Rheumatology 2000;39:1004–1008

Nitric oxide is a mediator of apoptosis in the rheumatoid joint

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Abstract

Objective. To study the role of nitric oxide (NO) derived from the inducible nitric oxide synthase (iNOS) pathway in the induction of apoptosis in the rheumatoid joint.

Methods. Joint tissue was obtained from four rheumatoid arthritis (RA) patients, three osteoarthritis patients and two patients with a fractured neck of the femur (NOF#), and apoptotic cells were identified in cryosections using the TUNEL (terminal dUTP nick end labelling) assay. Expression of iNOS was determined using immunohistochemistry. NO synthesis and the effect of NOS inhibitors on apoptosis levels were studied in explant cultures of RA cartilage and synovium.

Results. Numbers of apoptotic cells were greatly increased in rheumatoid synovium and articular cartilage compared with NOF# and osteoarthritic synovium. Immunohistochemistry showed co-localization of iNOS staining and apoptosis in the synovial lining layer and articular cartilage. The NOS inhibitor L-NMMA (L-N^G-monomethylarginine) strongly inhibited apoptosis in explant cultures of synovium and cartilage, and this was reversed by the NO donor *S*-nitroso-acetyl-penicillamine.

Conclusion. This study indicates that NO acts as a mediator of apoptosis in RA and suggests that NOS inhibitors reverse this process.

KEY WORDS: Rheumatoid arthritis, Cytokines, Nitric oxide, Apoptosis, Cartilage, Synovium.

Inflammatory diseases such as rheumatoid arthritis (RA) are associated with increased production of nitric oxide (NO), due to activation of the inducible nitric oxide synthase (iNOS) pathway [1–3]. Studies in animal models have suggested that NO plays a causal role in the pathogenesis of joint inflammation and tissue damage, since the severity of arthritis can be reduced by the administration of NOS inhibitors [4–6]. Several cell types present within the joint, including synovial fibroblasts, endothelial cells and chondrocytes, can be induced by pro-inflammatory cytokines to produce NO *in vitro* [7–10]. Moreover, localization studies have shown that there is up-regulation of iNOS expression in synovial lining cells, chondrocytes and blood vessels in joint tissues obtained from patients with RA [11–13]. The localization of iNOS expression to the synovial lining layer and cartilage is of interest in the light of other studies which have shown that apoptosis is increased in RA, particularly in the synovial lining layer and cartilage [14, 15]. The mechanisms responsible for apoptosis in the rheumatoid joint remain unclear, although previous workers have shown that expression of Fas antigen is increased in RA synoviocytes [15, 16] and that Fas antibody can stimulate the apoptotic death of synoviocytes *in vitro* [15]. In this study we investigated the hypothesis that activation of the iNOS pathway is also involved in stimulating apoptosis in the rheumatoid joint, since high levels of NO are known to stimulate apoptosis in many cell types *in vitro* [17–20].

Submitted 15 April 1999; revised version accepted 14 March 2000.
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Methods

Patients

Samples of synovium and cartilage were obtained from two patients undergoing surgery for fractured neck of the femur (females aged 79 and 74 yr), four RA patients (two males and two females aged between 38 and 72 yr) and three osteoarthritis (OA) patients (one male and two females aged between 59 and 80 yr) undergoing hip replacement surgery. Informed consent was obtained from all patients and the study was approved by the Grampian Research Ethics Committee. The tissue specimens were transported to the laboratory immediately after collection for explant culture, or were snap-frozen in TissueTek cryostat embedding medium (Miles Inc., Elkhart, IN, USA) and stored at -20°C for immunohistochemical studies.

Explant culture

Explants of RA synovium and cartilage were cultured on stainless steel grids in 12-well tissue culture plates (Costar, High Wycombe, UK) using α-MEM medium

supplemented with 10% fetal calf serum and penicillin/streptomycin (Gibco). The tissue explants were cultured for 24 h in the presence or absence of the NOS inhibitor L-N^G-monomethylarginine (L-NMMA) (1 mM) and/or the NO donor *S*-nitroso-acetyl-penicillamine (SNAP) (300 μM). At the end of the culture period, the explants were frozen in TissueTek cryostat embedding medium. Sections (7 μm) were cut on a cryostat and mounted on chrome gel-subbed glass slides. The sections were air-dried overnight and stored at -20°C until immunohistochemical analysis could be performed. Additional explants were fixed in 2% glutaraldehyde, embedded in Epon and examined by electron microscopy.

