MORPHOLOGY AND PATHOMORPHOLOGY

Nitroxide Synthase Activities at Different Stages of Adjuvant Arthritis

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 143, No. 5, pp. 576-579, May, 2007
Original article submitted March 23, 2006

The location and activities of nitric oxide synthases in synovial cells during different stages of inflammatory process were studied by the immunocytochemical method in animals with experimental rheumatoid arthritis. Direct involvement of the NOergic mechanisms in the development of adjuvant arthritis was demonstarted.

Key Words: *adjuvant arthritis; nitric oxide neuronal and inducible synthases; NADPH diaphorase*

Rheumatoid arthritis (RA) is a progressive uncontrollable inflammation of the articular synovial membrane [3]. Despite intensive studies, the involvement of various mediators in the formation of tissue destruction and "malignant" degeneration of the synovial membrane is poorly studied [1]. Recent studies were focused on the role of nitric oxide (NO) in the development of autoimmune synovitis [11,13,16]. It was found that nitrite concentration in the synovial fluid of RA patients was significantly higher than serum nitrite concentrations in the same patients, which illustrated high local level of NO synthesis in the joints involved in RA [11,14]. Despite the data on the nitroxidergic function of the synovial membrane, the dynamics of expression of NO synthase (NOS) in the articular tissues at different stages of RA remains not quite clear.

We studied the role of NOS at different stages of the development of synovial inflammation of autoimmune origin.

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MATERIALS AND METHODS

The study was carried out on C57Bl/6 mice (n=30) characterized by immune response with activation of Th1 lymphocyte subpopulation. This feature allows adequate modeling of adjuvant arthritis (AA) [4,8]. AA was induced by injection of 0.1 ml complete Freund's adjuvant (Difco) into the hind paw pad (n=15).

The animals were sacrificed on days 7, 14, and 30 after the adjuvant injection (5 per term). Control group consisted of 15 intact mice. All experiments were carried out in accordance with regulations on humane handling of laboratory animals (Supplement 4 to Order No. 755 issued by the Ministry of Health of the Russian Federation).

The material was collected 10 min after sacrifice. The hind paws were amputated and fixed in 3% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h, after which the preparations were stored at ambient temperature in decalcification solution (5.5 g EDTA, 90 ml distilled water, and 10 ml 10% formalin). Some specimens were washed in buffered 30% sucrose solution for 12-24 h at 4°C. Serial 50-μ sections for histochemical reaction for NADPH diaphorase (NADPH-d) and 20-μ sec-

0007-4888/07/1435-0638

tions for immunohistochemical reaction were prepared in the frontal plane on a cryotome. A part of the material was embedded in paraffin, stained with hematoxylin and eosin for interpretation of morphological changes.

Summary NOS activity in synovial membrane cells was evaluated by a previously described method [12], bearing in mind that NADPH-d is a specific topochemical NOS marker [6]. Activity of NADPH-d was evaluated by the density of histochemical precipitate in synoviocyte cytoplasm and expressed in optical density units.

The location of n-NOS and i-NOS was studied in specimens of the tibiotarsal joint synovial membrane. Immunocytochemical staining of sections included several successive steps: preincubation, treatment with first antibodies, treatment with second antibodies, and immunoperoxidase test. Treatment with second antibodies and the immunoperoxidase test were carried out according to the instruction for ImmunoCruz Staining System (Santa Crus). Quantitative data were statistically processed using Student's t test ($p<0.05$).

