and a new EcoRI site in the downstream primer (5′-tgg gaa ttc agg gca agg gca a-3′). The primer pair spanned 376 bases coding for the human mRNA of the osteocalcin gene (Accession No.: X51699) [30]. The PCR product as well as the cloning vector (pT3/T7α18 Life Science, Eggenstein, Germany) were digested with the restriction enzymes EcoRI and HindIII. Ligation, cloning, and amplification of the osteocalcin cDNA fragment were performed according to Sambrook et al. [31]. The amplified plasmid DNA was checked by multiple restriction digestions as well as by sequencing (Perkin-Elmer, Abiprism, Weiterstadt, Germany). For *in vitro* transcription and labeling of antisense and sense riboprobes, the plasmid DNA was digested with HindIII for the antisense probe or EcoRI for the sense probe. It was purified by phenol/chloroform extraction and transcribed using 1 μg of linearized plasmid DNA, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dUTP, and 0.35 mM digoxigenin-11-UTP in 40 mM Tris-HCl, pH 8.0, 6 mM MgCl2, 10 mM dithiotritol, 2 mM spermidine, and 40 U T7 RNA-Polymerase (for antisense probe) or 40 U T3 RNA-Polymerase (Boehringer Mannheim) for sense probe. The components were incubated for 2 hours at 37°C. Template DNA was removed with 20 U DNase I (Boehringer Mannheim). The reaction was stopped by adding 2 μl 0.2 M EDTA pH 8.0 RNA was further purified by ethanol precipitation and resuspended in 100 μl DEPC-treated H_2O. Transcripts were checked for integrity, length (379 nucleotides for anti-sense probe, 386 nucleotides for sense probe), and labeling efficiency by denaturing gel electrophoreses followed by Northern blotting and digoxigenin detection using the DIG-nucleic-detection-kit (Boehringer Mannheim). For *in situ* hybridization, approximately 100 ng of OC antisense or sense riboprobe were used for each tissue section. Immediately before use, riboprobes were incubated for 10 minutes at 96°C followed by 2 minutes at 4°C.

In Situ Hybridization

Tissue sections were deparaffinized in xylene and rehydrated through graded ethanol (100%–50%). Sections were incubated with Proteinase K, 50 μg/ml in 100 mM Tris, pH 7.5, with 50 mM EDTA, for 15 minutes at 37°C and postfixed in 4% (w/v) paraformaldehyde/PBS (phosphate-buffered saline) for 1 minute. Sections were briefly washed in DEPC-treated double-distilled water and acetylated two times for 5 minutes in 0.1 M triethanolamine by adding 0.3% (v/v) acetic acid anhydride. Sections were briefly incubated in PBS, dehydrated using increasing concentrations of ethanol (50%–100%), and then air-dried under dust-free conditions. Sections were prehybridized with a commercial hybridization buffer containing 0.5 M NaCl and blocking reagent (ECL gold hybridization buffer, ECL blocking reagent) (Amersham, Braunschweig, Germany). The buffer contained 8 M urea, which is equivalent to 50% (v/v) formamide to reduce the melting temperature of nucleic acid hybrids. Sections were carefully covered with Parafilm (Sigma, Munich, Germany) and prehybridization was carried out for 1 hour at 43°C for OC probe and 28S RNA probe. Fifty microliters of the hybridization solution containing 100 ng of the OC probe or 10 ng of the 28S RNA probe was applied to each section. The sections were then covered with parafilm and incubated in a humidified chamber overnight at 43°C. Sense strand *in situ* hybridization was also performed with an OC probe using similar conditions as for the anti-sense hybridization. After hybridization, the sections were washed with 1 × sodium chloride, sodium citrate (SSC) for 10 minutes at room temperature and with 0.3 × SSC at 40°C for 1 hour. Nonspecific binding was removed by RNase treatment (T1-RNase 25 U/ml combined with RNase A, 5 μg/ml, in 5 mM Tris, 0.5 M NaCl, pH 7.5, 5 mM EDTA) for 1 hour at 37°C. Sections were briefly washed in 1 × SSC at room temperature, then washed in 0.1 × SSC for 2 hours at 45°C. Bound probes were detected using a commercially available nucleic-acid-detection-kit (Boehringer Mannheim). Briefly, sections were incubated in blocking solution containing 10% (v/v) fetal calf serum. After blocking, an anti-digoxigenin ALP-labeled antibody was added and bound antibodies were detected after incubation with the substrate solution (X-phosphate/NBT) overnight. Sections

were counterstained with 0.5% (w/v) light green (Merck, Darmstadt, Germany) in 5% (v/v) ethanol and mounted in Entellan (Merck).

Controls

The specificity of the OC probe was verified by Northern blotting using mRNA from a human osteosarcoma cell line MG-63 (ECACC, Salisbury, UK) (data not shown). Cells were stimulated with $1\alpha,25(OH)_2$ vitamin D3 (10.4 μM) and Vitamin D2 (2.5 μM) (both Sigma) for 2 weeks. The OC riboprobe detected a single band of about 0.6 kilobases corresponding to the size of the OC mRNA. In addition, nucleic acid sequence homologies were tested using the FASTA program of the EMBLE Institute in Heidelberg, Germany. Homologies to listed proteins were less than 62% within an overlapping region of maximal 114 base pairs. Integrity of the mRNA in cartilage and bone was assessed using a 115 base pair antisense riboprobe for the human 28S RNA (nucleotide 4400–4415, Accession No.: M11167, Ambion, Austin, USA). Probe concentration and hybridization conditions were similar to those used for the OC probe. Unspecific binding of the probes to DNA sequences was excluded by an additional RNase treatment of the specimen before *in situ* hybridization. In all RNA predigested controls, no specific cellular staining could be observed by *in situ* hybridization using the OC or the 28S RNA probe.

