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Distribution of Protein Nitrotyrosine in Synovial Tissues of Patients with Rheumatoid Arthritis and Osteoarthritis

JAGDEEP KAUR SANDHU, SUSAN ROBERTSON, H. CHAIM BIRNBOIM, and ROSE GOLDSTEIN

ABSTRACT. Objective. Because nitric oxide related species have been found in the inflamed joints of patients with arthritis, we investigated whether protein nitrotyrosine (a marker of tissue exposure to peroxynitrite) is present in their synovial tissues.

Methods. Protein nitrotyrosine was detected immunohistochemically and by Western blot analysis. Synovial tissues removed surgically from 12 patients with rheumatoid arthritis (RA) (mean age 63.7 yrs) and 20 with osteoarthritis (OA) (mean age 66.6 yrs) were studied.

Results. Nitrated proteins were detected immunohistochemically in all of 18 tissues examined. Diffuse staining of the stroma was seen in all patients, with more extensive staining in RA than OA ($p = 0.008$). Intense staining was detected in some lymphocytes, but not in others, even within a single lymphoid aggregate. Neutrophils did not stain for nitrotyrosine. Vascular endothelial cells stained for nitrotyrosine but adjoining smooth muscle cells did not. Both cytoplasmic and nuclear staining was seen in macrophages, endothelial cells, and lymphocytes. Numerous bands of nitrated proteins were detected by Western blot analysis of 15 synovial tissue extracts. Inducible nitric oxide synthase (iNOS) was detected immunohistochemically in endothelial cells, macrophages, vascular smooth muscle cells, and synoviocytes.

Conclusion. Nitrotyrosine-containing proteins were found in essentially all synovia from RA and OA patients. The most prominent site of nitration in all cases was the stroma. iNOS, the likely source of the nitrating species, was found in a variety of cell types. (J Rheumatol 2003;30:1173–81)

Key Indexing Terms:
SYNOVIOCYTES NITROTYROSINE PEROXYNITRITE
NITRIC OXIDE DAMAGE INFLAMMATION

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease that often progresses to debilitating destruction of the joint. Although osteoarthritis (OA) has usually been considered to be a noninflammatory disease, mild to moderate inflammatory changes are also seen in OA synovium[1]. Inflamed tissues contain proinflammatory cytokines such as interleukin 1, tumor necrosis factor-α, and also inflammatory cells such as neutrophils and macrophages, which are sources of reactive nitrogen and oxygen species described in arthritis[2,3]. The inducible form of nitric oxide synthase (iNOS) is expressed at high concentrations by many cell types in the inflamed synovium of patients with RA and, to a lesser extent, with OA[4,5]. NOS generates nitric oxide (NO·), a short-lived radical that can convert to nitrite and nitrate. High concentrations of serum nitrite/nitrate and elevated urinary nitrate:creatinine ratios have been found in patients with RA[6-8]. Synovial fluid (SF) nitrite levels are higher than those found in serum, suggesting that NO· may arise within the inflamed joint[6,7]. Animal models of arthritis provide further evidence for a role of reactive nitrogen oxide species (RNOS) in the etiopathology of the disease[9-12]. There is appreciable evidence that RNOS is also present in the articular cartilage of arthritic joints, where it may contribute to tissue destruction[13,14]. Chrondrocytes can express high levels of the inducible and the neuronal forms of NOS (iNOS and nNOS)[5,15-17]. Further evidence of the clinical importance of NO and iNOS in RA comes from new insight into the mechanism of action of clinically important drugs. Many of our therapies either directly or indirectly significantly inhibit iNOS, including corticosteroids and minocycline[2] and/or NO production, as shown for methotrexate (MTX)[18].

Nitric oxide radical (NO·) can react with superoxide radical to form peroxynitrite, a potent nitrating and oxidizing chemical that can in turn react with tyrosine to form nitrotyrosine. The presence of protein nitrotyrosine in diseased tissues has been widely used as a marker for

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peroxynitrite[19-26], although other pathways of tyrosine nitration have been identified[27,28]. Nitrotyrosine-free amino acid has been identified in the SF and serum of patients with RA[29]. Only one study of protein nitrotyrosine in human RA synovia has been reported[30]; OA synovia have not previously been examined. Protein nitrotyrosine has been described in animal models[9,30]. We describe the first comparison of protein nitrotyrosine in synovial tissues of RA and OA patients using both Western blotting and immunohistochemistry.

MATERIALS AND METHODS

Patients. Synovial tissue was obtained from 2 groups of RA and OA patients. All RA patients met the criteria of the American College of Rheumatology (ACR)[31]. The protocol for obtaining synovial tissue samples was approved by the institutional ethics review board. Clinical evaluation included age, duration of disease, presence of rheumatoid nodules, and radiographic evidence of erosion. Laboratory findings included erythrocyte sedimentation rate (ESR; Westergren method), rheumatoid factor (RF; latex fixation), and C-reactive protein (CRP). Medications at the time of joint surgery were noted. For the immunohistochemical studies, synovial samples from 9 RA and 9 OA patients were available. For Western blot analysis, we used synovial samples from 3 patients with RA, 11 OA, and one systemic lupus erythematosus.