RESULTS

The minimum effective dose of complete Freund's adjuvant injected into the tibiotarsal joint caused autoimmune inflammation, which was comparable by morphological signs with RA in humans. The

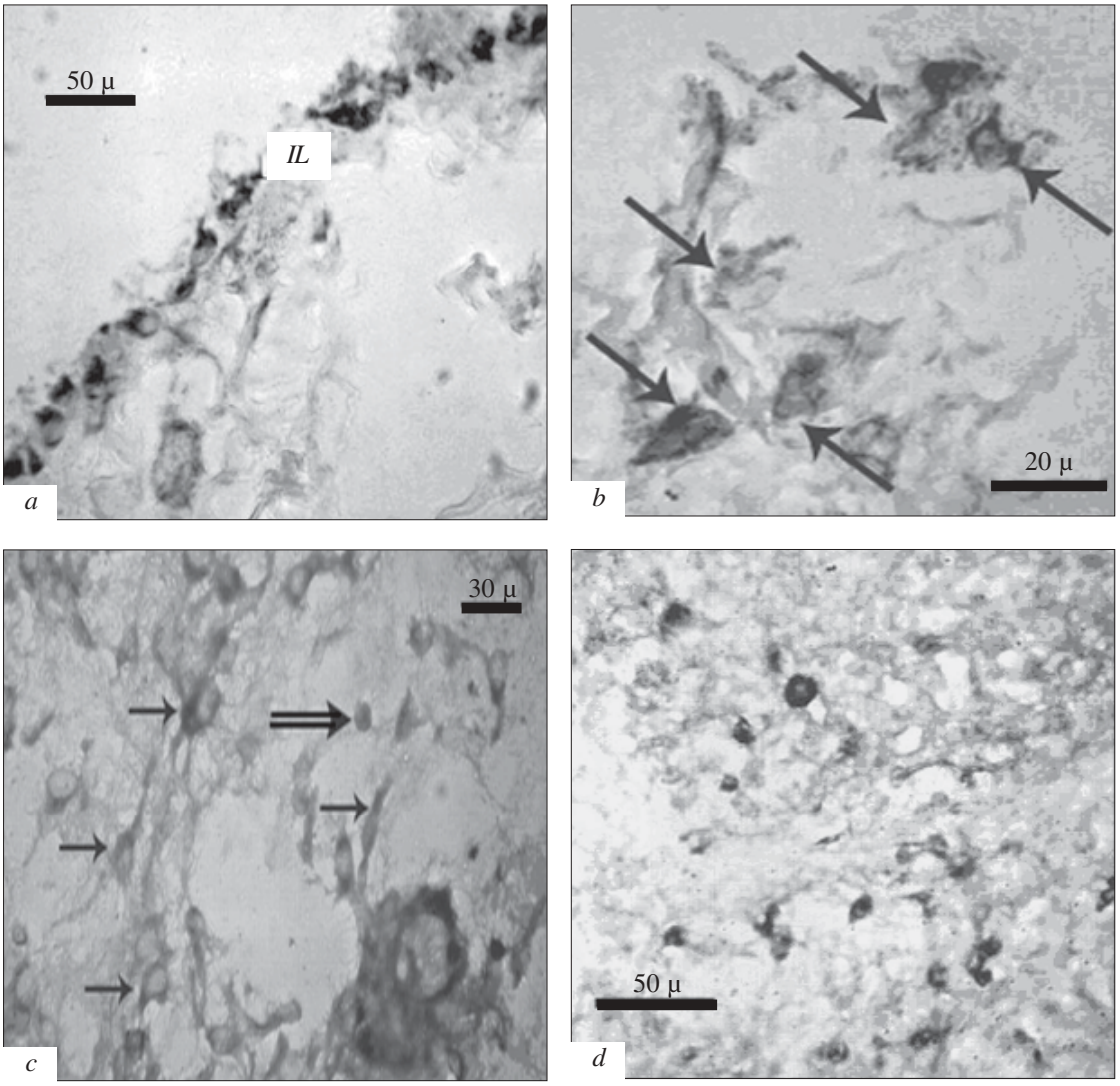

Fig. 1. NADPH-d, n-NOS, and i-NOS in tissues of mouse tibiotarsal joint at different stages of AA development. *a*) NADPH-d-positive synoviocytes of the surface layer (SL) at the early stage of AA; *b*) NADPH-d in mouse synovial membrane at the early stage of AA. Arrows show fibroblast- and macrophage-like cells of stromal layer; *c*) i-NOS-immunopositive synoviocytes of the synovial membrane stromal layer at the early stage of AA. Labeled fibroblast-like cells (arrow), macrophages and lymphocytes (double arrow); *d*) n-NOS in stromal layer synoviocytes at the stage of chronic inflammation (day 30 after adjuvant injection).

