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Activation of annexin II and V expression, terminal differentiation, mineralization and apoptosis in human osteoarthritic cartilage

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Summary

Objective: To test the hypothesis that terminal differentiation of chondrocytes in human osteoarthritic cartilage might lead to the failure of repair mechanisms and might cause progressive loss of structure and function of articular cartilage.

Design: Markers for terminally differentiated chondrocytes, such as alkaline phosphatase, annexin II, annexin V and type X collagen, were detected by immunohistochemical analysis of human normal and osteoarthritic knee cartilage from medial and lateral femoral condyles. Apoptosis in these specimens was detected using the TUNEL labeling. Mineralization and matrix vesicles were detected by alizarin red S staining and electron microscopic analysis.

Results: Alkaline phosphatase, annexin II, annexin V and type X collagen were expressed by chondrocytes in the upper zone of early stage and late stage human osteoarthritic cartilage. However, these proteins, which are typically expressed in hypertrophic and calcifying growth plate cartilage, were not detectable in the upper, middle and deep zones of healthy human articular cartilage. TUNEL labeling of normal and osteoarthritic human cartilage sections provided evidence that chondrocytes in the upper zone of late stage osteoarthritic cartilage undergo apoptotic changes. In addition, mineral deposits were detected in the upper zone of late stage osteoarthritic cartilage. Needle-like mineral crystals were often associated with matrix vesicles in these areas, as seen in calcifying growth plate cartilage.

Conclusion: Human osteoarthritic chondrocytes adjacent to the joint space undergo terminal differentiation, release alkaline phosphatase-, annexin II- and annexin V-containing matrix vesicles, which initiate mineral formation, and eventually die by apoptosis. Thus, these cells resume phenotypic changes similar to terminal differentiation of chondrocytes in growth plate cartilage culminating in the destruction of articular cartilage in osteoarthritis. © 2000 OsteoArthritis Research Society International

Key words: Annexin, Apoptosis, Cartilage, Mineralization.

Introduction

Synovial joints allow the movement of skeletal elements, such as those in the limbs, vertebral column and mandible. Normal joint function throughout life is largely dependent on the structural and functional integrity of articular cartilage and the phenotypic stability of articular chondrocytes, the cells that produce, maintain, remodel and repair the extracellular matrix of articular cartilage.

During endochondral ossification chondrocytes undergo a series of differentiation events, including proliferation, hypertrophy, terminal differentiation and eventually cell death (apoptosis). In addition, terminally differentiated chondrocytes release matrix vesicles, alkaline phosphatase, annexin II and annexin V-containing particles which initiate mineralization of the extracellular matrix.[1–4]

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In contrast, chondrocytes in healthy human articular cartilage maintain a stable phenotype. These cells do not proliferate but are capable of producing matrix components, such as type II, IX, XI collagen and aggrecan. Articular cartilage can be divided into four zones (upper, middle, deep, and calcified zone). Each zone has a different composition and structure depending on the different functional demands of these zones in articular cartilage.[5,6] For example, the collagen fibrils in the upper zone are thinner and the amount of aggrecan is lower than in the deeper zones. In addition, cells and collagen fibrils in the upper layer are arranged differently to those in the deeper layers.[5,6] The zone of calcified cartilage, the deepest zone of articular cartilage, provides a smooth transition from the noncalcified articular cartilage matrix to the highly calcified underlying bone matrix.[7] Chondrocytes in calcified articular cartilage, in contrast to cells in noncalcified articular cartilage, retain some properties of hypertrophic growth plate chondrocytes and synthesize hypertrophic marker proteins, such as alkaline phosphatase and type X collagen.[8,9]

Degenerative joint diseases, such as osteoarthritis, are characterized by an initial loss of proteoglycans from the upper zone followed by the degradation of the collagen network.[10,11] Several studies have provided evidence that synthesis of types II, VI, IX, XI collagen is significantly

increased in osteoarthritic cartilage compared to normal healthy articular cartilage, suggesting that articular chondrocytes try to repair the damaged matrix.[12–16] However, eventually the reparative processes fail and the breakdown of the cartilage matrix leads to a progressive loss of the various zones of articular cartilage. In addition, some studies have shown a switch in collagen synthesis to types I and III collagen in osteoarthritic cartilage, suggesting a 'dedifferentiation' of articular chondrocytes.[17–19] On the other hand, expression of type X collagen and annexin V has been demonstrated in human osteoarthritic cartilage, which would be indicative for chondrocyte hypertrophy as a further differentiation.[9,20–23] Apart from these findings, it has been demonstrated that chondrocytes in osteoarthritic cartilage upregulate the synthesis of proteolytic enzymes, suggesting that the loss of matrix in osteoarthritic cartilage reflects the action of these proteolytic enzymes.[24–27] However, the exact mechanisms which lead to a failure of the reparative processes, degradation of the extracellular matrix and loss of cartilage integrity, are not well understood.