Synovial tissues. Synovial tissue was obtained from patients with RA and OA at the time of joint replacement surgery at the Division of Orthopedic Surgery, Ottawa Hospital–General Campus. Synovial samples were obtained from hip or knee joints. Similar joint tissue from nonarthritic younger patients was not available. Synovial specimens were frozen in liquid nitrogen and stored at –100°C or fixed for 24 h in 10% neutral buffered formalin and embedded in paraffin.

Nitrotyrosine and iNOS immunohistochemistry. Synovial tissue fragments were cut into 4 µm sections and processed for immunohistochemistry[32]. Paraffin was removed with toluene and rehydrated in ethanol and water. Sections were then incubated with Tris-buffered saline (TBS; 50 mM Tris-HCl, 130 mM NaCl, pH 7.6) containing 3% H_2O_2 for 10 min at room temperature to inactivate endogenous peroxidase activity. Nonspecific IgG were blocked with 1% normal swine serum at room temperature for 30 min. Excess liquid was drained and sections were incubated with 0.5 µg/ml rabbit polyclonal anti-nitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY, USA) or in a humid chamber at 4°C for 16 h. Sections were then washed in TBS and incubated with Dako Envision peroxidase conjugated to goat anti-mouse/anti-rabbit immunoglobulins (Dako Envision System, Dako, Glostrup, Denmark) for 30 min at room temperature. Immunolabelling was detected using freshly prepared diaminobenzidine (DAB, 0.02%) and H_2O_2 (0.006%) for 10 min at room temperature, washed, and then counterstained with hematoxylin. Conditions for immunohistochemistry were first established using tissue from 2 patients. Subsequently, all OA and RA tissues were stained as a single batch. For all tissue samples, a positive control and 3 negative controls were carried out. The positive control (*in situ* chemical nitration of tissue proteins) involved treatment with 1 mM $NaNO_2$, 1 mM H_2O_2 in 100 mM sodium acetate at pH 5.2 for 20 min at room temperature. Specificity of the primary antibody for nitrotyrosine was shown as follows: (1) before incubation with tissue sections, the primary antibody was preincubated 1 h at room temperature with either 1 mM nitrotyrosine (Sigma) or 10 µg/ml nitrated bovine serum albumin (BSA); (2) tissue nitrotyrosine was eliminated by chemical reduction to aminotyrosine (two 20 s treatments with freshly prepared 1 M sodium hydrosulfite)[33]; (3) the primary antibody was omitted. Nitrated BSA was prepared by incubating a 6 mg/ml solution of albumin for 18 h at room temperature in the presence of 10 mM $NaNO_2$, 0.3% H_2O_2, 9.0 µM $FeCl_3$ in 20 mM sodium acetate at pH 5.6; nitrated albumin was precipitated with 4 volumes of ethanol at –20°C for 18 h. All controls were found to be equally effective in eliminating nitrotyrosine staining. Other controls included staining with rabbit polyclonal glial fibrillary acidic protein (Dako Diagnostics Canada Inc., Mississauga, ON, Canada; marker for astrocytes) and microtubule associated protein 2 (Chemicon International Inc., Temecula, CA, USA; marker for neurons) as irrelevant antibodies. No staining was observed in tissues from RA patients (n = 2); however, human brain tissues were positive. Normal rabbit serum used at the same dilution as the antinitrotyrosine antibody also tested uniformly negative.

Immunostaining for iNOS in synovial tissues was carried out as follows. Following treatments as described above to the primary antibody step, sections were incubated in a humid chamber with 2.5 µg/ml of rabbit polyclonal antibody to mouse macrophage iNOS (Transduction Laboratories, Lexington, KY, USA). After overnight incubation at 4°C, bound antibody was detected by incubation at room temperature for 30 min with 7.5 µg/ml goat anti-rabbit biotinylated IgG (Dimension Laboratories Inc., Mississauga, ON, Canada). The conjugate was detected with 5 µg/ml avidin-peroxidase (Dimension Laboratories). Immunolabelling was detected using DAB or nickel-DAB, followed by counterstaining with Mayer's hematoxylin.

