Ultrastructural changes of the articular cartilage in some arthropathies with special reference to chondrocyte cell death

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Abstract

Objective: To determine in situ using TEM the balance of apoptosis and necrosis in the articular cartilage of patients with inflammatory (rheumatoid arthritis and seronegative spondylarthritides) and degenerative (osteoarthritis) joint diseases and to establish possible correlation between the cell death rate and the matrix vesicles formation. Methods: Cartilage samples of the knee joint were obtained from patients with rheumatoid arthritis (RA, 18 cases), osteoarthritis (OA, 22 cases), Reiter’s disease (RD, 9 cases), peripheral form of the ankylosing spondylarthritides (AS, 6 cases) and psoriatic arthritis (PA, 6 cases) during arthroscopy or knee surgery. Normal samples taken from autopsy cases without a history of joint diseases were used as control. Samples were processed for TEM with subsequent semi-quantitative estimation of the cell death rate in the superficial, middle and deep zone of non-calcified articular cartilage, and computer-aided ultramorphometric evaluation of the matrix vesicles of different types. Results: Both apoptotic and necrotic cell death could be identified in the cartilage of patients with inflammatory joint diseases, including seronegative spondylarthritides and degenerative arthropathies. Apoptosis dominated over necrosis in all examined arthritides, including RA patients in which necrosis of the chondrocyte was the most frequent among arthropathies, while the highest apoptotic cell death rate was discovered in OA in which it correlated with the volume and numeric density of the matrix vesicles. These data provide evidence that apoptosis may contribute to the cartilage breakdown not only in RA and OA but also in the seronegative spondylarthritides, which had a significantly higher apoptotic rate than the normal cartilage.

Key words: articular cartilage, rheumatoid arthritis, Reiter’s disease, ankylosing spondylarthritides, psoriatic arthritis, osteoarthritis, electron microscopy, matrix vesicles, cell death.

INTRODUCTION

Cartilage destruction manifested by the loss of chondrocytes and matrix depletion is one of the most essential features in many arthropathies. Articular cartilage is comprised of a large amount of functional extracellular matrix maintained by a small number of chondrocytes, the sole resident cell type. It lacks perichondrium, nerves, blood and lymphatic vessels. Though metabolic processes may be very active in the articular cartilage, its regenerative potential remains very much limited.1-4 In both inflammatory and degenerative chronic joint diseases, chondrocytes of the different zones of articular cartilage undergo destruction, death and disintegration. Excessive loss of the chondrocytes results in irreversible cartilage breakdown; therefore a prevention of it becomes one of the most significant problems of modern arthrology.5-8

Recent investigations have demonstrated that chondrocytes die by apoptosis in rheumatoid arthritis (RA),9-12 osteoarthritis (OA)12-17 and traumatic arthropathies.18,19 Thorough knowledge of apoptotic cell death is of paramount importance for osteoarthrology, but despite many available ways of detecting apoptotic cell death, the analysis of apoptosis reveals considerable conceptual and technological problems.20 Many data from recent publications are controversial in terms of incidence of apoptosis in joint diseases and proportion of the cells dying by apoptosis and necrosis. Some of these discrepancies may be explained by application of different methods of cell death mode identification. In most of the papers apoptotic cells were estimated using transferase-mediated dUTP-biotin nick end labeling (TUNEL) method, although some investigators discovered that many necrotic cells are stained positively by this method, hence
overestimation of apoptosis becomes inevitable. Some of the investigations done by transmission electron microscope (TEM) do not take into account the stage of the cell death and the possibility of the development of secondary necrosis of chondrocytes what might provide another reason for the contradictions in the research data regarding cell death incidence in RA and OA. All the investigations of human articular cartilage with respect to apoptosis deal with either RA and OA, or traumatic arthropathies. Until now articular cartilage undergoing “milder” destruction in seronegative spondyloarthritides have not been investigated in terms of the cell death rate.