Nitrite measurement

Culture supernatants were assayed for nitrite, a stable metabolite of NO, using the Griess reaction, as described previously [10].

Detection of apoptosis

Apoptosis was detected by the terminal dUTP nick end labelling (TUNEL) method [21] using the ApopTag Plus *In Situ* Apoptosis Detection Kit (Oncor, Gaithersburg, MD, USA). Sections were fixed in formalin for 10 min, washed twice in phosphate-buffered saline (PBS) and post-fixed in ethanol/acetic acid (2:1) for 5 min at -20°C. Subsequently, the sections were washed twice in PBS at room temperature, incubated with equilibration buffer for 3 min followed by terminal deoxynucleotide transferase (TdT) reaction buffer for 1.5 h at 37°C. The TdT reaction was terminated by a 30 min incubation in stop buffer and three washes in PBS. The sections were then either (i) incubated for 30 min with fluorescein isothiocyanate (FITC)-conjugated anti-digoxigenin (DIG) (fluorescence detection) then washed and embedded, or (ii) incubated for 30 min with alkaline phosphatase (AP)-conjugated anti-DIG, washed in PBS, incubated with AP-black substrate (Vector Labs), then washed and embedded. Total numbers of nuclei were determined by counterstaining with DAPI (4′,6-diamidino-2-phenylindole; Sigma Chemical Company, Poole, UK) or haematoxylin. The percentage of apoptotic nuclei was then calculated in a minimum of four sections of each treatment for each patient sample. For synovium, 200–300 nuclei were counted per section, whereas for cartilage 100–200 nuclei were counted per section.

Immunohistochemistry

Staining for iNOS was performed using a well-characterized polyclonal rabbit anti-human iNOS antibody (a gift from Dr H. Oshima, International Agency for Research on Cancer, Lyon, France) as previously described [13]. In short, the sections were fixed in 50% acetone/PBS for 5 min followed by a 10 min incubation with 1% H_2O_2 in methanol to quench endogenous peroxide activity and washed twice in PBS. The sections were then incubated with the anti-iNOS antibody for 45 min, washed twice in PBS and incubated for 45 min with biotinylated goat anti-rabbit antibody. The biotinylated goat anti-rabbit antibody was detected using a Texas red-conjugated tyramide amplification kit (DuPont NEN Research Products, Boston, Massachusetts, USA) and visualized by fluorescence microscopy. For double staining for iNOS and apoptosis, the sections were first stained for iNOS as described, followed by the TUNEL stain using FITC-conjugated anti-DIG.

Statistical analysis

Differences between groups were analysed by univariate analysis of variance with Tukey's post-test using SPSS for windows. Differences were considered to be significant if the two-tailed P values were $\leqslant 0.05$.

Results

Many apoptotic cells were detected in the synovial lining layer ($42.2 \pm 9.1\%$; four sections from four patients) and in cartilage ($49.8 \pm 28.4\%$; four sections from three patients) of RA patients, as determined by the TUNEL assay (Fig. 1a, b) whereas apoptotic cells were sparse in lymphoid aggregates (Fig. 1c). The presence of apoptosis was confirmed by the finding of typical morphological changes on electron microscopic examination (Fig. 1d) and by DNA laddering (not shown). Apoptosis was virtually absent in osteoarthritic synovium ($<0.1\%$; four sections from three patients) (Fig. 1e) or normal synovium ($<0.1\%$; four sections from two patients) (not shown). These differences were highly significant ($P < 0.001$).