In this study we investigated the hypothesis that chondrocytes in the upper zone of human osteoarthritic cartilage undergo phenotypic changes, resulting in a progressive loss of articular cartilage structure and function. Using immunohistochemistry and electron microscopy we investigated whether chondrocytes of human osteoarthritic cartilage undergo terminal differentiation which might be a cause of progressive cartilage breakdown.

Methods

TISSUE SAMPLES

Human osteoarthritic knee cartilage from medial and lateral femoral condyles (56–85 year old donors) was collected from patients undergoing knee arthoplasty (14 samples) at the time of surgery. Clinical data of these patients were carefully reviewed to exclude secondary forms of OA, such as rheumatoid arthritis. Articular cartilage without signs of OA was obtained from human knees (34–59 year old donors, four samples) within 12–24 h after death. Sections from all cartilage specimens were stained with hematoxylin and safrain O staining to determine the severity of OA.[28] Based on histology the specimens were classified into early (no signs of fibrillation, some clustering of cells evident, overall thickness of cartilage preserved) and late (extensive fissuring and fibrillation, clustering of chondrocytes, loss of cartilage) forms of OA. This study included samples of original articular cartilage only, and excluded osteophytes and other repair cartilage. In addition, only areas of late stage osteoarthritic cartilage are shown in this study which show a largely preserved cartilage thickness.

The localization of annexin II and annexin V was also studied in 19-day-old chick growth plate cartilage.

IMMUNOHISTOLOGY

Human articular cartilage samples with underlying bone were fixed with 4% paraformaldehyde in phosphate buffered saline for 24 h and then decalcified using 0.2 M EDTA (pH 8.0) at 4°C for several weeks. Samples were embedded in paraffin and 5 μm thick sections were cut perpendicular to the cartilage surface. Before immunostaining, sections were treated with sheep testicular hyaluronidase (2 mg/ml; Sigma Chemicals Co., St Louis, MO) for 30 min at 37°C. Immunostaining was performed using the Histostain™ SP Kit (Zymed Laboratories Inc., San Francisco, CA) following the manufacturer's instructions. Briefly, sections were treated with peroxidase quenching solution to eliminate endogenous peroxidase activity, followed by incubation with a blocking solution for 10 min at room temperature. Sections were then incubated with primary antibodies for 3 h at room temperature followed by biotinylated secondary antibodies for 10 min at room temperature. After washing, sections were incubated with a streptavidin–peroxidase conjugate for 10 min at room temperature followed by a solution containing diaminobenzidine (DAB; chromogen) and 0.03% hydrogen peroxide for 5 min at room temperature. Sections were counterstained with hematoxylin, dehydrated, mounted and viewed under a Zeiss microscope.

To localize calcium deposits, nondecalcified sections were stained with 0.5% alizarin red S solution, pH 4.0, for 5 min at room temperature. Stained sections were washed three times with water and ethanol.

END LABELING OF TISSUE SECTIONS USING A TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE (TUNEL PROCEDURE)

End labeling of human cartilage sections was performed using TACS 2 TdT In Situ Apoptosis Detection Kit (Trevigen, Inc., Gaithersburg, MD). Sections were first treated with 1% Triton X-100 in phosphate buffered saline (pH 7.4) for 10 min at room temperature followed by treatment with proteinase K (20 μg/ml) for 15 min at room temperature and, to inhibit peroxidase activity, by incubation with 3% H_2O_2 in phosphate buffered saline for 5 min at room temperature. Prior to labeling, sections were equilibrated in a transferase labeling buffer for 5 min and then incubated in a reaction mixture containing biotin-labeled deoxynucleotides, Mn^{2+} and terminal deoxynucleotidyl transferase at 37°C. After 60 min the incubation was stopped and the nucleotides were detected using streptavidin-conjugated peroxidase and DAB as a color substrate. Sections were counterstained with methylen green, mounted and viewed under a Zeiss microscope.