Although biogenesis of matrix vesicles was found to be related to the chondrocyte cell death by apoptosis, many authors using TEM for the description of the articular cartilage stated that they could not view bona fide separation of the matrix vesicles from the surface of the apoptotic cells. Quantitative aspects of the balance between the incidence of the apoptotic cells on one side and formation of matrix vesicles on the other have not yet been described. The objective of the present study was to determine in situ using TEM a degree to which apoptosis and necrosis occur in the articular cartilage of the patients with inflammatory (RA and seronegative spondyloarthritis) and degenerative (OA) joint diseases and to establish a correlation between the incidence of the matrix vesicles and cell death rate.

MATERIALS AND METHODS

Human knee cartilage from donors without a history of arthritis was obtained at the autopsy (10 samples: 4 women and 6 men between 22 and 54 years old, average 39.4 +/- 4.5); arthritic cartilage samples were obtained from patients at the time of knee surgery or during arthroscopy performed for diagnostic and therapeutic indications. Among them were 18 patients with rheumatoid arthritis (RA): 12 female and 6 male patients, average age was 40.2 +/- 1.6; 22 patients with primary osteoarthritis (15 female and 7 male patients, average age 48.9 +/- 1.8) and 21 patients with seronegative spondyloarthritides: Reiter’s disease (RD; 9 cases: all male between 16 and 62 years old, average age 30 years); peripheral form of the ankylosing spondyloarthritides (AS; 6 cases: 5 male and 1 female aged between 21 and 50 years, average 34.0 +/- 1.94) and psoriatic arthritis (PA; 6 cases: 4 female and 2 male patients aged between 25 and 42, average age 32.2 +/- 2.3 years).

Only those patients who had never received corticosteroids in the joint cavity of the examined joint prior to investigation were included in the quantitative assessment group. Samples were processed for TEM and embedded in Araldit resin. Semithin sections were stained with Taenzer-Unna stain. Ultrathin sections were stained with uranyl acetate-lead citrate and viewed under transmission electron microscope JEM 100S. The percentage of apoptotic cells was then estimated in the superficial, middle and deep zones of the non-calcified areas of the articular cartilage. Between 30 and 100 cells were evaluated in each zone of the cartilage in every specimen. To view sufficient amounts of cells for statistically significant results we needed to evaluate up to 10 blocks of tissue from every specimen, because in many case cartilage was considerably destroyed, the superficial layer was absent, the middle layer was partly destroyed and many lacunae appeared to be empty. For the ultramorphometric analysis of the matrix vesicles calibrated images of the articular cartilage zones were assessed using TRIM software (UOP, Russia) according to common morphometric principles.

Statistical analysis

Results are expressed as the mean +/- SEM. Statistical comparisons were performed with the unpaired 2-tailed t-test (parameters of matrix vesicles) and the percentage discrimination test (cell death rates). Correlation was estimated by Spearman’s rank correlation coefficient, Fisher’s transformation being used where necessary.