Nitrotyrosine staining index. The percentage of each tissue section that stained for nitrotyrosine was estimated from digital photomicrographs (×200 power, 1600 × 1200 pixel) of 3 fields selected at random. Analysis for brown pixels was carried out with the aid of ImagePro Plus, version 4.0, software (www.mediacy.com/ipe.htm) with color sensitivity = 4/5, color selection = 1 × 1 pixel. A strongly-staining tissue section was first selected to establish the minimum shade of brown (i.e., DAB precipitate) that would be considered positive. Once this ImagePro Plus template was prepared, it was used for the analysis of all images. The nitrotyrosine staining index is an estimate of the percentage of tissue sections that stain for nitrotyrosine and is calculated from the total number of brown pixels in the 3 images divided by 19,200. This index reflects the extent, and not total intensity, of staining.

Western blot analysis. Frozen synovial tissue (Patients 19–33, Table 1) was thawed and homogenized on ice in radioimmunoprecipitation buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% sodium dodecylsulfate (SDS), 1% sodium deoxycholate] containing PMSF (1 mM) and protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA). Protein was quantified by the Bradford assay[34]. Extracts were diluted with SDS sample buffer (2% SDS, 10% glycerol, 1% 2-mercaptoethanol, 0.0005% bromophenol blue, 125 mM MOPS, pH 6.8) and boiled at 100°C for 5 min. Protein samples (25 µg per lane) were subjected to SDS-12% polyacrylamide gel electrophoresis at 200 V for 1 h and electrophoretically transferred to polyvinylidene membrane (Millipore) for 16 h at 15 V at 4°C using an improved transfer procedure (Haqqani AS, Birnboim HC, unpublished data). Membranes were blocked with TBST (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 8.0) containing 5% nonfat milk for 1 h at room temperature. They were probed with 1:2000 dilution of rabbit polyclonal anti-nitrotyrosine antibody in TBST containing 2% nonfat milk for 1 h at 37°C. Membranes were washed with TBST and then incubated with a 1:2500 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in TBST containing 2% nonfat milk for 1 h at 37°C. Phosphatase was detected using BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt/nitroblue tetrazolium) substrate (KPL, Gaithersburg, MD, USA).

Statistical analysis. The nitrotyrosine staining index in tissue sections from RA and OA synovia was compared using a 2-tailed Mann-Whitney U test. Correlation between nitrotyrosine staining index and various disease variables was by Spearman rank correlation test. All statistical analyses were carried out using GraphPad Prism, version 3.02 (www.graphpad.com). P = 0.05 was considered statistically significant.

RESULTS

Patients' clinical details. Characteristics of patients from

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Table 1. Demographic information and clinical variables for patients with RA and OA. Western blot analysis was performed on Patients 19–33.

Patient	Sex	Age, yrs	Diagnosis	Disease Duration, yrs	Nitrotyrosine Staining Index, %
1	F	68	RA	8	14.6
2	M	71	RA	20	18.8
3	F	45	RA	20	12.0
4	M	66	RA	6	33.0
5	F	54	RA	—*	20.1
6	F	74	RA	19	18.5
7	F	66	RA	19	18.0
8	M	74	RA	7	23.8
9	F	50	RA	29	21.5
10	F	64	OA	—	21.5
11	F	85	OA	—	15.9
12	F	78	OA	—	11.3
13	F	79	OA	—	6.8
14	M	66	OA	—	10.0
15	F	71	OA	20	11.0
16	M	68	OA	—	17.3
17	M	61	OA	—	14.3
18	F	58	OA	> 15	11.8
19	M	58	OA	20	—
20	M	81	OA	1	—
21	F	63	OA	12	—
22	F	41	OA	5	—
23	F	68	OA	—	—
24	M	64	OA	6	—
25	F	68	OA	—	—
26	F	43	OA	—	—
27	M	76	OA	—	—
28	F	54	SLE/OA	1	—
29	M	74	RA	—	—
30	F	70	RA	—	—
31	M	74	OA	—	—
32	F	66	OA	4	—
33	M	52	RA	—	—

* Not available. SLE: systemic lupus erythematosus.

whom samples were available are summarized in Table 1. The mean age of RA patients was 63.7 ± 10.5 (mean ± SD) years and the mean age of OA patients was 66.6 ± 11.3 years. The difference in mean age of patients in the 2 groups was not statistically significant. All RA patients except Patient 8 had erosions and RF. All RA patients except Patient 3 had rheumatoid nodules. Of the 12 RA patients, 2 were taking MTX, hydroxychloroquine and oral prednisone, 2 were taking MTX and hydroxychloroquine, 3 were taking only MTX, 2 were taking only prednisone, one was taking hydroxychloroquine and azathioprine, and one was taking hydroxychloroquine and prednisone. No patient with OA was receiving any long-acting rheumatic drug at the time of the study.

Synovial tissues. As expected, synovia from RA patients showed evidence of extensive disease. Inflammatory cells, including neutrophils and synoviocytes, lymphocytes and plasma cells, macrophages, and mast cells were present to varying degrees in most cases. Representative examples are shown in Figure 1, panels A–F. Other features of diseased synovia include neovascularization, hyperplasia, and the presence of multinucleated giant cells (panels G–I). OA synovia showed only mild, nonspecific changes without marked inflammatory cell infiltrate and no synovial hyperplasia (not shown).