SEMI-QUANTITATIVE ANALYSIS OF CHONDROCYTES SHOWING CELL-ASSOCIATED IMMUNOSTAINING OR TUNEL-LABELING

To gain some insight into the extent of expression of terminal differentiation marker proteins or apoptosis in osteoarthritic cartilage, the percentage of stained cells in osteoarthritic cartilage was determined. We counted 200–300 chondrocytes in each zone (upper, middle, deep, calcified) of separately stained sections from different donors (four normal articular cartilage samples, four early stage and five late stage osteoarthritic cartilage samples). Data are expressed as the mean±SD of the percentage of total cells which show cell-associated staining. For apoptosis, TUNEL-positive single cells and cells in clusters were counted separately.

ELECTRON MICROSCOPY

Tissue was fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4) containing 2% tannin and post-fixed in 1% osmium tetroxide. The tissues were dehydrated in a

graded series of ethanol and embedded in epoxy resin. Ultrathin sections were contrasted with uranyl acetate and lead citrate. Specimens were examined in a transmission electron microscope (100CX II, JEOL U.S.A., Peabody, MA) operated at 80 kV. Samples were also analysed by an electron dispersive microanalysis system using Quantex software (Kevex Corp., San Carlos, CA).

ANTIBODIES

The preparation and specificity of polyclonal antibodies against chicken annexin II and annexin V were described elsewhere.[29] These antibodies do not cross-react with other annexins and matrix proteins.[29] Using extracts from human cartilage we were able to show that these antibodies react with human annexin II and annexin V, respectively (data not shown). The preparation of polyclonal antibodies against human type X collagen was described elsewhere.[30] This antibody has been shown to be specific and does not cross-react with other collagens or matrix proteins.[30] Monoclonal antibodies against human bone and liver alkaline phosphatase were obtained from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA) and were described by Lawson *et al*.[31]

Results

EMBRYONIC GROWTH PLATE CARTILAGE

In embryonic chick growth plate cartilage cellular staining for annexin II and V was obtained in prehypertrophic (PHC), early hypertrophic (EHC) and hypertrophic cartilage (HC), while in calcified cartilage (CC) both cellular and matrix staining was detected (Fig. 1A–G). Matrix staining in calcified cartilage is due to the release of matrix vesicles containing annexin II and V into the extracellular matrix.[3,4] Only a few chondrocytes in the proliferative zone were positive for annexin II and V, while most of the proliferative chondrocytes showed no staining (Fig. 1B,E). The cartilage zones immunostained for annexin II and V corresponded to those immunostained for type X collagen (data not shown).

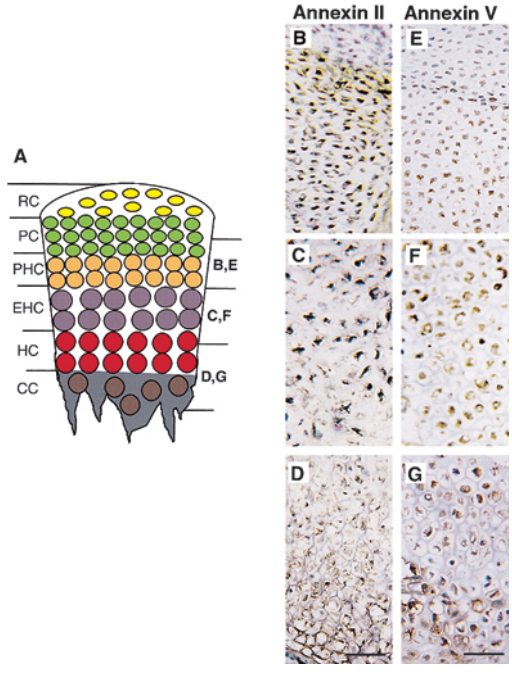

Fig. 1. Immunohistochemical analysis of annexin II (AnII, B–D) and annexin V (AnV, E–G) in sections of 19-day-old embryonic chicken growth plate cartilage. (A) Schematic representation of the 19-day-old embryonic chick growth plate; yellow cells represent the resting chondrocytes (RC), green cells the proliferative chondrocytes (PC), orange cells the prehypertrophic chondrocytes (PHC), blue cells the early hypertrophic chondrocytes (EHC), red cells the hypertrophic chondrocytes (HC) and brown cells the calcified chondrocytes (CC). (B, E) Immunostaining for annexin II (B) and annexin V (E) in the zones of proliferative (PC) and prehypertrophic (PHC) chondrocytes. While most of the proliferative chondrocytes showed no staining for these molecules, cellular staining was observed in prehypertrophic chondrocytes. (C, F) Immunostaining for annexin II (C) and V (F) in the zones of early hypertrophic (EHC) and hypertrophic chondrocytes (HC). Note the cellular staining for annexin II and V in both zones. (D, G) Immunostaining for annexin II (D) and V (G) in the hypertrophic (HC) and calcified zones (CC). While only cellular staining was obtained in hypertrophic cartilage, cellular and matrix staining was obtained in calcifying cartilage. Bar, 100 μm.