Immunolocalization of OC

Immunolocalization of OC was performed on decalcified cartilage/bone sections as well as on nondecalcified cartilage sections. Tissue sections were deparaffinized in xylene and rehydrated. The sections were then digested with 2 mg/ml hyaluronidase (Merck) in PBS, pH 5.5 for 15 minutes at 37°C and with 1 mg/ml pronase (Boehringer, Mannheim) in PBS pH 7.4 for 30 minutes at 37°C. After two washes in PBS, sections were incubated in PBS containing 5% (w/v) bovine serum albumin (BSA). Two well-characterized anti-OC monoclonal antibodies, 2H9 and 8H12, were used [32]. Both antibodies recognize full-length human OC and large N-terminal OC mid-fragment. Antibody 8H12 binds to the N-terminal region (amino acid 7–19) and 2H9 binds to the mid-region (amino acid 20–43) of OC. The antibodies recognize both γ-carboxylated and undercarboxylated OC forms. Protein G-purified antibodies (200 ng) were diluted in 1% (w/v) BSA/PBS and incubated overnight at 4°C. After three washes in Tris-buffered saline (TBS), a biotin-labeled donkey anti-mouse antibody (Dianova, Hamburg, Germany) diluted 1:80 in 1% (w/v) BSA/TBS was added and incubated for 30 minutes at room temperature. After that, ALP-labeled streptavidin was added according to the protocol of the manufacturer (Dako, Hamburg, Germany). After three short washes in TBS, bound antibodies were visualized using Fast Red (Sigma) as a color substrate. To exclude nonspecific background staining, sections were incubated with mouse antisera (Dako, Hamburg, Germany) and immunohistochemical detection was performed as described above.

Immunolocalization of Type X Collagen and Alkaline Phosphatase

For type X collagen detection, decalcified cartilage/bone sections were enzymatically pretreated with 0.02 mg protease (type XXIV) (Sigma) for 60 minutes at 37°C, followed by hyaluronidase treatment as described above. The monoclonal anti-type X collagen antibody (clone 53) characterized and described previously [33] was used for the detection of type X collagen. Sections were incubated with 1 μg/ml antibody in 1% (w/v) BSA/PBS overnight at 4°C. Detection of bound antibodies was performed according to the detection of the anti-OC antibodies. For ALP immunohistochemistry, hyaluronidase and pronase pretreatment were used according to the OC protocol described above. A monoclonal antibody against ALP (clone B4–78) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, USA). Specificity of this antibody against bone ALP was described previously [34]. Sections were incubated with 1 μg antibody diluted in 1% (w/v) BSA/PBS overnight at 4°C. The protocol for the visualization of bound antibodies was according to the OC protocol described above. For collagen type X and ALP, negative controls were performed using a mouse antisera.

Results

OC Expression in the Subchondral Bone Plate

Osteoarthritic sections and sections without any histological signs of osteoarthritis were incubated with monoclonal OC antibodies and analyzed for OC protein staining. Normal samples (5 out of 5) and samples with minor osteoarthritic lesions (10 out of 10) showed a homogenous staining within the bone matrix (Fig. 1A). The margins of the subchondral bone plate stained more intensively, as compared to the bone matrix (Fig. 1B). When analyzing moderately and severely affected cartilage/bone sections, a thickening and condensation of the subchondral bone plate was observed in the central weight-bearing areas. Staining of the OC-protein was almost homogeneously distributed within the bone trabeculae (Figs. 2A, 2B). The staining of OC was intense, especially at the margins of the bone trabecular, but no significant differences could be observed when compared with the protein deposition shown in normal or slightly affected cartilage/bone samples.

OC, a 5.8 kDa protein, may diffuse within this tissue during the long process of decalcification. To evaluate the significance of the observed protein staining pattern, OC gene expression was analyzed using *in situ* hybridization. When analyzing normal and mildly affected cartilage/bone sections, only two sections of the mildly affected group showed a faint signal in osteoblasts, which were lining the subchondral bone trabeculae (Fig. 1D). In moderately affected cartilage/bone sections, 6 out of 10 samples showed expression of OC mRNA in osteoblasts (data not shown). In all severely affected samples, OC gene expression could be seen in the osteoblasts (Fig. 2C). In all three tissue samples from end-stage osteoarthritis, which had total loss of the cartilage layer, bone cysts within the subchondral bone plate could be observed. Osteoblasts, lining the margin of these cysts, showed strong expression of OC mRNA (Figs. 3A,B). To exclude a degradation and loss of mRNA during decalcification and fixation, samples were incubated with the 28S RNA probe as a positive control. As shown, in normal cartilage/bone sections, mRNA signal could be observed in all nucleated cells (Fig. 1E).

OC Expression in Cartilage

OC may be released from bone and diffuse into the cartilage layer during the decalcification procedure and produce false-positive results. Therefore, only cartilage samples without the subchondral bone plate, which had not undergone the decalcification procedure, were used to investigate the OC protein expression in cartilage. Both OC antibodies used in this study failed to detect OC protein in all normal cartilage or in cartilage samples with slight osteoarthritic lesions (10 out of 10) (data not shown). However, in moderate OA, 4 out of 10 samples showed a cellular staining for OC (Figs. 4A,B). The staining intensity was faint and vis-

