IMMUNOLOCALIZATION OF INDUCIBLE NITRIC OXIDE SYNTHASE IN SYNOVIIUM AND CARTILAGE IN RHEUMATOID ARTHRITIS AND OSTEOARTHRITIS

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SUMMARY

Nitric oxide has been implicated as a mediator of inflammatory arthritis, and recent work has shown that pro-inflammatory cytokines stimulate NO production in vitro by activation of the inducible nitric oxide synthase (iNOS) pathway. In order to identify the cellular sources of NO production within the joint, we have used immunohistochemical techniques to study the distribution of iNOS in synovium and cartilage from normal and diseased joints. iNOS was most strongly expressed in the synovial lining layer, subsynovium, vascular smooth muscle and chondrocytes from patients with rheumatoid arthritis (RA). Analysis of serial sections, coupled with double immunofluorescent staining, showed that the CD68* macrophages in the synovial lining layer and, to a lesser extent, fibroblasts were the predominant source of iNOS within synovium, whereas T cells, B cells and neutrophils were negative. A similar pattern of iNOS staining was seen in osteoarthritis, but fewer cells were iNOS positive and the intensity of staining, particularly in cartilage, was much weaker than in RA. In contrast, no evidence of iNOS was detected in non-inflammatory synovium or in cartilage derived from normal joints (fractured neck of femur). In conclusion, these data support the hypothesis that synovium and cartilage are important sources of increased NO production in patients with inflammatory arthritis. Localization of iNOS at these sites within the inflamed joint raises the possibility that increased local production of NO may contribute to the pathogenesis of inflammatory arthritis by increasing synovial blood flow and by modulating cellular function within synovium and articular cartilage.

KEY WORDS: Nitric oxide, Rheumatoid arthritis, Synovitis, Cartilage.

Nitric oxide is a free radical with a short biological half-life, generated enzymatically from L-arginine by a family of NO synthase isoenzymes (reviewed in [1, 2]). Two constitutively expressed calcium/calmodulin-dependent forms of NO synthase produce small quantities of NO in vascular endothelial cells (eNOS) and nervous tissue (nNOS) where it acts as a mediator of smooth muscle relaxation [3, 4] and of neurotransmission [5] via stimulation of cyclic guanosine monophosphate (cGMP) production. A third isoform of NO synthase (iNOS) is expressed by many other mammalian cells when they are exposed to bacterial endotoxin or inflammatory cytokines including interleukin 1 (IL-1), tumour necrosis factor (TNF) and interferon gamma (IFN-γ) [1, 2]. This form of NOS is calcium independent and, once activated, produces large amounts of NO which have been implicated in the control of host defence mechanisms [6] and immunoregulation [7].

Recent studies have suggested that activation of the iNOS pathway may be important in the pathogenesis of inflammatory arthritis [8]. Increased serum and urinary levels of the NO breakdown products nitrate and/or nitrite have been found in patients with rheumatoid arthritis (RA) [9, 10], and synovial fluid nitrite levels have been shown to be higher than those in serum [9], suggesting that NO may be derived from within the joint in this situation. In keeping with this hypothesis, pro-inflammatory cytokines have been found to stimulate NO production by cultured human chondrocytes, synovial fibroblasts and osteoblasts in vitro [11–13], identifying these cells as potential sources of NO within inflamed synovium. Although there have been few studies on the possible sources of NO production by joint tissues in vivo, a recent study showed evidence of iNOS expression in chondrocytes, endothelial cells, fibroblasts and synovial macrophages in RA [14]. In order to investigate further the possible role of iNOS activation in the pathogenesis of inflammatory arthritis, we used immunohistochemical techniques to determine the sites of iNOS expression in normal synovium and diseased synovium from patients with RA and osteoarthritis (OA).

PATIENTS AND METHODS

Samples

Seventeen patients were studied; five had classic or definite RA, as defined by the American College of Rheumatology (formerly the American Rheumatism Association) criteria, seven had OA and five patients had osteoporosis (OP) with no history of inflammatory or degenerative joint disease. Samples of synovium and cartilage were collected into phosphate-buffered saline
(PBS) during joint replacement surgery or internal fixation of fractures and transported to the laboratory within 15 min. Specimens were trimmed, frozen in OCT compound (Tissue-Tek, Elkhart, USA) and used to prepare cryostat sections of 7 μm which were air dried on chrome gel subbed slides, and stored at −20°C until further use.

**Immunohistochemistry**

Staining for iNOS was performed using a polyclonal rabbit anti-iNOS specific antibody which has been previously characterized [15, 16]. Normal rabbit serum was used as a negative control.