Immunohistochemical studies showed that there was a close correlation between the presence of iNOS staining and apoptosis in the synovial lining layer, as detected by double staining of RA sections for the expression of both iNOS and DNA strand breaks (Fig. 2). Diffuse staining for iNOS (red) and apoptotic nuclei (green) was widespread in the synovium. Most apoptotic nuclei were adjacent to cytoplasmic iNOS-stained tissue, and several apoptotic nuclei appeared yellow, indicating double staining for both iNOS and apoptosis. In view of the fact that apoptotic cells were closely associated with iNOS immunoreactivity, we went on to study the effects of an NOS inhibitor and an NO donor on apoptosis in short-term cultures of synovial and cartilage explants from patients with RA.

In the cartilage explants, the 24 h medium NO_2^- level varied between 4.4 and 27.1 μM. In a typical explant culture, L-NMMA reduced the NO_2^- level from 6.1 ± 1.8 to 1.8 ± 0.2 μM ($P < 0.05$). NO_2^- was not detectable in the synovium explants, probably because of the small tissue/culture volume ratio. Analysis of synovial explants which had been cultured for 24 h in medium alone showed high numbers of apoptotic cells, whereas apoptosis was greatly reduced in matched explants cultured in the presence of the NOS inhibitor L-NMMA (Fig. 3A). Apoptosis increased once again when explants were cultured in the presence of both L-NMMA and the NO donor SNAP. Identical results were observed in cartilage explants that had been