Immunohistochemical staining for iNOS and nitrotyrosine. NO is a free radical produced at sites of inflammation such as the RA joint by a family of enzymes, the NO synthases. The inducible form (iNOS) was detected in the synovial tissues of RA patients (Figure 1, panels J–L). This enzyme was present in synoviocytes, macrophages, vascular smooth muscle cells, vascular endothelial cells, and occasionally in cells of the subsynovium. Staining was also detected in macrophages and synovial lining layer (not shown). No staining of lymphocytes, plasma cells, or neutrophils was seen.

NO can react with superoxide (produced by phagocytic cells) to form peroxynitrite and other species that can nitrate

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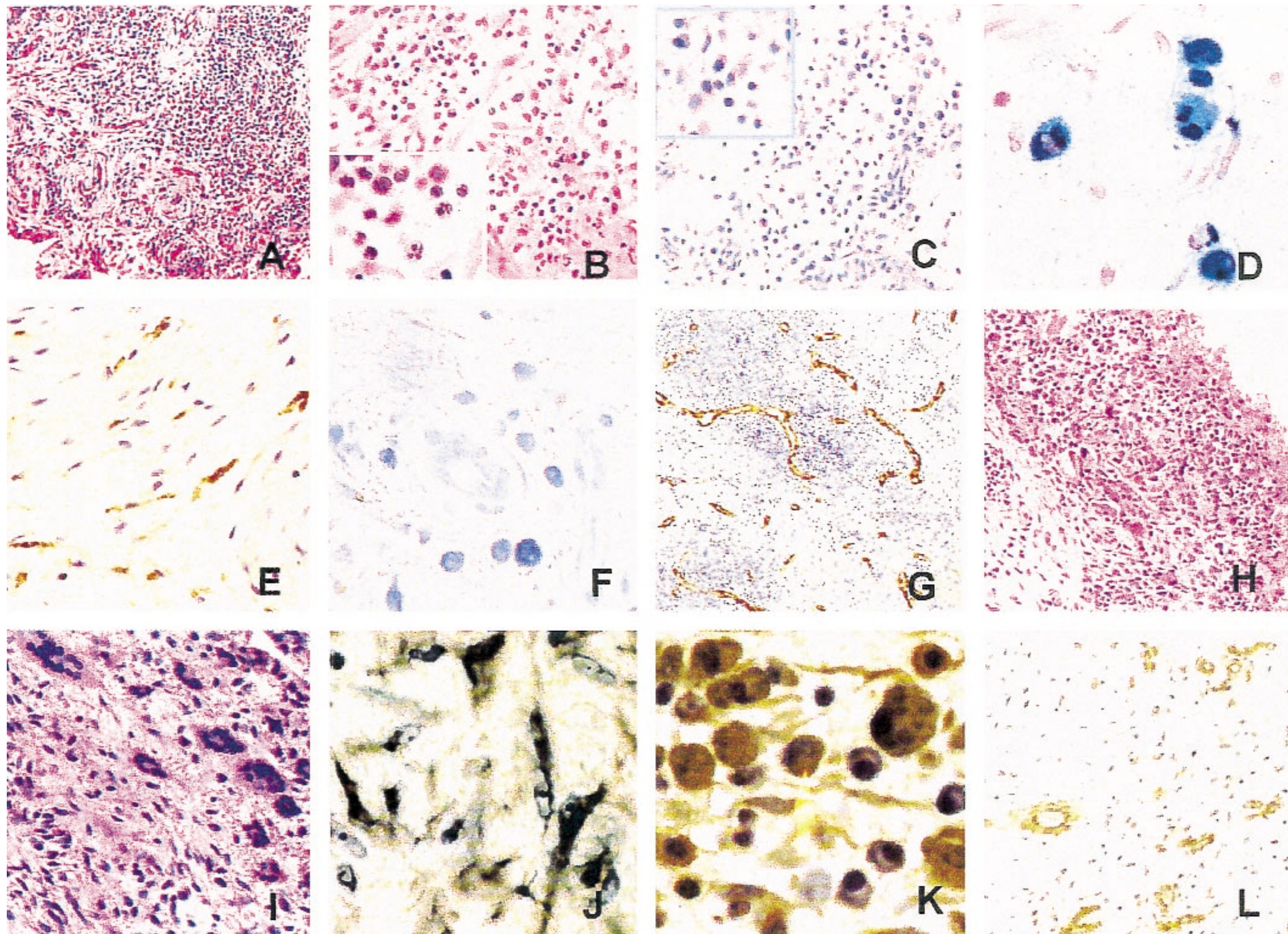