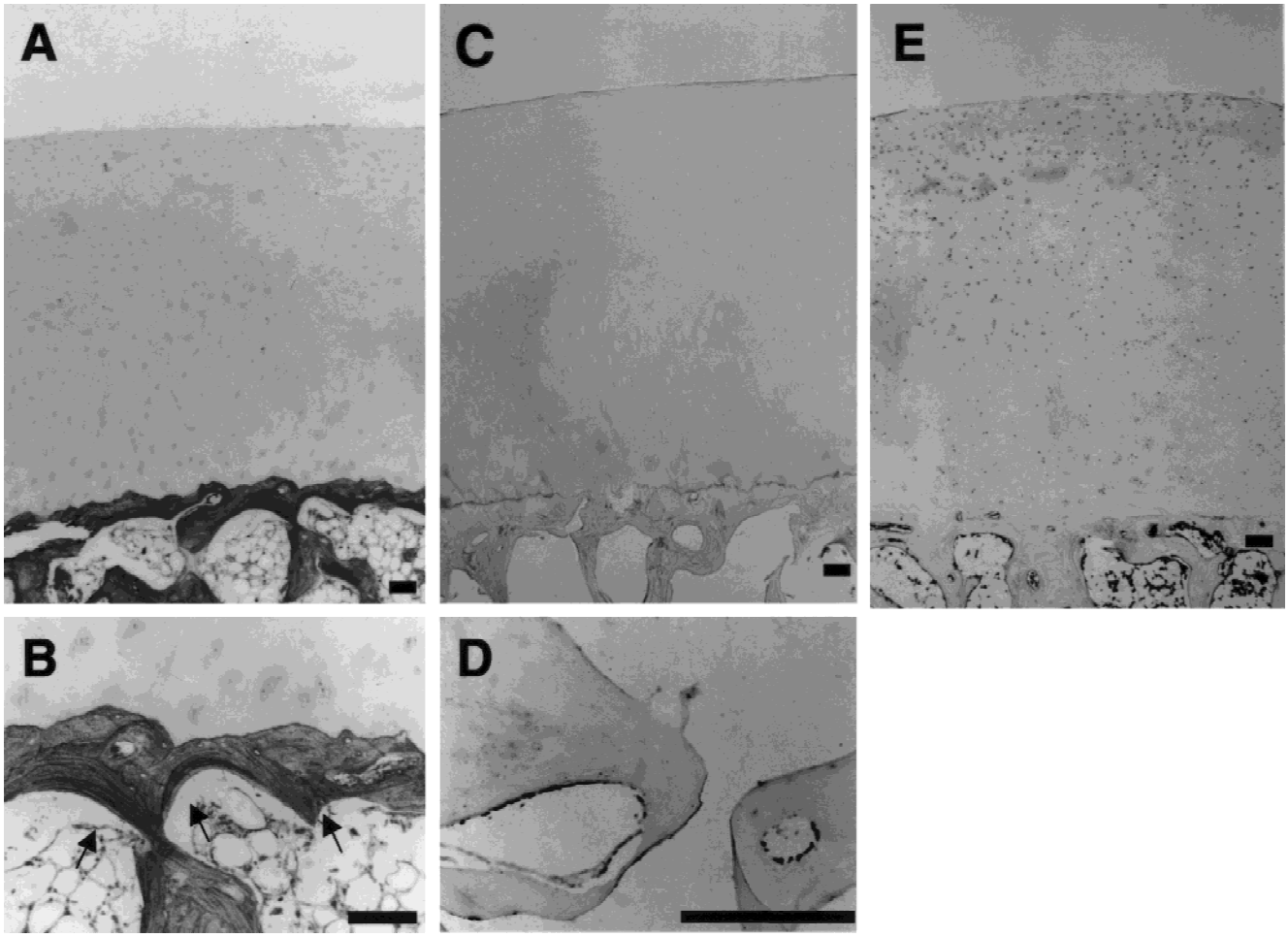

Fig. 1. Decalcified paraffin sections from femoral knee cartilage and bone from the central weight-bearing area (54-year-old man; original magnifications A, C, E: 30×; B, D: 125×, bars = 0.4 mm). **(A)** Staining for OC using a monoclonal antibody against recombinant human OC (8H12) as described in Materials and Methods. OC is distributed homogeneously within the bone matrix. No staining is seen in the cartilage matrix. **(B)** Higher magnification of the subchondral bone plate of A. Slightly enhanced deposition of OC is notified at the margins of the subchondral bone plate (→). Deposition of OC also visible in the bone marrow may be caused by diffusion during the decalcification procedure. **(C)** *In situ* hybridization using a digoxigenin-labeled antisense riboprobe (see Materials and Methods). No specific mRNA signal is detected in chondrocytes. **(D)** Higher magnification of the subchondral bone shows OC gene expression in osteoblasts lining the bone trabeculae. **(E)** Control *in situ* hybridization with the 28S mRNA probe. mRNA signal is observed in all nucleated cells.

ible only in the middle and adjacent areas of the deep zone. Due to the loss of the superficial zone in this state, chondrocytes from the middle zone are now localized near to the articular surface. In severe stages, parts of the upper middle zone are also lost. In the remaining cartilage layer, staining intensity was markedly increased in all samples (Fig. 5A). Proliferating chondrocytes especially, arranged in clusters showed a strong cellular staining. Deposition of OC protein within the cartilage matrix was not observed. The staining intensity strongly depended on the previous enzymatic digestion. Without hyaluronidase/pronase treatment, no or only a faint staining was observed. Incubation of samples with mouse sera as a negative control produced no staining (data not shown).

Gene expression was analyzed on nondecalcified cartilage sections along with decalcified cartilage/bone sections. In chondrocytes of normal cartilage, no staining for OC mRNA was observed (5 out of 5) (Fig. 1C). The 28S RNA probe used as a positive control demonstrated that mRNA was present (Fig. 1E). In moderately affected samples, 5 out of 10 were positive. The distribution of a specific OC mRNA signal was limited to the lower middle zone and deep cartilage zone (Fig. 4C). In severely affected samples, chondrocytes from the deep zone again produced a strong signal (7 out of 10) (Fig. 2C, 5B). Chondrocyte clusters, present in moderately and severely affected osteoarthritic cartilage, demonstrated a prominent OC mRNA signal (8 out of 10) (Fig. 5C). OC mRNA expression was similar in cartilage sections and cartilage/bone sections, which had undergone the decalcification procedure.

To further characterize the phenotypical status of normal and osteoarthritic chondrocytes, ALP and type X collagen expression was investigated. The staining pattern is schematically summarized in Table 1. Alkaline phosphatase protein expression was observed in the extracellular cartilage matrix of normal and osteoarthritic samples (Fig. 6). In normal cartilage, ALP staining was limited to the territorial matrix of chondrocytes from the deep zone (4 out of 5) (Fig.