NORMAL ARTICULAR CARTILAGE

Four different adult human articular cartilage samples showing no signs of OA were investigated. Articular cartilage can be divided into four zones: the upper zone, middle, deep and calcified zone. The calcified cartilage zone provides a transition from the noncalcified deep articular cartilage zone to the underlying mineralized subchondral bone. The cartilage samples had a smooth, intact cartilage surface (Figs 2A,F and 3A,F,K). They were uniformly stained with safrain O, indicating no loss of proteoglycans, and with antibodies specific for type II collagen (data not shown). However, the upper, middle and deep zones of these specimens did not immunostain with antibodies to annexin II (AnII, Fig. 2A), annexin V (AnV, Fig. 2F), alkaline phosphatase (AP, Fig. 3A), and type X collagen (X, Fig. 3F; see also Table I). Some immunostaining for annexin II, annexin V, alkaline phosphatase and type X collagen was detected in areas of calcified cartilage (see Table I), which is in agreement with previous studies showing that these molecules are predominantly expressed in hypertrophic and calcifying cartilage.[8,9,32] In addition, treatment of sections with alizarin red S to detect mineral deposits revealed only staining in bone and the calcified cartilage zone but not in the upper, middle and deep zones of normal human articular cartilage (data not shown).

When sections of normal cartilage samples were treated with the TUNEL procedure to detect apoptotic cells, none of the nuclei of the chondrocytes in the upper, middle (Fig. 3K) and deep zones were stained (see also Table I).

EARLY STAGE OF OSTEOARTHRITIS

Four samples of early stage osteoarthritic cartilage with a preserved overall thickness of articular cartilage (except the loss of the 2–3 top layers of elongated cells in some areas of the upper zone), but loss of proteoglycans in the upper zone were investigated. Staining of these sections with antibodies to annexin II (An II, Fig. 2B,C), annexin V (AnV, Fig. 2G,H) or alkaline phosphatase (AP, Fig. 3B,C) demonstrated that 60–80% of chondrocytes in the upper zone were immunopositive for these molecules, while no staining or only few stained cells were observed in the deeper zones (see also Table I). Immunostaining for

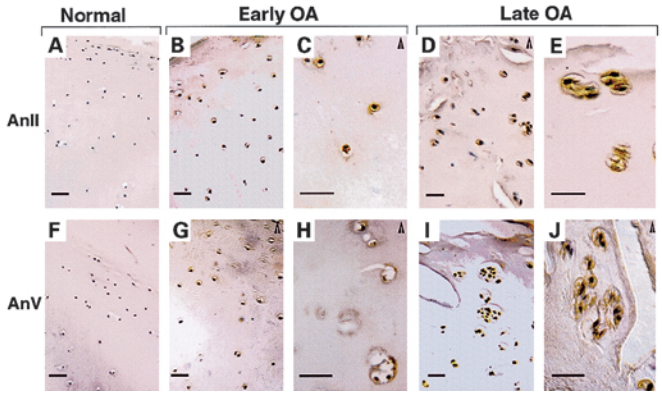

Fig. 2. Immunohistochemical analysis of annexin II (AnII, A–E) and annexin V (AnV, F–J) in sections of human normal and osteoarthritic articular cartilage. (A, F) Note the absence of staining with antibodies against annexin II (A) and annexin V (F) in human normal articular cartilage from femoral condyle (male, 34 years old). (B, C, G, H) A significant number of chondrocytes in the upper zone of early stage osteoarthritic cartilage show immunostaining for annexin II (B, C) and annexin V (G, H). (C, H) High magnification showing that staining for annexin II and V is restricted to the cell. (B, C) femoral condyle, male aged 56 years. (G, H) femoral condyle, female aged 73 years. (D, E, I, J) Immunostaining for annexin II (D, E) and annexin V (I, J) in the upper zone of late stage osteoarthritic cartilage. (E, J) High magnification shows that staining for annexin II and V was found not only on the cell surface but also in the surrounding pericellular matrix. (D, E) femoral condyle, female aged 67 years. (I, J): femoral condyle, female aged 73 years. Bar, 100 μm.

annexin II, annexin V and alkaline phosphatase was mostly restricted to the cell surface (Figs 2C,H and 3C). In addition, immunostaining was detected for type X collagen (X, Fig. 3G,H) in the upper zone of early stage human osteoarthritic cartilage, but was absent in the deeper zones.