Figure 1. Histological features of diseased RA synovium and immunostaining for iNOS. A–C, H, I, H&E stained sections. A: Inflamed synovium showing pronounced inflammatory cell infiltrate. B: neutrophils and synoviocytes. C: lymphocytes and plasma cells. D: macrophages (stained with Pearl's Prussian blue and counterstained with neutral red). E: macrophage-like synoviocytes (stained for CD68). F: mast cells (stained with toluidine blue). G: neovascularization (stained for factor VIII). H: hyperplasia of the synovial layer. I: multinucleated giant cells. Immunostaining for iNOS in (J) synoviocytes, (K) macrophages, (L) vascular smooth muscle cells and vascular endothelium in the subsynovium. A and G, original magnification ×100; H and L, ×200; B, C, E, F, and I, ×400; D, J, and K, ×600.

tyrosine residues in proteins. Synovial tissues of RA and OA patients and controls were examined immunohistochemically using an antibody that detects nitrotyrosine (Figure 2). The antibody used was found to be specific for nitrotyrosine (Figure 2, panels A–C). Using serial sections, the nitrotyrosine signal could be blocked by competing with nitrated albumin (panel B), and *in situ* chemical nitration produced a strong signal (panel C). Diffuse staining of the stroma was seen in RA (Figure 2, panels A and D) and, to a lesser degree, in OA synovium (Figure 2, panel E and F).

Synovial tissues were examined in detail to identify regions and cell types that stained for nitrotyrosine. Examples from RA Patient 6 are shown in Figure 3. Strong immunostaining of the stroma was found in areas of the subsynovium, often adjacent to lightly-staining areas (Figure 3A, 3B). Strong staining of vascular endothelium (both cytoplasmic and nuclear) was seen in large and medium-size blood vessels. Interestingly, immediately adjacent vascular smooth muscle cells did not stain, but surrounding stroma was strongly stained (Figure 3A, 3B). Similarly, some lymphocytes in a lymphoid aggregate were strongly stained, while other lymphocytes in adjacent areas of the same aggregate did not stain (Figure 3C). Nitrotyrosine staining of this lymphoid aggregate was eliminated by chemical reduction (Figure 3F). Intense nitrotyrosine staining was observed in plasma cells, macrophages, and synoviocytes (Figure 3D). In most samples, the stroma stained strongly, while staining of neutrophils and fibrin was never seen (Figure 3E). In general, stroma of the synovial layer stained more lightly than stroma of the subsynovium. Nuclear staining of synoviocytes was seen in some specimens (not shown). A similar pattern of nitrotyrosine staining was seen in OA patients, but the intensity of staining was less (Figure 2E). The knee synovium of a single patient with

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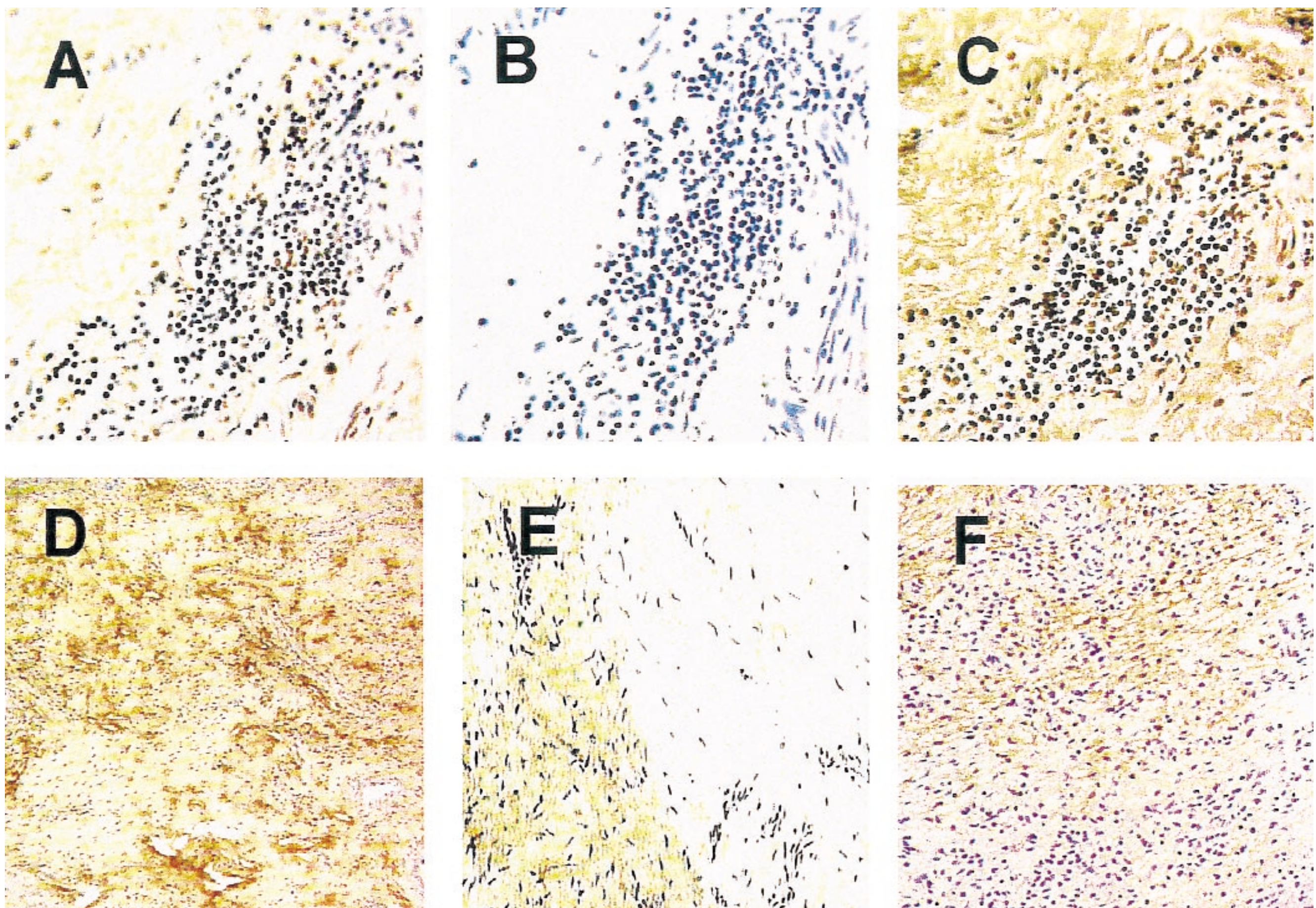