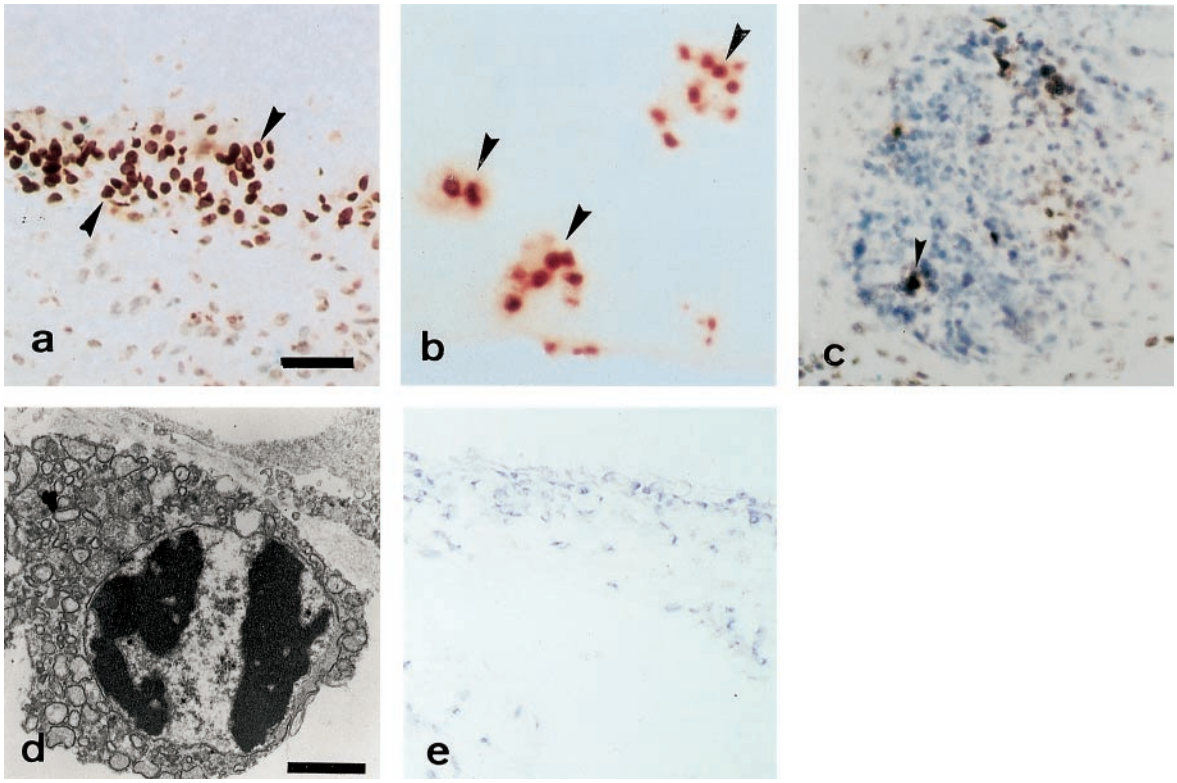

FIG. 1. Apoptotic cells in synovium and cartilage of RA patients. Apoptotic cells were identified using the TUNEL stain as described. Large numbers of apoptotic nuclei (arrowheads) were present in cryosections of RA synovium (a) and cartilage (b), whereas hardly any were observed in lymphoid aggregates (d) or OA synovium (e). Apoptosis was confirmed using electron microscopy (c, RA synovium). Scale bar = 80 μm in a, b, c and e; 3 μm in d.

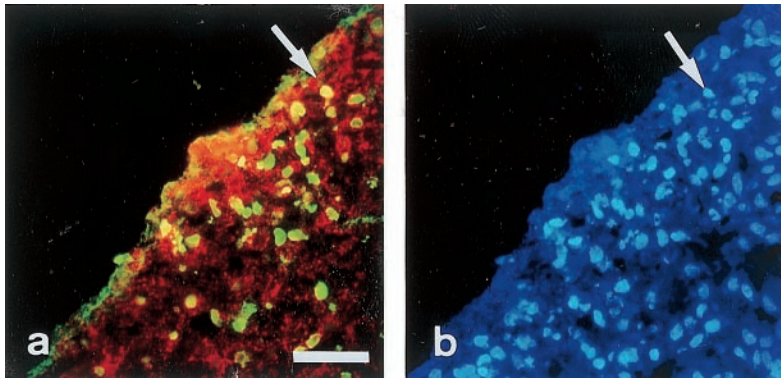

FIG. 2. Co-localization of iNOS and apoptosis. (a) Cryosections of RA synovium were double-stained for iNOS (red) and apoptosis (TUNEL, green). The synovial lining layer is clearly positive for both iNOS and apoptosis. The outer edge of the tissue also appears to be stained, but this is due to non-specific staining. (b) The same area of tissue stained using DAPI, showing the nuclei of all cells. Scale bar = 80 μm. An example of a cell showing dual staining is indicated by the white arrowhead.

cultured under the same conditions (Fig. 3B). Taken together, these experiments show that NO is necessary to stimulate synovial cell and chondrocyte apoptosis under these experimental conditions.

Discussion

In this study we found increased numbers of apoptotic synovial lining cells and chondrocytes in tissue samples from patients with rheumatoid arthritis, compared with similar samples from osteoarthritic and normal joints. These data are in keeping with the results of previous workers and confirm that inflammatory disease is associated with up-regulation of apoptosis within the joint [14, 15]. The distribution of apoptotic cells closely mirrored that of iNOS expression, and double staining for the presence of iNOS and DNA strand breaks showed that many of the cells which were undergoing