The distribution of iNOS staining was related to phenotypic markers which were detected using the following murine antibodies (supplied by Dako, Glostrup, Denmark or the Scottish Blood Transfusion Service Antibody Production Unit, Law Hospital, Carluke): anti-CD68 (macrophage); anti-prolyl-4-hydroxylase (fibroblast); anti-neutrophil elastase (neutrophil); mouse anti-CD22 (B cell) and anti-CD2 (T cell). Biotinylated porcine anti-rabbit IgG secondary antibodies were purchased from DAKO, sheep anti-rabbit Ig/Cy3 from Sigma (Poole, Dorset), streptavidin–fluorescein isothiocyanate (FITC) conjugate from Amersham (Little Chalfont) and goat biotinylated anti-rabbit IgG, horse biotinylated antimouse IgG, VECTASTAIN Elite ABC kit and the VECTASTAIN VIP substrate kit were from Vector Laboratories (Peterborough).

Immediately prior to staining, cryostat sections of synovium were fixed in acetone for 10 min, air dried at room temperature and rehydrated in PBS containing 10% fetal bovine serum (FBS) for 10 min. Acetone-fixed sections of cartilage were incubated for 30 min in a humidified chamber with 100 μl hyaluronidase (5 mg/ml; Sigma, Poole), washed three times for 5 min in PBS and once for 10 min in 10% FBS after each incubation. Primary and secondary antibodies were diluted in 10% FBS. Sections of synovium and cartilage were incubated with primary and secondary antibodies for 30 min at room temperature and washed twice for 5 min in PBS. VECTASTAIN ABC reagent and VIP substrate solutions were used in accordance with the manufacturer’s instructions. Substrate development was terminated after 5 min, and sections were counterstained with Harris’s haematoxylin, dehydrated and mounted in DePeX mounting medium (BDH Ltd, Poole). For double immunofluorescence studies, sections of synovium were incubated serially with: rabbit anti-iNOS, sheep anti-rabbit Ig/Cy3 conjugate, mouse anti-CD68, biotinylated horse anti-mouse Ig and, finally, streptavidin–FITC conjugate. All incubations were for 30 min and were followed by three washes for 5 min in PBS. In control incubations where either specific antibody was omitted, no cross-reactivity was observed between the secondary reagents.

**RESULTS**

The distribution of iNOS was studied in sections of synovium and cartilage from five patients with RA (two knee, three hips), seven with OA (three knee, four hips) and five with femoral neck fractures. There was strong immunostaining for iNOS in the lining cells of synovium in all individuals with RA (Fig. 1A) and to a lesser extent in 5/7 individuals with OA (Fig. 1B). In contrast, iNOS was absent from the synovial lining in normal synovium from patients with hip fracture (Fig. 1C). The patterns of staining were similar in synovium derived from knee and hips in OA and RA patients (not shown).

Strong iNOS staining was also observed in the vascular smooth muscle in medium-sized blood vessels in all RA samples studied, whereas lymphoid aggregates in RA synovium were uniformly negative for iNOS (Fig. 1D). Staining for iNOS was also observed in synovial vascular smooth muscle from patients with OA in 5/7 cases, but again was weak or absent in vessels from normal synovium (data not shown). iNOS was strongly expressed in chondrocytes from RA cartilage (Fig. 1E), whereas staining was weaker in cartilage from OA patients (Fig. 1F) and completely absent from normal cartilage (not shown).

In order to characterize further the cells responsible for iNOS expression, serial sections were stained for iNOS and monoclonal antibodies directed against a range of cell lineage-specific markers, including CD2, CD22, CD68, prolyl-4-hydroxylase and neutrophil elastase. Areas of synovium containing cells reactive for CD2, CD22 and neutrophil elastase were uniformly negative for iNOS staining, demonstrating that lymphocytes and neutrophils are not a prominent source of iNOS in RA synovium (not shown). In agreement with previous data [17], we found that the majority of cells in the synovial lining layer were positive for the macrophage marker CD68+ or fibroblast marker prolyl-4-hydroxylase (data not shown). Since the synovial lining layer was the predominant site for iNOS expression, we went on to perform double immunofluorescent staining for iNOS and CD68 (Fig. 2A–C). This confirmed co-localization of iNOS in CD68+ cells. While most CD68+ cells were positive for iNOS in the section shown, analysis of multiple sections showed that iNOS staining was undetectable in between 10 and 20% of CD68+ cells from both RA and OA synovial samples.