RESULTS

Most of the articular cartilage cells whose nucleus-containing profiles were assessed for the cell death rate had intact appearance with a predominance of the euchromatin in the nucleus and well-developed organelles (Fig.1). Cells undergoing both types of cell death, apoptosis and necrosis, could be found in almost every specimen (Figs 2-5). Different stages of apoptosis (the earlier and the later ones) were discovered in the damaged joints. The incidence of apoptosis and necrosis among the cells of the articular cartilage in different arthropathies and age-matched control group is shown in the Fig.6. Assessment of the nucleus-containing profiles of the chondrocytes showed that cell death rate was the highest in OA, being significantly higher compared to the joint diseases with milder cartilage destruction (AS, p<0.01 and RD,
FIG. 1: Electron micrograph of the knee joint cartilage: (a) chondrocyte of the middle zone of the normal cartilage with numerous large mitochondria (M) intermingled with cisternae of RER (E) and prominent Golgi apparatus (G). A few vacuoles and lysosomes (L) are visible. Chromatin is finely dispersed in the nucleus (N). Initial magnification 14,000x. (b) middle zone of the articular cartilage from the patient with AS. Isogenic group of chondrocytes with the two intact cells (C) surrounded by lacunar matrix lacking proteoglycan granules, with the remnants of the dead cell (D) in the interterritorial matrix. Initial magnification 3,000x
FIG. 2: Electron micrograph of the knee joint cartilage: (a) apoptotic middle zone chondrocyte (advanced stage of apoptosis) from the patient with PA with misshapen nucleus (N), marginated chromatin and accumulation of glycogen (GI) in the cytoplasm. The integrity of membranes seems to be retained despite apparent disorganization of cell structure. Initial magnification 5,000x. (b) advanced stage of apoptosis in the articular cartilage of the patient with RD. Patchy pattern of heterochromatin (H) condensation and margination within a large apoptotic body surrounded by a narrow rim of cytoplasm. A number of smaller apoptotic bodies are located nearby. Initial magnification 15,000x.
FIG. 3: Electron micrograph of the knee joint cartilage: (a) typical cap-like margination and fragmentation of chromatin resulted in the formation of rosette-like apoptotic bodies (AB) in the articular cartilage of the patient with RA. CF – collagen fibrils. Initial magnification 4,200x. (b) the two large apoptotic bodies in the cartilage of the patient with OA. Chromatin (Ch) is homogenized and strongly condensed, as is the surrounding cytoplasm. Initial magnification 6,000x.
FIG. 4: Electron micrograph of the knee joint cartilage from a patient with OA: (a) deep zone of the articular cartilage at the site where superficial and middle zones are completely destroyed, and lacuna is connected with the deep fissure, while lacunar matrix is absent. Chondrocyte is undergoing necrosis with karyopiknosis, homogenization of chromatin (Ch), rupture of plasmatic membrane and membranes of the organelles, destruction of RER cisterns, and vacuolization of cytoplasm. Initial magnification 4,200x. (b) Isogenous group of chondrocytes under destruction: cell (C) in the late stage of apoptosis preceding the formation of the post-apoptotic matrix vesicles conglomerations (MV). L lacuna. Initial magnification 1,400x.
FIG. 5: Electron micrograph of the knee joint cartilage from a patient with RD: (a) a front of erosion on the surface of the articular cartilage (arrow). Fragments of the dead cells are displayed, one of them with apparent budding of apoptotic bodies (Ch). Initial magnification 1,400x. (b) the chondrocyte undergoing initial stage of apoptosis with apparent formation of matrix vesicles by budding (arrow). Aside from typical patchy pattern of chromatin (Ch) the cell displays condensed cytoplasm accommodating accumulations of glycogen (Gl) and cytoplasmic fibrils (CF) obliterating the cytoplasm and other organelles. Initial magnification 20,000x.
TABLE 1: Mean diameter and volume density of matrix vesicles in articular cartilage of normal and arthritic joints

<table>
<thead>
<tr>
<th>Group</th>
<th>Diameter of matrix vesicles, mcm</th>
<th>Volume density of matrix vesicles (%)</th>
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<tbody>
<tr>
<td>Rheumatoid arthritis</td>
<td>0.185 ± 0.003</td>
<td>0.94</td>
</tr>
<tr>
<td>Osteoarthritis</td>
<td>0.208 ± 0.004***</td>
<td>1.98</td>
</tr>
<tr>
<td>Normal cartilage</td>
<td>0.161 ± 0.007***</td>
<td>0.26</td>
</tr>
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*** p < 0.001 compared to RA

p < 0.05). In AS this index was significantly lower than in RA (p < 0.05). Other seronegative arthritides’ cell death rates did not differ significantly, either compared to each other or to RA and OA. Therefore total cell death rate may not be considered a very informative index, although it was significantly lower in the age-matched control group than in any arthropathy under investigation.