Figure 2. Representative examples of synovia stained for nitrotyrosine. A–C: serial sections of synovium from RA Patient 7, knee joint. A: synovium stained for nitrotyrosine. B: negative control, competitive inhibition of anti-nitrotyrosine antibody with nitrated BSA. C: positive control, *in situ* chemically nitrated tissue section. D: RA Patient 4, knee joint. E: OA Patient 18, knee joint. F: OA Patient 10, knee joint. A–C, original magnification ×400; D–F, ×100. Patients' details given in Table 1; other details in Materials and Methods.

Sjögren's syndrome showed similar immunoreactivity (data not shown).

Control synovia from 3 cadaver cases taken from the left shoulder of individuals with no known history of arthritis were also studied for nitrotyrosine staining. Either no staining or very weak staining of the stroma was seen in all cases; no staining of any cell types was seen (data not shown).

Nitrotyrosine staining index. Quantitative results of the extent (percentage of tissue) of nitrotyrosine staining are shown in Figure 4. The nitrotyrosine staining index indicated that tissues from RA patients stained more extensively than OA patients (median 18.8% vs 11.8%, respectively). This difference is statistically significant (Mann-Whitney U test; 2-tailed $p = 0.008$).

Nitrotyrosine staining index and clinical variables. Clinical variables of RA and OA patients were examined to determine if any correlated with the nitrotyrosine staining index (Table 1). No correlation was found between age or disease duration in RA synovia ($p > 0.1$). ESR and CRP were available for only 3 patients, too few to test for a statistical correlation. However, it is notable that RA Patient 4, who had the highest ESR (116 mm/h) and CRP (44 mg/l), also had the highest nitrotyrosine staining index (33%). The staining index did not correlate with the medication taken by RA patients at the time of the study (data not shown).

Detection of nitrated proteins by Western blot analysis. Western blot analysis to detect the sizes of nitrated proteins in synovial tissue homogenates in a group of RA and OA patients was carried out. The conditions of Western blotting were not quantitative for stromal proteins, therefore no correlations with immunohistochemistry of nitrotyrosine in these samples can be determined. Examples of Western blots probed for nitrotyrosine from 3 RA and 11 OA patients are shown in Figure 5. Several prominent nitrated protein bands between 20 and 80 kDa of unknown identity were seen in every case.

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DISCUSSION

Reactive nitrogen oxide species (RNOS) are consistently found at sites of chronic or recurrent inflammation. The source of these reactive species is nitric oxide synthase, expressed by activated inflammatory cells. In the inflamed RA and OA synovium, the inducible NOS (iNOS) isoform has frequently been identified[5,15,35-37]. Our studies of iNOS are in close agreement with these studies. In RA synovium, we observed iNOS expression in synovial lining cells, macrophages, vascular endothelium, and vascular smooth muscle. No iNOS expression was detected in synovial neutrophils, plasma cells, or lymphocytes. Interestingly, others have reported that iNOS is expressed in lymphocytes taken from SF, although a direct comparison with lymphocytes from synovial tissue was not carried out[38]. Thus, as expected from the inflammatory nature of these diseases, iNOS is expressed at high concentrations by many cell types in the inflamed RA and OA joint.