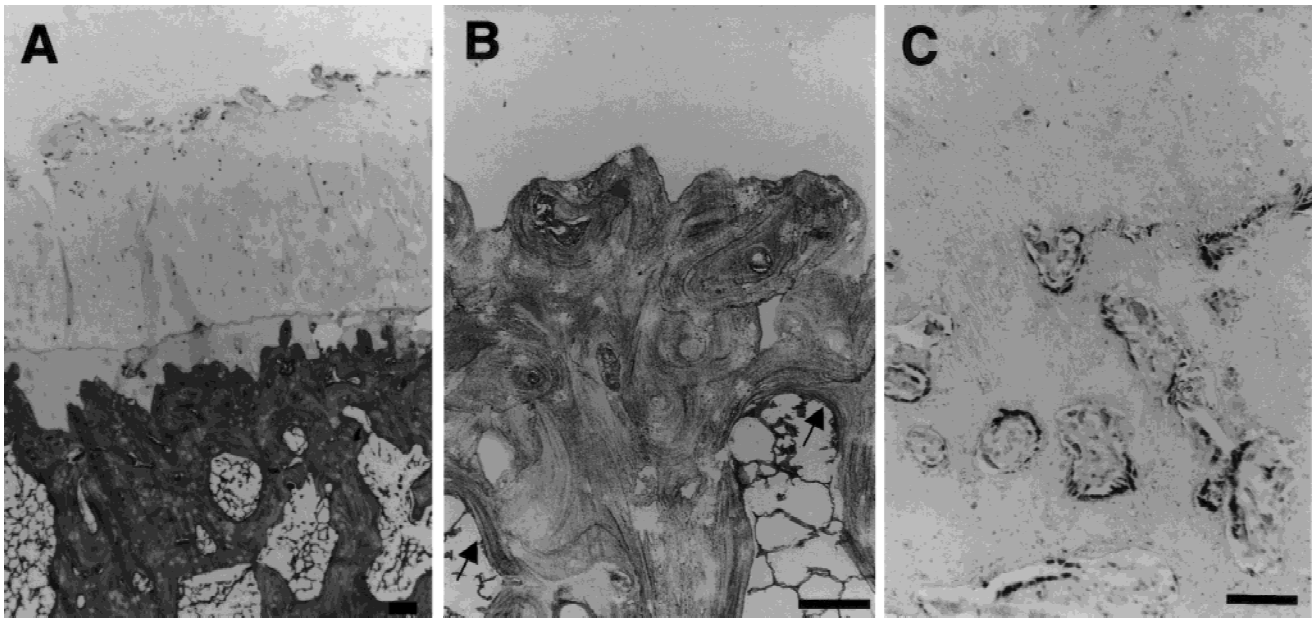

Fig. 2. Decalcified paraffin sections of femoral knee cartilage and subchondral bone with severe OA (72-year-old woman; magnifications A: 30×; B, C: 125×, bars = 0.4 mm) characterized by the loss of superficial tangential cartilage zone and fissures down to the middle zone. **(A)** The subchondral bone mass is increased (same magnification as in Fig. 1A). Samples were immunostained with OC as described in Materials and Methods. Chondrocytes show staining for OC, which may result from diffusion and unspecific deposition of OC during the decalcification procedure. The subchondral bone stains homogeneously. **(B)** Higher magnification the subchondral bone plate of 2A. Enhanced deposition of OC is notified on the margins of the subchondral bone compartment (→). Deposition of OC is also visible in the bone marrow. **(C)** Localization of OC mRNA is shown by *in situ* hybridization in osteoblasts lining a bone trabecula and in chondrocytes from the deep cartilage layer.

6A). Osteocytes and the margins of the subchondral bone trabeculae also stained for ALP. In samples with moderate and severe signs of OA, chondrocytes from the deep cartilage layer showed both cellular and diffuse territorial staining (Fig. 6B). Diffuse cellular and territorial staining were also detected in clusters of proliferating chondrocytes (Fig. 6C).

Protein expression of type X collagen in normal and osteoarthritic cartilage was restricted to the lower deep cartilage layer and the adjacent calcified cartilage zone (Fig. 7). Immunohistochemistry of chondrocytes from normal cartilage (5 out of 5) and from cartilage with minor osteoarthritic lesions (10 out of 10) showed a pericellular deposition of type X collagen but no interterritorial staining (Fig. 7A). Within the deep zone and calcified zone, staining for type X collagen was observed in a focal distribution; 20% of the chondrocytes in these zones stained positive. Staining for type X collagen was increased in late osteoarthritic lesions. In addition to increased cytoplasmatic staining for type X collagen, diffuse extracellular matrix staining was observed in the lower deep and calcified zone (Fig. 7B).

Discussion

Osteocalcin, a marker that characterizes mature and fully differentiated osteoblasts, was analyzed immunohistochemically and by *in situ* hybridization using normal and osteoarthritic cartilage and subchondral bone. For the protein detection, two well-characterized monoclonal antibodies against OC were used [32]. Both antibodies (2H9 and 8H12) recognize full-length and large N-terminal mid-fragment of OC, independent of γ-carboxylation degree of OC. Immunolocalization of OC in the subchondral bone of normal and osteoarthritic samples failed to detect quantifiable morphological differences. However, our results, obtained by *in situ* hybridization, clearly demonstrated an increase in the OC mRNA in moderate and severe osteoarthritic lesions.