Incidence of apoptosis was significantly higher in OA than in any inflammatory joint disease (RD, p < 0.001; AS, p < 0.05; RA and PA, p < 0.05). At the same time cells with the ultrastructural features of necrosis were more frequent in RA than in OA, despite the fact that even in RA apoptosis was more common than necrosis. Therefore apoptosis was a predominant mode of cell death in all the arthropathies under investigation.
FIG. 7: Electron micrograph of the knee joint cartilage: (a) aggregation of different types of the matrix vesicles: empty (thin arrow), amorphous (A) and crystalloid (C) in the interterritorial matrix of deep zone of the articular cartilage in RA. Some of the matrix vesicles reveal dense outer membrane (thick arrow). Initial magnification 15,000x. (b) a conglomerate of the large crystalloid matrix vesicles (C) with prominent prickle of crystals (arrow) in the deep zone of osteoarthritisic articular cartilage. A amorphous matrix vesicles. Initial magnification 20,000x.
investigation and in the normal joints while apoptotic cell death rate proved to be a very informative index discriminating not only normal and affected articular cartilage but inflammatory and degenerative joint diseases as well. Regarding distribution of the dying chondrocytes in the layers of the non-calcified cartilage, it was discovered that in the superficial zone not destroyed by pannus, chondrocytes died mainly by necrosis, while in the middle and deep zone apoptosis was dominant. In OA apoptosis was dominant in all the three layers, although in most cases under investigation the superficial zone was destroyed with rare necrotic cells discovered mainly in the middle layer, while in the deep layer they appeared at the sites where a deep slot connected a lacuna with the joint cavity (Fig. 4a).

It would not be sufficient to estimate the incidence of cell death only with respect to nucleus-containing profiles of the dying cells (Fig. 2a). As macrophageal cells are not present in the cartilage to remove the apoptotic bodies the latter may persist in the lacunae for a long time. Very often we could find aggregations of the matrix vesicles resembling the contours of the chondrocytes and representing sites of their disintegration (Fig. 4b). Rather frequently we could view matrix vesicles separating from the surface of a chondrocyte undergoing apoptosis (Fig. 5b). It was presumed long ago that the matrix vesicles represent apoptotic bodies, the presumption being supported later on by the other investigators, though evidence of the identity of the biochemical composition of matrix vesicles and apoptotic bodies was provided recently. We undertook ultramorphometric investigation of the incidence of matrix vesicles in the undamaged joints and those affected by inflammatory and degenerative joint diseases in terms of their type, size and numeric density. Matrix vesicles were classified into empty, amorphous and crystalloid type (Fig. 7).

As shown in Table 1 and Fig. 8, matrix vesicles had larger diameters in OA than in RA, while in the normal joints they were smaller than in both arthropathies (p<0.001). There was more than a 2-fold difference in the volume density of the matrix vesicles between OA and RA, and in both arthropathies this parameter greatly exceeded the one for the unaffected joints (Table 1). In OA there was a highly significant positive correlation between the numeric density of the matrix vesicles and the number of apoptotic cells as well as between the volume density of matrix vesicles and apoptotic index (Fig. 9). Regarding the distribution of the vesicles of different types in the normal articular cartilage, empty, amorphous and crystal vesicles comprised 28%, 43% and 29% respectively. Crystal vesicles were encountered only in the deep layer of the non-calcified cartilage. There

![FIG. 8: Size distribution of matrix vesicles in articular cartilage of normal and arthritic joints](image)
was definite correlation between the type and size of the vesicles: crystal ones were the largest among the matrix vesicles, therefore only rather large vesicles might become the centers of mineralization. Thus the size and the number of matrix vesicles reflect the character of cartilage breakdown; in OA both parameters were higher than in RA. These results are in accordance with the data regarding higher calcification of the articular cartilage in OA compared to the inflammatory arthropathies.30

FIG. 9: Correlation between the apoptotic chondrocyte rate and numeric density and volume density of matrix vesicles in osteoarthritis