**DISCUSSION**

This study confirms the findings of previous work which has shown that iNOS is strongly expressed in synovium and cartilage of patients with inflammatory joint disease [14]. While iNOS expression was strongest in the synovium from RA patients, this finding was not disease specific, since iNOS was also expressed in the synovial lining layer, blood vessels and, to a lesser extent, cartilage from patients with OA. While OA is not generally considered an inflammatory disease, previous work has shown evidence of mild to moderate inflammatory changes in OA synovium [18], consistent with the fact that the pro-inflammatory cytokines IL-1 and TNF have also been detected in OA synovial fluids [19]. Our demonstration of iNOS in OA synovium and
The absence of iNOS in non-inflammatory synovium derived from patients with femoral neck fractures, however, indicates that iNOS expression in synovium is not physiological, but rather, is a pathological reaction in joint inflammation, irrespective of the underlying cause.

The pattern of staining found here is in general agreement with that observed by Sakurai et al. [14], who studied iNOS expression in patients with RA and OA by *in situ* hybridization and immunohistochemistry. In the study cited above, iNOS staining was detected in macrophages, synovial fibroblasts and chondrocytes, but as also reported here no evidence of iNOS was found in T or B lymphocytes. A major difference between the studies, however, was that Sakurai reported detection of iNOS in endothelial cells, whereas our observations clearly show localization of iNOS within vascular smooth muscle. Non-specific staining of the vessel wall with our antibody cannot be invoked as a reason for these differences since we observed no staining in smooth muscle in normal or OA synovium. Accordingly, we believe that these

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**Fig. 1.**—Photomicrographs showing immunoreactivity for iNOS in articular tissues. There was intense iNOS staining in the synovial lining layer in RA synovium (A), weaker staining in OA synovium (B) and no iNOS staining in normal synovium (C). Strong iNOS staining was also observed in vascular smooth muscle in RA synovium (small arrowheads), in occasional cells in the subsynovium (small arrows), but not in lymphoid aggregates (D). While iNOS was clearly detected in RA cartilage (E), staining was weak or absent in OA chondrocytes (F).
Photomicrographs showing double staining for CD57 "green" and iNOS "red" in RA synovium. (A) CD68-positive cells (green); (B) iNOS-positive cells (red); (C) cells positive for both markers (yellow; strongly staining cells indicated by white arrows). While all CD68+ cells in this section stain positive for iNOS, analysis of multiple sections showed that iNOS staining was undetectable in between 10 and 20% of CD68+ cells from both RA and OA synovial samples.

Fig. 2.

Differences may be largely those of interpretation, since examination of Sakurai’s figures also shows clear evidence of iNOS staining in vascular smooth muscle. We cannot, however, exclude the possibility that iNOS may also be expressed in endothelium in view of the very close apposition of these cell types which can both be induced to produce iNOS by inflammatory mediators in vitro [1].

It is of interest that the detection of iNOS in macrophage-like cells in both studies contrasts with in vitro experiments which have shown that it is difficult to induce human monocytes/macrophages to express iNOS and produce NO, even using combinations of pro-inflammatory cytokines and endotoxin [20, 21]. Although the reasons for this remain unclear, it is probable that for effective stimulation, cytokines must be present in the context of a co-stimulatory signal which might only be effectively provided by factors such as cell–cell or cell–matrix contact.

Whilst the present studies do not address the functional role of NO in inflammatory arthritis, there is ample evidence to suggest that NO may act as an important mediator of joint damage in this situation. Thus, previous studies have shown that inhibitory effects of IL-1 on matrix production by cultured chondrocytes are partly mediated by NO [22, 23], and that NO activates metalloproteinases in cultured chondrocytes [24]. We have previously found that IL-1-induced bone resorption in mouse calvarial organ cultures is potentiated by NO [25] and that high levels of NO also inhibit osteoblast proliferation [12]. These actions together might be expected to contribute to the increased bone loss which is an early feature of RA [26, 27]. Other potential pro-inflammatory actions of NO include changes in the vascular permeability of inflamed tissues [28, 29], potentiation of TNF and IL-1 release by leucocytes [30], and stimulation of angiogenic activity by human monocytes [31]. The well-established vasodilating effects of NO [3, 4], coupled with clear demonstration of iNOS expression in vascular smooth muscle within synovium, also raises the possibility that NO may also play a role in the pathogenesis of joint swelling and vasodilatation which accompany inflammatory arthritis. The pathogenic role of NO in arthritis is certainly supported by the observation that inhibitors of NO synthase suppress the development of disease in animal models such as adjuvant arthritis and streptococcal cell wall arthritis [32, 33]. Although the pathophysiological role of NO in humans with inflammatory arthritis remains to be addressed, our data confirm that iNOS is strongly induced at specific sites within the inflamed joint, supporting the hypothesis that efforts to inhibit NO production may provide a new therapeutic approach to the treatment of inflammatory arthritis.

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REFERENCES