Labeling of early stage osteoarthritic cartilage sections with the TUNEL procedure revealed no staining of the nuclei of chondrocytes throughout the entire cartilage (Fig. 3L, Table I). Treatment of these samples with alizarin red S showed no staining of the upper and deeper zones (data not shown).

LATE STAGE OF OSTEOARTHRITIS

Late stage osteoarthritic cartilage samples (five samples) showed extensive fissuring, fibrillation, clustering of cells, and major loss of proteoglycans and cartilage. Between 70 and 90% of chondrocytes in the upper zone of late stage human osteoarthritic cartilage showed strong immunostaining for annexin II (AnII, Fig. 2D,E), annexin V (AnV, Fig. 2I,J) and alkaline phosphatase (AP, Fig. 3D,E). Staining was not restricted to the cell surface but was also found in the pericellular matrix (Figs 2E,J and 3E). Type X collagen staining was detected in the extracellular matrix surrounding chondrocytes (Fig. 3I,J). No staining for these proteins was detected in deeper zones (see Table I).

TUNEL staining of late stage human osteoarthritic cartilage samples revealed labeling of nuclei of single chondrocytes and cells in clusters located in the upper zone, indicating that these cells undergo apoptosis (Fig. 3M,N). No staining of cells was detected in deeper zones (data not shown). In some clusters only a few chondrocytes TUNEL stained (Fig. 3M), while in other clusters most of the cells were labeled (Fig. 3N).

Treatment of late stage osteoarthritic cartilage samples with alizarin red S showed staining not only of calcified cartilage but also of upper areas, while no staining was detected in deeper zones (Fig. 3O), indicating the formation of mineral deposits in the upper zone of late stage human osteoarthritic cartilage.

Table I represents a semi-quantitative analysis of the staining results. The data are presented as the percentage of total cells, which are immunopositive or TUNEL-positive in each zone. While normal human articular cartilage showed no immunostaining for the marker proteins of terminal differentiation, between 50 and 80% of the cells in the upper zone of early stage osteoarthritic cartilage stained for annexin II, annexin V, alkaline phosphatase, and type X collagen. The percentage of immunopositive cells in the upper zone slightly increased in late stage osteoarthritic cartilage. Only between 0 and 10% of cells in the middle and deep zones of early and late stage osteoarthritic cartilage showed immunostaining for these proteins. In contrast, only cells in the upper zone of late stage osteoarthritic cartilage were labeled by the TUNEL procedure. 61% of cells in clusters and 60% of cells in areas that showed no cluster formation were apoptotic.

ELECTRON MICROSCOPIC ANALYSIS OF LATE STAGE HUMAN OSTEOARTHRITIC CARTILAGE

Electron microscopic analysis of five knee specimens of late stage human osteoarthritic cartilage revealed mineral crystals present in the upper zone in all investigated samples. Matrix vesicles with round shape and various size were found in the matrix of the upper zone of osteoarthritic cartilage (Fig. 4A). Matrix vesicles were associated with calcified areas (Fig. 4B). Electron dispersive X-ray microanalysis of these mineralized areas, as seen in Fig. 4B, revealed strong calcium and phosphate peaks, confirming that these areas indeed contain calcium phosphate crystals (Fig. 4C).

Discussion

In this study, we provide evidence that articular chondrocytes in the upper zone of human osteoarthritic cartilage undergo terminal differentiation, release matrix vesicles, which mineralize the extracellular matrix, and undergo cell death. In the upper zone of human osteoarthritic cartilage

