Apoptosis as a Mechanism of Autoimmune Inflammation in Human Knee Joint

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Apoptosis in the cartilage and synovial membrane of the knee joint in patients with rheumatoid arthritis was studied using the immunocytochemical TUNEL method. The degree of apoptosis correlated with the duration of inflammation. The process predominated in the chondroblast population, lymphocytic infiltration, and synovial membrane fibroblasts.

Key Words: *apoptosis; chondroblasts; synovial membrane; rheumatoid arthritis*

The mechanisms of apoptosis are now intensely studied among the pathogenetic aspects of rheumatoid arthritis (RA). Activity of this process essentially modulates the time course of inflammatory process [4,7, 8,13]. Apoptosis limits proliferative activity of T cells and restricts the zone of inflammatory infiltration in joints of rats with adjuvant arthritis [13]. However, during the acute period of experimental arthritis these effects are inverted [9,14]. Studies of apoptosis should be carried out at different stages of inflammation, but the known models not always correspond to human RA [1,5]. Cell elements of the joints liable to apoptosis are still under question. Here we studied apoptosis of the cartilage and cells of the synovial fluid involved in the development of RA in human knee joints.

MATERIALS AND METHODS

Specimens of the cartilage and synovial membrane of the knee joint from 2 female and 3 male patients with RA (age 25-46 years) were studied. The patients were divided into 2 groups: with early (up to 2 years) and late (more than 10 years) RA. The material was collected during arthroscopy and synoviocapsulectomy, which were carried out according to medical indications. Articular tissue from a man with trauma of the inner meniscus without history of rheumatic disease served as the control.

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Apoptosis was studied by the immunocytochemical method TUNEL (terminal deoxynucleotidil transferase-mediated dUTP nick end-labeling), based on the detection of fragmented DNA. The material was fixed for 2 h at 4°C in cold 4% paraformaldehyde prepared on 0.1 M phosphate buffer (pH 7.4). The samples were washed for 24 h in 0.1 M phosphate buffer which was replaced 7-8 times, after which were plunged in 15% buffered sucrose solution. Cryostat sections (20 μ) were mounted on slides, postfixed in cold ethanol-acetic acid solution for 5 min at -20°C, and washed (2×5 min) in phosphate buffer. Leveling buffer (75 μl) was applied onto slides and left for at least 10 sec at ambient temperature. TUNEL-stained structures were detected using ApopTag Fluorescein In Situ Apoptosis Detection Kits (Chemicon). The sections were incubated according to the standard protocol in a humid chamber for 1 h at 37°C, after which were plunged in stop buffer (10 min). The sections were washed 3 times in phosphate buffer (5 min in each portion) and then incubated for 1 h with Fab-fragment of porcine second antibodies to FITC-conjugated rabbit Ig (Dako). The sections were thoroughly washed in phosphate buffer, embedded in glycerol, and examined through a FITC type filter (B1 450-490 nm) under a Polyvar microscope. Some sections were stained with Toluidine Blue.

Quantitative evaluation of TUNEL-positive nuclei was carried out using an ocular morphometric grid applied onto 100×100 μ sites of the cartilage and synovial tissue. The apoptotic index (AI) was estimated as

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the ratio of total number of TUNEL-positive nuclei (N_{TUNEL}) to the number of cells stained with Toluidine Blue with visible non-pyknotic nucleus (N_T) by the $AI=(N_{TUNEL}\times100)/N_T$ formula [11]. The data were processed by variation statistics methods using Student's *t* test ($p<0.05$).

RESULTS

The TUNEL method selectively detects apoptotic cells by the presence of fluorescent label in the nuclei with signs of DNA fragmentation: condensed chromatin, pyknosis, karyorrhexis (Fig. 1). Morphological signs of apoptosis and necrosis detected on the cartilage and synovial sections are mutually exclusive. TUNEL-positive elements are characterized by intact cell membrane without foci of inflammatory infiltration close to them.