APOPTOTIC INDEX

Numeric Density of Matrix Vesicles

r=+0.74; z=+0.95; p<0.001

Volume Density of Matrix Vesicles

r=+0.71; z=+0.89; p<0.001

ARTICULAR CARTILAGE IN ARTHROPATHIES

DISCUSSION

The present investigation provided new data on the incidence of the cell death rate (apoptosis and necrosis) in the articular cartilage of patients with inflammatory (RA, RD, AS, PA) and degenerative (OA) arthropathies compared to the normal joints basing on the semi-quantitative TEM analysis. The highest rate of apoptosis was detected in OA while lowest among arthritides was in the peripheral form of AS. Our data are in agreement with the results of other investigations where high apoptosis rate in OA were discovered by TUNEL method, flow cytometry and immunohistochemical identification of apoptotic cells.14,15,24,31 Contrary to the data of some other investigations,6,11,17 which found that apoptosis was not a widespread phenomenon in OA or that in RA the apoptotic index was higher than in OA, we discovered that in RA it was significantly lower, compared to OA, and remained the dominating mode of cell death in both arthropathies. The reasons for these discrepancies were explained in a very impressive recent comment by T. Aigner20 regarding “the confusing complexity of results” of assessment of the cell death in the articular cartilage which “might be partly due to technical variability or insufficiency; more likely, however, this provides evidence that most if not all approaches thought to be ‘specific’ for apoptotic cell death address different aspects of cell death, have different sensitivities and specificities”. Until now most investigators using TEM for examination of the articular cartilage consider it to be one of the most reliable methods of cell death discrimination,5,9,24,32,33 but an insufficient amount of cases examined ultrastructurally by some researchers (not more than 3 or 4 cases per group) did not allow them to make conclusions regarding quantitative aspects of apoptosis in the articular cartilage by means of TEM. This remark is equally applicable to the explanation of the discrepancies in the distribution of the apoptotic cells between the layers of the non-calcified articular cartilage. According to our data apoptosis was dominant in all the three layers of the articular cartilage in OA, while other investigators13,31 confine it mainly to the superficial layer. As for normal cartilage, evaluating as many as 300 cells per specimen we could identify apoptotic cells in each of them, the range being between 0.7% and 3% contrary to the data of some other investigators who did not find apoptotic cells in the normal articular cartilage.31 Regarding the balance between apoptotic and necrotic cells, apoptosis was found to be dominant even in RA, in which necrotic rate was the highest among arthropathies. It has been stated33 that cells which are clearly necrotic as revealed by TEM may also stain positively for TUNEL thus indicating the risk of using the latter alone for the assessment of cell viability, apoptotic and necrotic processes in the whole-tissue specimens. In the present investigation we verified apoptosis
and necrosis by TEM basing on the major distinguishing features of the two types of cell death.\(^\text{25}\) Most of these features were common for all types of the dying cells in terms of the chromatin margination and condensation, nuclear disintegarion, formation of blebs, shrinkage and disintegration of the cytoplasm. At the same time early stages of the chondrocyte apoptosis displayed some rather specific ultrastructural features such as accumulation of glycogen and cytoplasmic filaments (Figs. 3a and 6b), while the loss of glycogen was typical for the necrobiotic stage. Contrary to some other investigators\(^\text{9,18}\) we could find bona fide apoptotic bodies accompanied by external membrane blebbing (Fig.5b). It remains controversial as to how biogenesis of matrix vesicles is related to apoptosis; recently it has been shown that 24-hour treatment with an apoptosis-inducing agent does not increase matrix vesicle protein or alter the calcifying activity of vesicles.\(^\text{24-28}\) In the present investigation we discovered a highly significant correlation in OA between apoptotic death rate and incidence of the matrix vesicles in terms of their volume and numeric density. This observation gives extra evidence of the relationship between chondrocyte apoptosis and matrix vesicles biogenesis and corresponds to the data of the other investigators regarding calcification of the articular cartilage in OA.\(^\text{16,28,30}\)

We obtained new data on the cell death rate in seronegative spondyloarthritis which to the best of our knowledge has not been previously assessed. These data reveal evidence that apoptosis is implicated in cartilage breakdown even in the arthropathies characterized by milder joint destruction compared to that in OA and RA. These data may provide better understanding of the nature of joint destruction in different arthropathies and promote the development of new therapeutic strategies aimed at prevention of excessive cell death and irreversible cartilage breakdown.

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