When using the monoclonal OC antibodies on decalcified cartilage/bone sections, we did not observe a difference in deposition of OC in chondrocytes from normal or osteoarthritic cartilage. However, nondecalcified paraffin-embedded cartilage sections indicated no staining for OC protein in the normal and mildly affected specimens. OC can easily be extracted with EDTA [35], therefore it was assumed that the decalcification procedure with 0.2 M EDTA was responsible for the observed OC staining in cartilage. Since the decalcification of bone is necessary to cut the paraffin section, all immunohistological data presented on the OC deposition in cartilage were obtained from peeled off cartilage layers without the subchondral bone. Without decalcification, OC expression was absent in normal cartilage and cartilage with minor osteoarthritic lesions such as slight fibrillations of the articular surface. Interestingly, in sections obtained from progressive osteoarthritis, we found expression of OC protein in cartilage. When using immunohistological data alone, the interpretation of these results is incomplete, as it has been shown that OC is also present in the synovial fluid [36–38]. A small protein of only 49 amino acid residues can easily diffuse into the cartilage matrix and accumulate there. Therefore, *in situ* hybridizations for OC mRNA were done on the same cartilage/bone and bone sections to identify OC-expressing

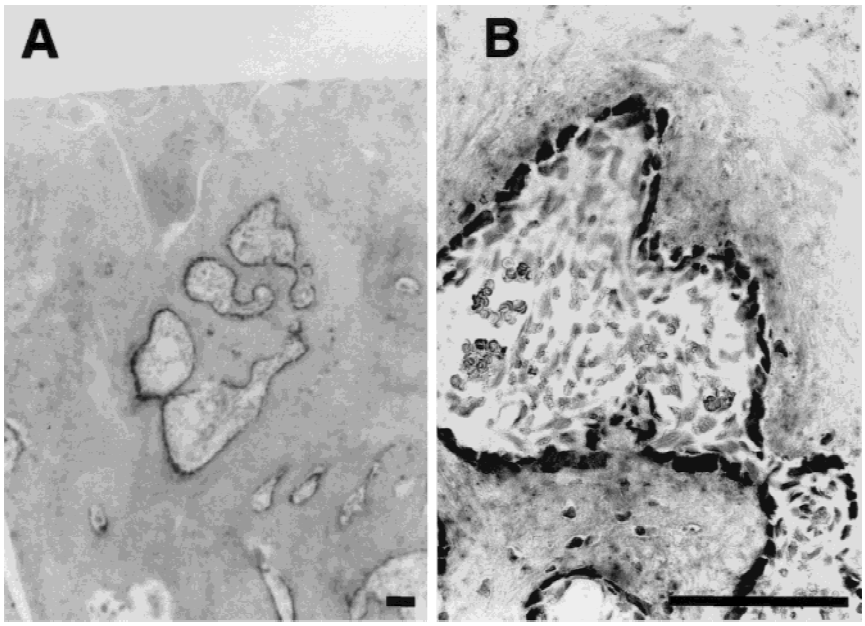

Fig. 3. Decalcified paraffin sections of femoral bone with end stage OA (72-year-old man; magnifications A: 30×, B: 310×, bars = 0.4 mm). **(A,B)** The cartilage layer is completely lost. Localization of OC mRNA is shown by *in situ* hybridization using a digoxigenin-labeled antisense riboprobe (see Materials and Methods). Osteoblasts along the margin of the bone cyst show strong expression of the OC mRNA.

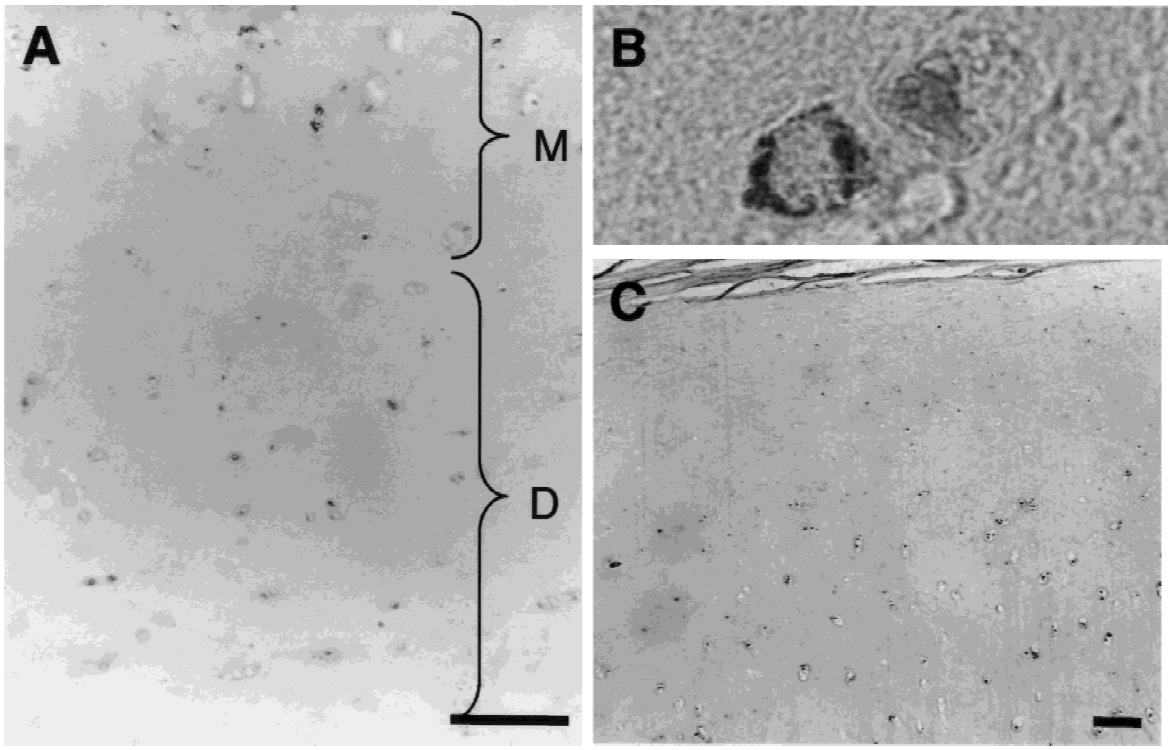

Fig. 4. Paraffin sections of femoral knee cartilage with moderate OA, no decalcification (68-year-old woman; original magnifications A: 125×, B: 1250×, C: 50×, M = middle zone, D = deep zone, bars = 0.4 mm). **(A)** Specimen was immunostained for OC using a monoclonal antibody against recombinant human OC (8H12) as described in Materials and Methods. Chondrocytes from the middle and deep cartilage zone show a faint cellular staining for OC protein. **(B)** High magnification of a single chondron from A shows intracellular deposition of OC. **(C)** *In situ* localization of the OC mRNA, using a digoxigenin-labeled antisense riboprobe (see Materials and Methods). OC expression is seen in chondrocytes from the lower middle and deep cartilage zones.