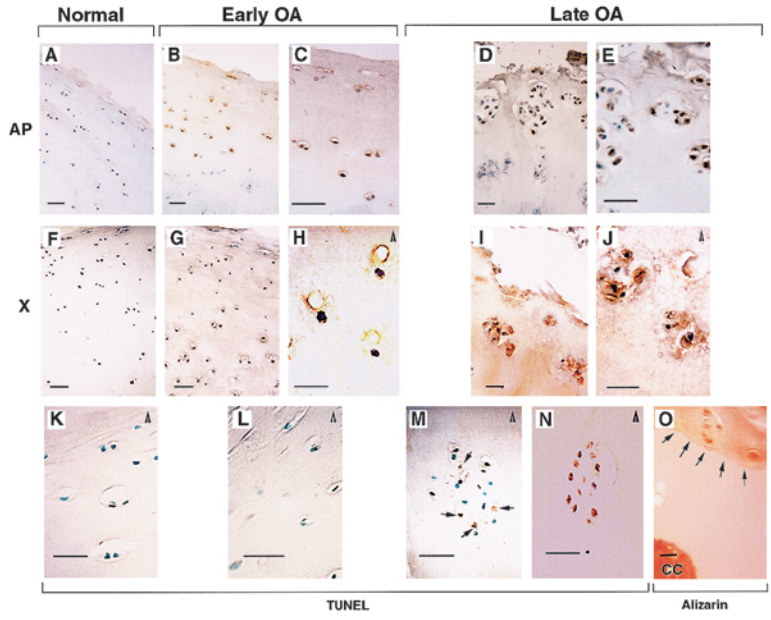

Fig. 3. Immunostaining for alkaline phosphatase (AP, A–E), type X collagen (X, F–J), TUNEL staining (K–N) and alizarin red S staining (O) of sections of human normal and osteoarthritic cartilage. Note the absence of immunostaining for alkaline phosphatase (A) and type X collagen (F) in normal human articular cartilage, while staining for alkaline phosphatase (B–E) and type X collagen (G–J) was detected in the upper zone of early stage and late stage human osteoarthritic cartilage. (A, B, D, F, G, I) Low magnification; (C, E, H, J) High magnification. (A, F) femoral condyle, male aged 34 years; (B, C) femoral condyle, male aged 56 years; (G, H) femoral condyle, female aged 70 years; (D, E) tibial plateau, female aged 69 years; (I, J) femoral condyle, male aged 79 years. (K–N) TUNEL staining revealed no labeling of chondrocytes in normal (K) and early stage osteoarthritic (L) cartilage, but note the presence of TUNEL positive cells in the upper zone of late stage osteoarthritic cartilage (M, N). In some cell clusters only a few chondrocytes were stained (M, arrow), while in other clusters most of cells were TUNEL positive (N). (O) Alizarin red S staining of late stage osteoarthritic cartilage. Note the staining not only in calcified cartilage (CC) but also in the upper zone (arrows). (K) tibial plateau, male aged 59 years; (L) femoral condyle, female aged 73 years; (M, N) femoral condyle, female aged 73 years; (O) tibial plateau, female aged 69 years; Bar, 100 μm.

chondrocytes in clusters and individual chondrocytes producing alkaline phosphatase, annexin II, annexin V and type X collagen were identified. These molecules have been shown to be normally expressed in hypertrophic and mineralizing growth plate cartilage (see also Fig. 1).[4,8,32,33] The zone of alkaline phosphatase, annexin II, annexin V and type X collagen expressing chondrocytes in osteoarthritic cartilage corresponds to the zone of loss of proteoglycans. In early stage human osteoarthritic cartilage between 50 and 80% of chondrocytes of the upper zone showed immunostaining for these molecules, while in late stage osteoarthritic cartilage between 70 and 90% of the cells were immunopositive. Few cells in the deeper zones showed immunostaining for these molecules. Ultrastructural analysis revealed matrix vesicles and needle-like crystals emanating from these particles in the upper zone of late stage osteoarthritic cartilage. In addition, we found that ~60% of cells in upper areas undergo cell death. From these findings we conclude that chondrocytes in the upper zone of human osteoarthritic cartilage initiate inappropriate terminal differentiation, leading to cell death and loss of structure and function of articular cartilage.

Chondrocytes in the upper zone of human osteoarthritic cartilage not only activate the expression of molecules which are predominantly expressed by hypertrophic chondrocytes, but these cells initiate the mineralization process. Matrix vesicles are associated with mineralized areas in upper regions of osteoarthritic cartilage. Matrix vesicles have a critical role in initiating the mineralization process in many hard tissues.[34] In growth plate cartilage only terminally differentiated, post-hypertrophic chondrocytes release mineralization-competent, alkaline phosphatase, annexin II, annexin V and type X collagen containing matrix vesicles, while hypertrophic chondrocytes release matrix vesicles, which do not contain these components, and thus fail to mineralize.[4] Our findings here indicate that chondrocytes in the upper zone of human osteoarthritic cartilage activate the expression of alkaline phosphatase, annexin II and V. Interestingly, immunostaining for these proteins is restricted to the cell surface in early stage of OA, while it is also found in the matrix of late stage osteoarthritic cartilage. Thus, chondrocytes in the upper zone of osteoarthritic cartilage release matrix vesicles which contain alkaline phosphatase, annexin II and V into the extracellular matrix, and therefore are able to initiate the mineralization process leading to mineral depositions in regions where no calcification should occur. Our data are in agreement with previous findings showing calcium-phosphate deposition in human osteoarthritic cartilage and the presence of matrix vesicles with enhanced alkaline phosphatase activity and the ability to mineralize.[35–39]