NO, the product of NOS, is a short-lived free radical that is difficult to measure in tissues. Thus, more stable surrogate markers are commonly used. Elevated concentrations of nitrite and nitrate have been reported in the serum, SF, and urine of patients with arthritis[7]. S-Nitrosothiol, a moderately stable protein-nitric oxide adduct, has been found in the serum and SF of patients with RA[39]. The most frequently used *in vivo* marker of tissue RNOS is 3-nitrotyrosine. This modified amino acid is produced in a reaction between peroxynitrite and tyrosine or tyrosine residues in proteins[33]. Neutrophil myeloperoxidase plus nitrite is another potential nitrating source[27]. Elevated levels of 3-nitrotyrosine as the free amino acid have been reported in serum and SF from patients with RA[29]. The availability of a specific antibody that detects nitrotyrosine residues in protein has allowed the tissue distribution of these modified proteins to be studied immunohistochemically[33].

In our study, the most reproducible histological feature of diseased synovial tissue was widespread staining of the stroma. Although the stroma contained no iNOS, diffuse staining for nitrotyrosine was always observed. In many instances, the stroma stained strongly, while cells within the same area of the stroma did not. Only macrophages (cells that produce both superoxide and nitric oxide) stained consistently for nitrotyrosine. Other cells either did not stain or stained variably for nitrotyrosine. Neutrophils (which produce superoxide but not NO) did not stain, while surrounding stroma did. The half-life of neutrophils in tissues is very short compared to the half-life of proteins in the stroma, and therefore nitrotyrosine in stroma may represent its accumulation over a long period of time. We observed striking heterogeneity of staining of lymphocytes for nitrotyrosine. In the stroma, some lymphocytes stained very intensely, while others immediately adjacent did not. This heterogeneity was even more prominent in lymphoid aggregates. This may be explained by trafficking of cells to and from the synovium. For example, nitrotyrosine-containing lymphocytes may have originated in the SF, where they were exposed to RNOS prior to migrating into lymphoid aggregates. Vascular endothelial cells, like macrophages, produce both NO and superoxide[28,35,40]; these cells also stained intensely for nitrotyrosine. Interestingly, immediately adjacent vascular smooth muscle cells did not stain for nitrotyrosine. Since smooth muscle cells express high levels of extracellular superoxide dismutase[41], it is possible that this enzyme protects these cells by intercepting superoxide and preventing the formation of the nitrating agent peroxynitrite.

Only one other immunohistochemical study of protein nitrotyrosine in RA synovium has been reported[30]. These authors also found strong nitrotyrosine immunoreactivity in macrophages; other inflammatory cell types stained for nitrotyrosine, but the cells were not characterized. Although they report no staining of endothelial cells, some nuclear staining appears to be present. There is one very conspicuous difference between our findings: their specimens showed strong staining of vascular smooth muscle cells, while our specimens showed no staining. We cannot provide any explanation at this time for this difference. Stromal staining was a prominent feature of our specimens, but it required several types of controls to provide convincing evidence of the specificity of the staining. Mapp, *et al*[30] did not comment on whether staining of stroma could be detected. Our findings, together with those of Mapp *et al*, provide strong evidence for the presence of protein nitrotyrosine in arthritic tissue.

Additional information about the nature of nitrotyrosine-containing proteins in synovial tissue was provided by Western blotting. Prominent protein bands at 78 kDa and 33 kDa were detected. The 78 kDa band was seen in most RA and OA specimens, while the 33 kDa band was seen only in some specimens. Bands of other sizes were observed in one or more specimens. The identity of these nitrated proteins remains unknown. Nitrated proteins have been detected by Western blot analysis in many disease conditions in humans and animals[42-45]. Combined with our immunohistochemical findings, these results strengthen the evidence that RNOS are generated in the arthritic joint and that nitrotyrosine may be useful as a marker of their presence.

Our observations of OA synovium were similar to RA synovium, but overall staining for nitrotyrosine was less extensive, 37% lower than for RA synovia. The number of nitrotyrosine-staining cells was lower in OA, reflecting less inflammatory cell infiltrate. Less intense staining for iNOS in OA compared to RA synovium has been reported[5]. These findings support the notion that similar but milder disease processes are occurring in OA compared to RA synovium.