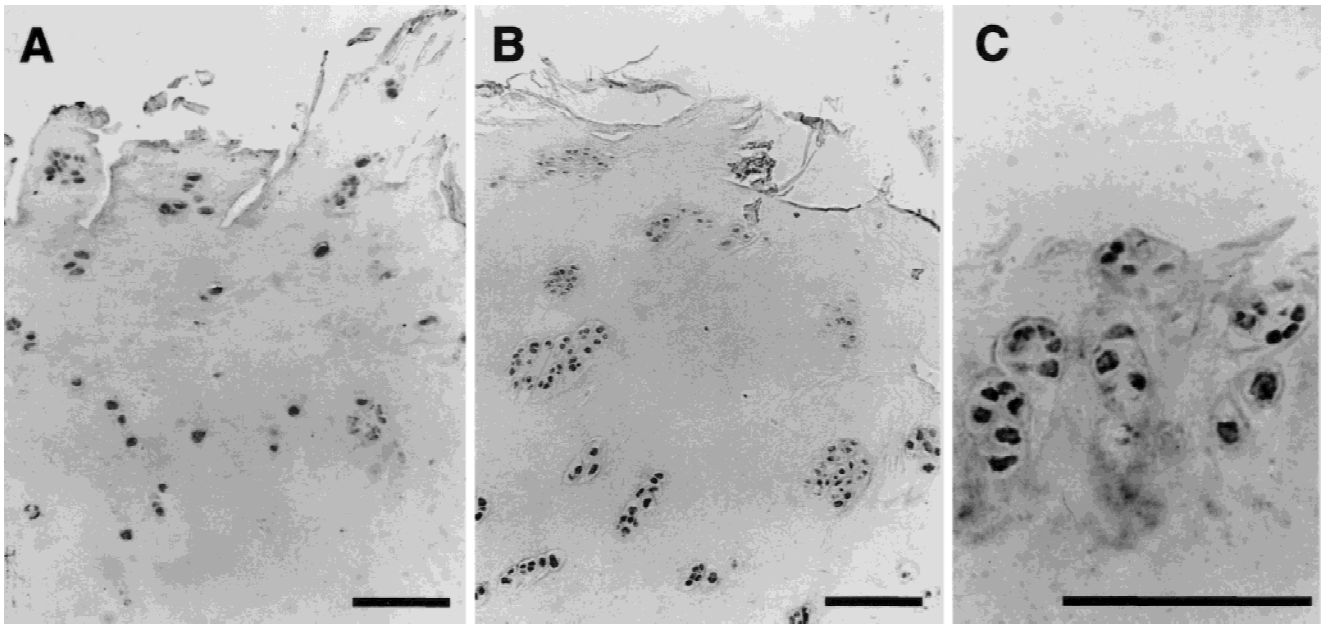

Fig. 5. Paraffin sections of femoral knee cartilage with severe OA (66-year-old woman; magnifications A, B: 125×, C: 312×, bars = 0.4 mm). The superficial tangential zone and upper middle zone of cartilage are lost and the lower middle cartilage now forms the cartilage surface. **(A)** Specimen was immunostained for OC using a monoclonal antibody against recombinant human OC (8H12) as described in Materials and Methods. Chondrocytes from the lower middle and deep cartilage zone and chondrocytes arranged in clusters show cellular staining for OC protein. **(B,C)** Localization of OC mRNA is shown by *in situ* hybridization using a digoxigenin-labeled antisense riboprobe (see Materials and Methods). Clusters of chondrocytes and chondrocytes from the deep cartilage zone show a strong signal for OC mRNA.