Once mineralization-competent matrix vesicles are released into the extracellular matrix, they rapidly accumulate mineral ions leading to the formation of the first crystal phase inside the vesicle lumen.[34] The annexins (II and V) are channel forming proteins which enable the rapid influx of calcium into matrix vesicles.[40,41] In addition, annexin V binds to types II and X collagen thereby anchoring matrix vesicles to the extracellular matrix, and binding of these collagens to matrix vesicles stimulates their calcium uptake and intralumenal crystal growth.[42] Crystal deposits in osteoarthritic cartilage drastically alter its normal physical properties and impair its function. In addition, crystal deposition in articular cartilage leads to inflammation,[35]

Table I

Percentage of cells in the upper, middle, deep and calcified zones of normal articular cartilage, early and late stage osteoarthritic cartilage showing immunostaining for annexin II, annexin V, alkaline phosphatase (APase), type X collagen, or TUNEL labeling

	Surface zone		Middle zone		Deep zone		Calcified zone	
Annexin II								
Normal	0		0		0		58.1±5.9	
Moderate	80.3±4.9		3.5±1.6		3.3±1.9		39.5±13.2	
Severe	88.8±2.5		6.3±5.3		4.6±2.5		63.1±1.9	
Annexin V								
Normal	0		0		0		29.4±8.5	
Moderate	70.0±16.6		5.3±0.5		1.5±2.1		21.4±4.3	
Severe	69.8±12.8		4.1±1.1		5.0±6.7		24.3±1.5	
APase								
Normal	0		0		0		57.9±5.6	
Moderate	63.2±6.3		0		0		57.7±12.4	
Severe	75.2±4.1		4.3±1.4		1.7±0.4		60.8±7.4	
Type X								
Normal	0		0		3.8±2.1		80.4±4.5	
Moderate	49.8±6.2		5.2±2.3		7.1±3.2		73.2±3.2	
Severe	78.5±7.6		3.8±1.8		9.9±4.1		73.8±1.0	
Apoptosis	*Single cells*	Clusters	Single cells	Clusters	Single cells	Clusters	Single cells	Clusters
Normal	0	0	0	0	0	0	0	0
Moderate	0	0	0	0	0	0	0	0
Severe	60.3±3.6	61.3±6.2	0	0	0	0	0	0
Mineralization								
Normal	No		No		No		Yes	
Moderate	No		No		No		Yes	
Severe	Yes		No		No		Yes	

200–300 chondrocytes in upper, middle, deep and calcified zones of normal articular cartilage, early and late stage osteoarthritic cartilage from four different donors were counted as described in Methods. Data are expressed as the mean±SD of the percentage of total cells which show cell-associated staining. For apoptosis, TUNEL-positive single cells and cells in clusters were counted separately.