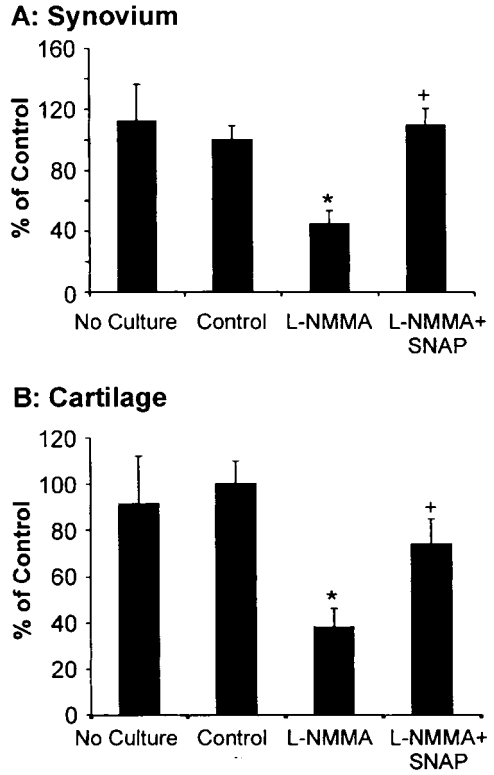

FIG. 3. Apoptosis in rheumatoid synovium and cartilage is NO-dependent. Apoptosis expressed as percentage of control ± S.E.M. in RA synovium (A) and cartilage (B) explants before culture, and after culture in the absence or presence of L-NMMA or L-NMMA plus the NO donor SNAP ($n = 4$; $^{*}P < 0.05$ *vs* control, $^{+}P < 0.01$ *vs* L-NMMA). Control levels of apoptosis were 61.1 ± 13.8% for the synovial lining layer and 43.9 ± 33.5% for cartilage.

apoptosis were also expressing iNOS. We went on to study the effects of NOS inhibitors and NO donors on apoptosis *ex vivo*, using short-term explant cultures of synovial tissue and cartilage from patients with RA. These experiments showed that the NOS inhibitor L-NMMA exerted dramatic inhibitory effects on apoptosis in explants of both synovium and cartilage, which were reversed by the NO donor SNAP. Although high levels of apoptosis were also observed in explants cultured under control conditions for 24 h, the percentage of apoptotic cells was similar to those in freshly isolated explants, which excludes the possibility that the apoptosis observed was merely an artefact of the culture process. Taken together with the co-localization of iNOS expression and apoptosis in tissue sections, these data strongly suggest that iNOS activation and increased NO production contribute to the high levels of apoptosis which are observed in RA. These data are consistent with those of other workers who have shown that NO induces apoptosis in cultured chondrocytes [19] and synovial fibroblast-like cells *in vitro* [22]. The difference between these studies and those reported here is that our studies used a tissue explant system in which the cells were studied under conditions more closely resembling their normal environment. Furthermore, we studied the role of NO produced by the tissue itself, whereas Aupperle *et al.* [22] studied the effects of NO generated by pharmacological NO donors. It has been suggested that other factors, such as dysregulation of p53 [22] and increased Fas expression [15, 16], may also contribute to the stimulation of apoptosis in this situation. It has been found previously that inactivation of p53 inhibits NO-induced apoptosis of synovial fibroblast-like cell lines, indicating that activation of the p53 pathway is involved in regulating NO-induced apoptosis [22]. Our results do not exclude a contributory role for Fas or p53 in this situation. Rather, they indicate that NO plays an important role as an effector of apoptosis in synovial lining cells and chondrocytes under the conditions studied. Data from Kobayashi *et al.* [23] have shown that tumour necrosis factor α (TNF-α) may sensitize synovial cells to the apoptotic effects of FasL. This is of interest in the light of the fact that TNF-α also acts as a potent inducer of the iNOS pathway in synoviocytes and chondrocytes [9]. It is therefore possible that NO plays a role in this sensitization to Fas-mediated apoptosis, although further studies would be needed to elucidate this.

Nitric oxide has several effects which may be relevant to the pathogenesis of tissue damage in RA, including stimulation of blood flow, inhibition of matrix production by chondrocytes [24], activation of metalloproteinases [25], modulation of the immune response [26], suppression of osteoblast activity [10] and enhancement of cytokine-induced osteoclastic bone resorption [27]. This study has shown that NO also acts as a key mediator of apoptosis within the joint. Whilst the role of apoptosis in the pathogenesis of joint destruction is incompletely understood, chondrocyte apoptosis would presumably be detrimental to cartilage integrity. In contrast, synovial cell apoptosis could be considered to be either beneficial in limiting synovial hypertrophy or detrimental in promoting the release of autoantigens or pro-inflammatory cytokines [28, 29]. While our results do not address the consequences of apoptosis for the regulation of disease activity in RA, they demonstrate that activation of the iNOS pathway promotes apoptosis within the joint. Clarification of the interrelationships between iNOS activation, apoptosis and disease activity in RA will, however, require further studies to be performed with NOS inhibitors *in vivo*.

Acknowledgement

This research was supported by a grant from the Arthritis Research Campaign.

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