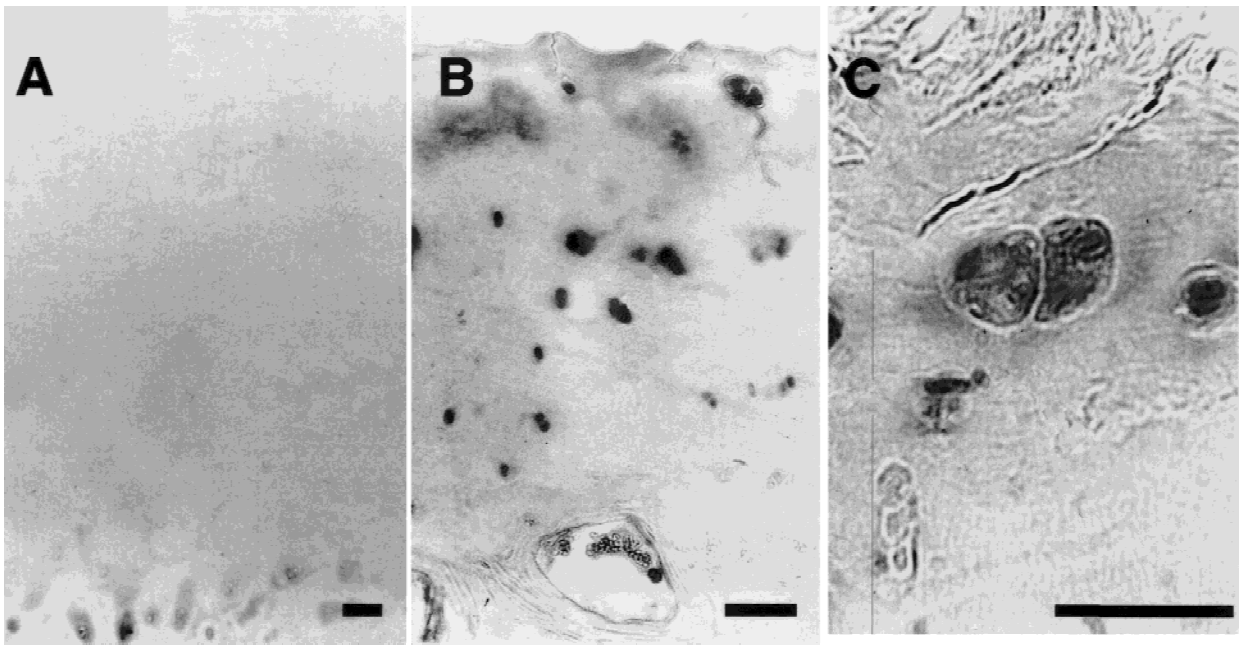

Fig. 6. Decalcified paraffin sections of normal **(A)** and severe femoral cartilage/bone **(B,C)** incubated with a monoclonal anti-alkaline phosphatase antibody (original magnifications A: 50×, B: 125, C: 310×, bars = 0.4 mm). **(A)** Chondrocytes from the deep zone show a faint territorial staining. **(B)** In severe OA, middle and deep zone chondrocytes show increased pericellular staining. **(C)** In clusters of proliferating chondrocytes, alkaline phosphatase staining is detectable.

cells. These *in situ* hybridization results clearly showed the expression of the bone-specific product OC in the chondrocytes of moderate and severe osteoarthritic cartilage and confirmed the immunohistological data. The colocalization of the mRNA signal in osteoblasts further indicates the specificity of the OC mRNA signal.

For the first time these results describe OC expression in differentiating adult human chondrocytes *in vivo.* The po-

Table 1. Expression of OC mRNA and protein (P), type X collagen (X), and alkaline phosphatase (ALP)[a]

	No OA				Moderate OA				Severe OA			
	OC		X	ALP	OC		X	ALP	OC		X	ALP
	mRNA	P	P	P	mRNA	P	P	P	mRNA	P	P	P
Tangential zone	−	−	−	−								
Middle zone	−	−	−	−	+	+	−	++				
Deep zone	−	−	+	+	+	+	+	++	+++	+++	++	++
Calcified zone	−	−	+	−	−	−	+	−	−	−	++	−
Subchondral bone	+	+++	−	+	++	+++	−	+	+++	+++	−	+

[a] Schematically summarized for the different zones of normal cartilage/bone and moderate and severe stages of OA. In moderate osteoarthritic stages, the superficial and several stages and the middle cartilage layers are lost and the joint line is then formed by the middle or deep zone.

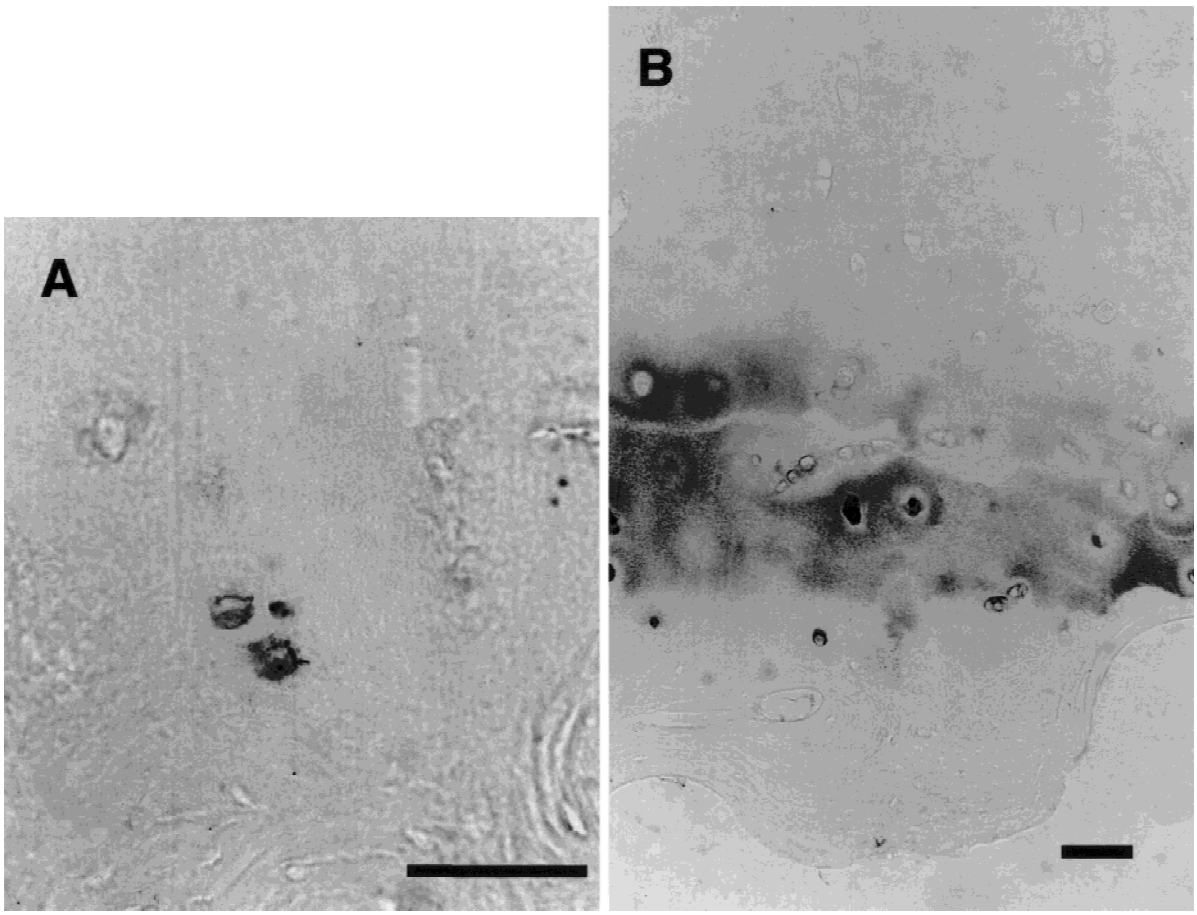

Fig. 7. Decalcified paraffin sections of normal **(A)** and severe femoral cartilage/bone **(B)** incubated with a monoclonal anti-type X collagen antibody (original magnifications A: 310×, B: 125×, bars = 0.4 mm). **(A)** Figure shows the cellular deposition of type X collagen in chondrocytes from the calcified cartilage. **(B)** In severe OA, type X collagen deposition is restricted to the lower deep and calcified zone.