Intensive fluorescence of apoptotic cell nuclei was detected in all studied RA cases. No TUNEL reaction was detected in the control. Chondroblasts at late stage of RA development were characterized by the highest AI values (Table 1). These cells located mainly in the surface zone of the cartilage formed isogenic groups containing 1-3 nuclei of apoptotic cells (Fig. 1, *a*). TUNEL labeled solitary chondrocyte nuclei were extremely rarely detected in the depth of the cartilage, where activity of the proliferative processes was much lower.

In the synovial membrane apoptosis developed in cell elements. TUNEL-positive cells were concentrated mainly in the perivascular spaces; their location coincided with that of inflammatory infiltration (Fig. 1, *b*). Lymphocytes were identified on sections stained with toluidine blue. Capillary walls did not react with TUNEL. Fluorescence of fragmented nuclei of solitary synoviocytes was observed in some cases (Fig. 1, *c*).

The prevalence of apoptosis correlated with the duration and severity of synovial inflammatory infiltration. The highest AI values were found at the late stage of RA (Table 1). Morphological profile of apoptotic structures in such cases was shifted towards hypertrophic synovial membrane fibroblasts (Fig. 1, *d*).

Apoptosis develops as a result of mutual effects of regulatory factors, but the process is triggered by

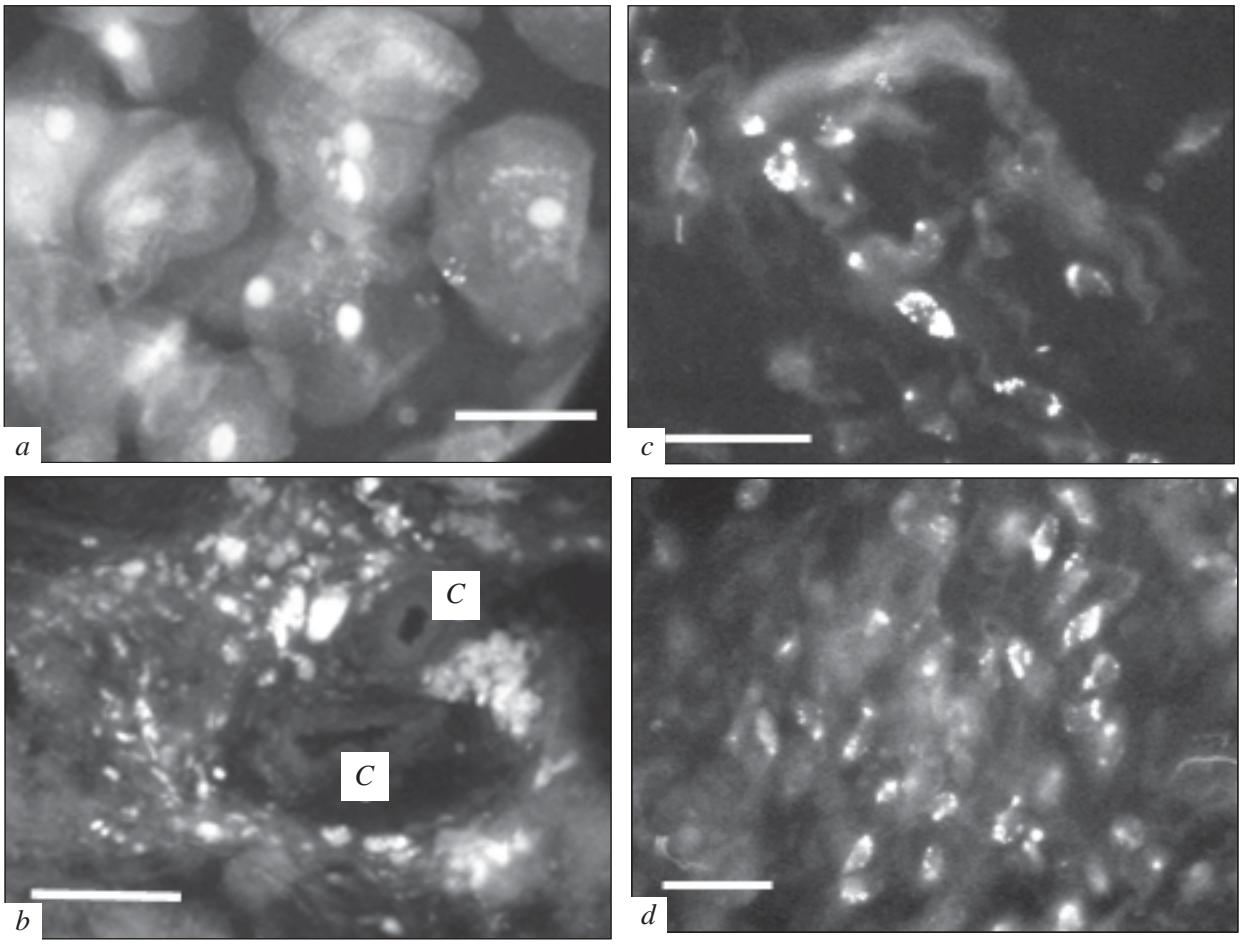

Fig. 1. Distribution of TUNEL-positive cells in structures of human knee joint in rheumatoid arthritis. *a*) chondroblasts with signs of apoptosis forming isogenic groups; *b*) TUNEL-immunoreactive lymphocytes of synovial membrane. Apoptosis of synoviocytes (*c*) and synovial fibroblasts (*d*). *C*: capillary lumen. Scale: 25 μ.

TABLE 1. Human Knee Joint Cartilage and Synovial Membrane Cell AI in RA

Cell elements of the joint	Early RA	Late RA
Chondroblasts	0.07±0.05	0.40±0.09
Chondrocytes	N.d.	0.01±0.03
Synovial lymphocytes	2.1±0.5	5.8±1.2
Synovial fibroblasts	0.2±0.1	0.7±0.2
Synoviocytes	N.d.	0.03±0.01

Note. "N.d.": not detected.