TABLE 1. NADPH-d (opt. dens. units) in Mouse Joint Synoviocytes in AA (*M*±*m*)

Synovial structure	Control	Day 14	Day 30
Macrophages	57.2±1.4	69.2±1.3*	60.1±3.4*+
Fibroblasts	51.1±2.1	66.1±1.4*	59.4±3.2*+

Note. Here and in Table 2: $p<0.05$ *compared to the control, +between the groups.

histological picture of AA can be divided into two stages: early arthritis (up to day 14 after the adjuvant injection) and late or chronic arthritis (day 30 of inflammation).

Activity of NADPH-d in intact C57Bl/6 mice was detected in the synoviocytes of the surface and stromal layers of the synovial membrane. In the surface layer, NADPH-d-positive synoviocytes formed a tight layer of elements of the same structure and shape. Three groups of NADPH-d-positive elements were distinguished by the intensity of cell staining: with high, moderate, and low activity of the enzyme.

Synoviocytes with moderate activity of the enzyme (42-60 opt. dens. units) predominated in the surface layer. No NADPH-d-positive cells were detected in the subsurface layer. The neuronal isoform of NOS activity was detected in synoviocytes of the surface, subsurface, and stromal layers of the synovial membrane. The percent of n-NOS-positive synoviocytes was 51.2±1.1% ($p<0.05$). No expression of i-NOS was detected in synovial membrane cells of control animals.

NADPH-d-positive synoviocytes of the surface, subsurface, and stromal layers with high activity of the enzyme were detected in the synovial membranes of mice with AA (Fig. 1, *a*, *b*). The numbers of labeled cells, predominantly with high level of enzyme activity, increased by on average 30.4% on days 7 and 14 of the experiment. Enzyme activity reached a high level (69.2±1.3 opt. dens. units), virtually no cells with moderate or low content of NADPH-d were detected (Table 1). On day 30 these values decreased significantly approaching the control level.

Immunocytochemical study of NOS showed increasing activity of i-NOS in the synoviocytes at the early stage of AA. The majority of cells were located in the surface and stromal layers (Fig. 1, *c*). The chronic phase of inflammatory reaction was characterized by reduction of i-NOS-immunoreactive staining of cells and more intensive n-NOS staining (Fig. 1, *d*). Comparative analysis showed that the content of i-NOS-immunopositive cells differed significantly from the number of cells containing n-NOS (Table 2). The dynamics of inflammation was associated with changes in not only i-NOS activity, but also typological pattern of labeled cells: mainly pleiomorphic synoviocytes of the surface and subsurface layers were detected during week 1 of AA, while later mainly stromal layer cells were detected with predominating i-NOS-immunoreactive fibroblasts and lymphocytes. Heterogeneous localization of the constitutive and inducible NOS indicated heterogeneous participation of NO in the development of AA.

Hence, we revealed direct involvement of NOergic mechanisms in AA development. These mechanisms are determined by the synoviocyte function, NOS activity in these cells being heterogeneous: i-NOS predominates during the acute phase, while n-NOS is synthesized incessantly during the acute and chronic phases of arthritis. All this suggests the protective function of i-NOS and pathological role of n-NOS.

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TABLE 2. i-NOS and n-NOS in Synovial Structures of Mouse Tibiotarsal Joint in AA

Cells	n-NOS/i-NOS control	i-NOS-positive cells, %		n-NOS-positive cells, %	
		day 14	day 30	day 14	day 30
Lymphocytes	0/0	93.2±1.2*	36.3±3.2+	97.2±2.2*	52.4±3.1*+
Synoviocytes	51.3±1.1/0	74.4±2.1*	23.2±1.1+	68.3±3.1*	56.2±2.2*+

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