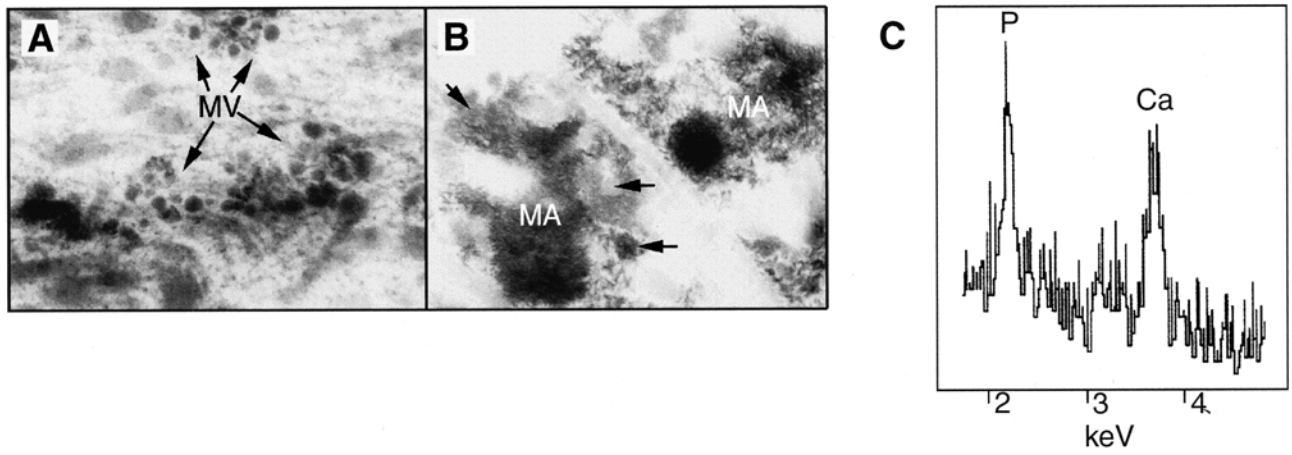

Fig. 4. Electron microscopy of late stage human osteoarthritic cartilage. (A) Electron micrograph showing matrix vesicles (MV) in the matrix of the upper zone. (B) Mineralized areas (MA) in upper regions of late stage osteoarthritic cartilage. Note that matrix vesicles (arrows) are associated with crystal deposits. (C) Energy dispersive X-ray microanalysis of mineralized areas as shown in (B). Note the presence of calcium (Ca) and phosphate (P) peaks. Tibial plateau, male aged 70 years (×72500).

and induces mitogenesis and matrix metalloprotease production in chondrocytes,[43] thereby accelerating cartilage destruction. We have evidence that the 1,4-benzothiazepine derivative K-201, a compound that selectively blocks annexin channel activities,[44,45] inhibits calcium uptake and mineralization of matrix vesicles (T. Kirsch *et al*., submitted). Thus, the interference with annexin channel activities might prevent crystal deposition in osteoarthritic cartilage.

As shown in this study, chondrocytes in the upper zone of osteoarthritic cartilage undergo a series of differentiation events, eventually leading to cell death or apoptosis. These results are supported by previous studies demonstrating apoptotic cells in upper layers of osteoarthritic human and rabbit cartilage.[37,46,47] Programmed cell death or apoptosis was also found in mineralizing growth plate chondrocytes close to the chondro-osseous junction.[2,48] Our findings would explain why articular chondrocytes in the upper zone of human osteoarthritic cartilage fail to repair the extracellular matrix. These cells lose their stable phenotype, undergo terminal differentiation and eventually cell death. They also, instead of synthesizing a type II collagen- and

aggrecan-rich matrix required for the proper function of articular cartilage, produce a type X collagen-rich, mineralizing matrix similar to the matrix found in hypertrophic and calcifying cartilage during endochondral ossification.[4,49–52]

Terminal differentiation of growth plate chondrocytes is controlled by a variety of factors. For example, we and others have shown that differentiation of hypertrophic chondrocytes to terminally differentiated, post-hypertrophic mineralizing chondrocytes requires cues, such as vitamin A or C.[4,41,50,52,53] On the other hand growth factors, such as FGF, PTHrP and TGFβ, were shown to inhibit maturation of chondrocytes.[54–59] Human articular chondrocytes of the upper zone initiate alkaline phosphatase and type X collagen synthesis when cultured in suspension in the presence of fetal calf serum, suggesting that these cells are arrested in an early stage of differentiation, but upon receiving the appropriate stimuli then can undergo terminal differentiation.[60] Interestingly, it has been demonstrated that conditioned media from immature chondrocyte cultures prevent hypertrophy, and that this effect is abolished by addition of monoclonal antibodies to TGFβ.[58] In addition, a previous study has indicated that the loss of responsiveness to TGFβ promotes terminal differentiation of chondrocytes and results in the development of joint diseases, such as OA.[59] Thus, it can be speculated that human articular chondrocytes physiologically synthesize factors, such as TGFβ, which inhibit their terminal differentiation. During pathogenesis, however, articular chondrocytes in the upper zone stop producing these factors leading to terminal differentiation, cell death, and cartilage destruction. In addition, osteoarthritic chondrocytes are exposed to growth factors synthesized by synovial cells and released into the joint cavity, which might further promote terminal differentiation of these cells.

In conclusion, our study provides evidence that terminal differentiation is the main fate of chondrocytes in the upper zone of human osteoarthritic cartilage. These cells resemble a phenotype similar to terminally differentiated, post-hypertrophic mineralizing chondrocytes of the epiphyseal growth plate. They activate the expression of alkaline phosphatase, annexin II, annexin V, type X collagen, and the release of matrix vesicles, components which play major roles in the initiation of mineralization.[8,41,42] The inappropriate terminal differentiation of articular chondrocytes in OA not only leads to cell death and failure of tissue repair, but is also accompanied by the deposition of hydroxyapatite crystals. Crystal deposition in articular cartilage can lead to inflammation and stimulation of metalloprotease production,[35] which could positively feedback or accelerate terminal differentiation of articular chondrocytes and cartilage destruction. Thus, prevention of terminal differentiation and matrix mineralization in human osteoarthritic cartilage might be a novel therapeutic target in OA.

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