the metabolic conditions of the nearest microenvironment. Cytoplasmic caspases mediate irreversible activation of apoptosis. Caspase activity leads to DNA fragmentation, subcellular organelle proteolysis, cell autointoxication and death [2]. Impairment of apoptosis induction mechanisms leading to inhibition or, vice versa, to hyperactivation of this process can be an important factor in the pathogenesis of RA. For example, apoptosis of synovial cell is effectively blocked by application of TNF and IL-1β [14]. Endogenous inhibitor of caspases FLIP protein (FLICE-inhibitory protein) suppresses activity of caspase-8 and transfer of the apoptotic signal from p75 family "death receptors". High level of FLIP is expressed in the synovial macrophage cytoplasm in patients with only early stages of RA development [3]. Our data indicate that apoptosis primarily involves the fibroblast population. Death of these cells inhibits organization of inflammatory infiltration and, presumably, prevents the formation of sclerotic changes in the synovium at late stages of RA.

During the late period of RA the balance between the connective tissue proliferation and apoptosis is shifted towards apoptosis, while the early stage of RA is characterized by opposite relationships. The data of pharmacological histochemical studies suggest that the morphotypical heterogeneity of TUNEL-reactive cells is determined by different mechanisms of their programmed death [4,10]. Presumably, apoptosis is triggered via alternative pathways [6,15]. For instance, mitochondrial pathway is induced by production of transcriptor factor p53; the defect in this factor leads to tumor-like growth of synoviocytes and fibroblasts [7]. Another mechanism of apoptosis develops in lymphocytes and is blocked by Bcl-2 factor [12]. Presumably, the expression of Bcl-2 at the early stage of RA limits the number of apoptotic cells and creates favorable conditions for proliferation, survival, and active functioning of inflammatory elements.

Pathological reorganization of articular tissues is paralleled by the decrease in chondrocyte compactness, which are subjected to apoptosis under conditions of long rheumatoid inflammation. Presumably, these effects are initiated by previous synovitis and can limit the growth and regeneration of the cartilage.

Hence, alteration of strictly programmed phases of selective cell death as a result of apoptosis correlates with progressive development of RA and is an important component in the pathogenesis of autoimmune inflammation.

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