tential of chondrocytes to differentiate into a posthypertrophic cell has been investigated in other biological systems. In cell culture experiments, using mainly chicken chondrocytes, expression of OC and other bone-related proteins such as osteopontin, ALP, and type X collagen was observed with the onset of chondrocyte hypertrophy [14, 15, 39–42]. Furthermore, in *in vitro* models of fracture healing, mesenchymal cells of the fracture callus also showed a bipotential pattern in synthesizing bone-related matrix components and cartilaginous proteins [19–21]. A further approach showed that mouse epiphyseal chondrocytes, which were transplanted in an interrenal compartment of syngenic animals, changed their phenotype from a primarily cartilage synthesizing cell type to a bone synthesizing cell type [43].

Our *in vivo* data indicate the expression of the bone-related proteins, OC, and ALP in human articular chondro-

cytes in the pathophysiological conditions of OA. These findings, together with the observed increase in type X collagen detection, raise the question about their role in osteoarthritis and the potential factors inducing this further chondrocyte differentiation. Our *in vivo* results in context with different *in vitro* models of chondrocyte hypertrophy raise two issues. First, in all *in vitro* models and *in vivo* investigations chondrocytes showed high metabolic activity, which is reflected in increased synthesis and turnover of matrix components. In OA, increased expression of collagens and proteoglycans is observed [23, 27]. Despite the high metabolic activity of chondrocytes in early OA, the increased synthesis cannot compensate for the degradation of cartilage by proteases. The newly synthesized extracellular matrix is insufficient to withstand the mechanical forces, leading to the disintegration of the matrix and thus, a continuous loss of cartilage. In OA, the metabolic activity is also accompanied by the proliferation of chondrocytes, whereas mitotic activity in normal adult articular cartilage is very low. In OA, the proliferation of chondrocytes leads to the formation of so-called "chondrocyte clusters." Chondrogenic cells in these clusters also have a very high metabolic activity and not only show an increased synthesis of physiological matrix components such as type II collagen and type VI collagen [44–46], but begin to express bone-related proteins such as OC, ALP, and also type X collagen, which marks chondrocyte hypertrophy. This alters the composition of the pericellular matrix and the microenvironment of chondrocytes, which is suggested to play a critical role in the proliferation and differentiation of chondrocytes and the induction of hypertrophy (reviewed in [47]).

Second, hypertrophic chondrocytes of the fetal growth plate and in fracture healing models are regulated by systemic mediators. Glucocorticoids and members of the transforming growth factor-β family, such as the bone morphogenic proteins, are thought to influence the differentiation of chondrocytes [42, 48–50]. In OA, these physiological mediators may also be important for the further differentiation of chondrocytes to a posthypertrophic phenotype. Due to the loss of articular cartilage, as it occurs in osteoarthritis, chondrocytes from the middle and deep cartilage zones become in closer contact to the joint space. It could be assumed that the exposure to cytokines and growth factors from the synovial fluid leads to a modified cell behavior. The precise role of cytokines and growth factors, modulating the chondrocyte phenotype towards cell hypertrophy in osteoarthritis, requires more clarification.

The mineralization process has been extensively investigated in the embryonic growth plate where ossification and reconstruction of the cartilaginous tissue is a physiological step in long bone growth development. One facet of this complex process is the expression of bone-associated proteins such as OC and ALP in the hypertrophic chondrocytes along with the occurrence of type X collagen. Involvement of these proteins in the regulation of mineralization has been proposed. For example, the Ca-binding properties of type X collagen indicate a putative role in calcification [51]. Expression of ALP can be found in the epiphyseal growth plate, but with cessation of growth, ALP and type X collagen expression is largely reduced or absent in adult articular cartilage [33, 52]. Under the pathophysiological circumstances of OA, the chondrocyte phenotype again changes towards hypertrophy, accompanied by the increased expression of type X collagen and constitutive cartilage macromolecules, such as type II collagen and proteoglycans [11, 53–55]. The altered expression pattern of chondrocytes is likely to influence the biochemical and biomechanical properties of the cartilage matrix. Therefore, the expression of OC, ALP, and type X collagen in late stages may mark a critical point in the process of OA when chondrocytes have lost their potential to maintain or restore an intact cartilage matrix.

The immunohistochemical expression pattern of OC and ALP showed a cellular and pericellular staining, whereas interterritorial matrix staining was absent. The pericellular matrix of chondrocytes varies qualitatively and quantitatively when compared with the interterritorial matrix. According to these findings, it is hypothesized that the pericellular matrix is also responsible for compensation of mechanical loading and transmission of biochemical and mechano-inductive signals towards the cellular and intracellular compartments. Increased expression of OC, ALP, and type X collagen may alter these pathways and support or maintain the un-physiological differentiation pattern seen in the osteoarthritic chondrocytes.

Our data indicate that the expression of OC marks the differentiation of chondrocytes into a hypertrophic phenotype in OA. Until now, this differentiation pathway has not been shown to be reversible and it may characterize a disease stage where cartilage repair mechanisms are no longer successful in maintaining functional cartilage architecture. The hypertrophy of chondrocytes in the fetal growth plate and in fracture callus is a physiological and necessary process followed by mineralization, whereas a physiological role of OC expression in OA is doubtful. Chondrocyte hypertrophy and differentiation in OA are found in late stages of the disease, which is characterized by a progressive disintegration of the matrix. The induction of a bone-like gene expression in OA is likely to mark a point in the disease process, where cellular behavior is dysregulated and repair mechanisms have failed.

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