One consequence of RNOS appears to be nuclear damage to cells in the arthritic joint. We have previously shown that T lymphocytes from the synovium of arthritic patients

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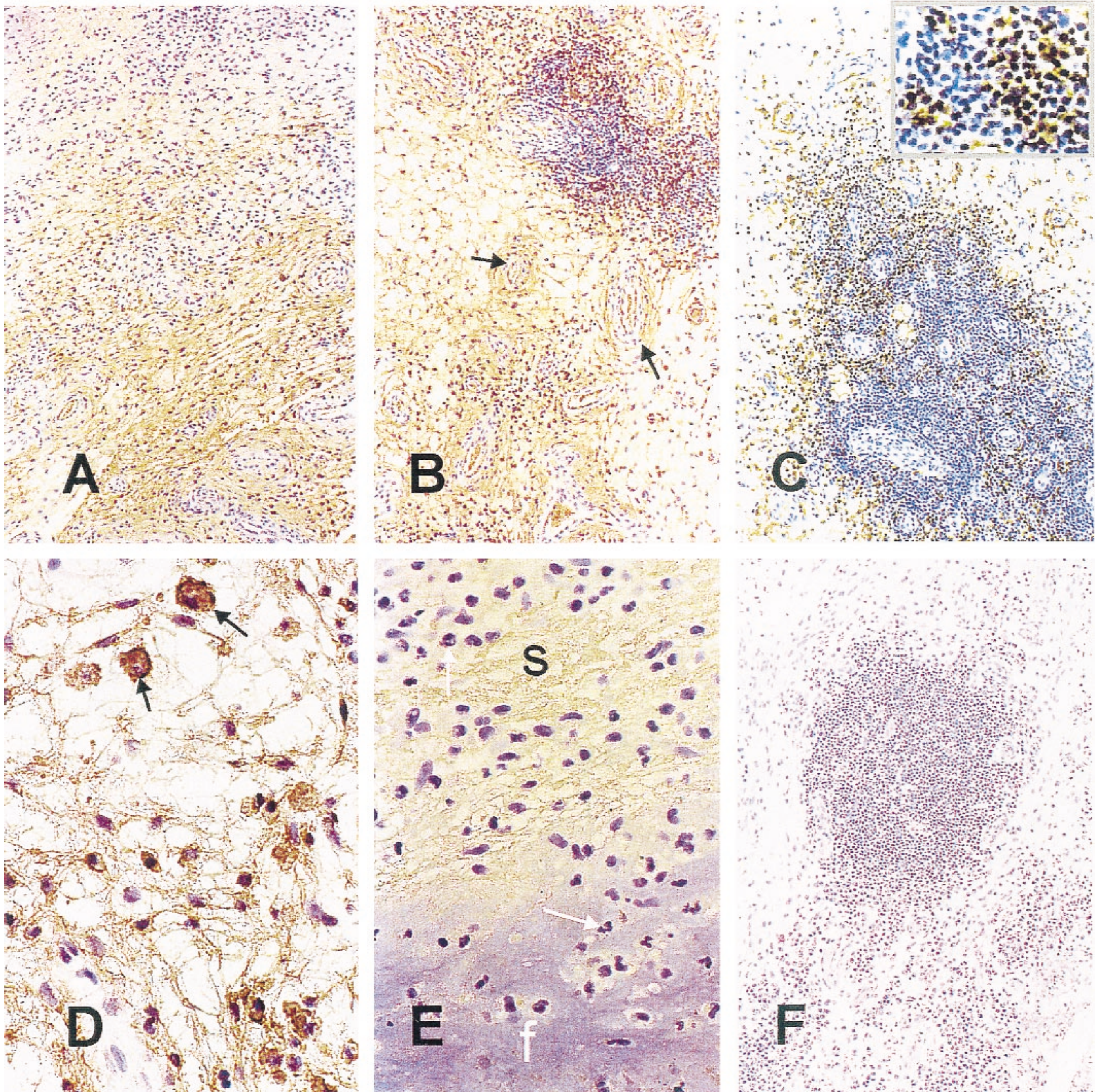

Figure 3. Staining of synovium from RA Patient 6 for nitrotyrosine. A: region showing intense stromal staining. B: arrows indicate staining of vascular endothelium and perivascular regions. C: lymphoid aggregate in which some lymphocytes are stained and others are not. D: arrows indicate macrophage-like cells showing intense staining. E: arrows indicate absence of staining of neutrophils, "f" indicates unstained fibrin and "s" indicates strongly stained stroma. F: negative control, *in situ* chemical reduction of nitrotyrosine to aminotyrosine. A–C, F, ×100; D–E, ×400. Other details in Materials and Methods.

exhibited a high mutation frequency[46]. Mutations in the p53 gene have also been reported in the RA synovium[47]. In this study, we found nuclear staining for nitrotyrosine in lymphocytes, endothelial cells, macrophages, and interstitial cells in the synovium. This suggests that histones may be targets of RNOS. Similar nuclear staining for nitrotyrosine was seen in a mouse tumor model[32]. Recent findings from our laboratory have provided unequivocal mass spectrometry evidence for nitrotyrosine in histones in the same murine tumor model[43]. Because stromal proteins and histones are stable, they may accumulate nitrotyrosine compared to rapidly turning over proteins and therefore appear as prominently nitrated. Whatever the mechanism, attention should be given to these proteins as

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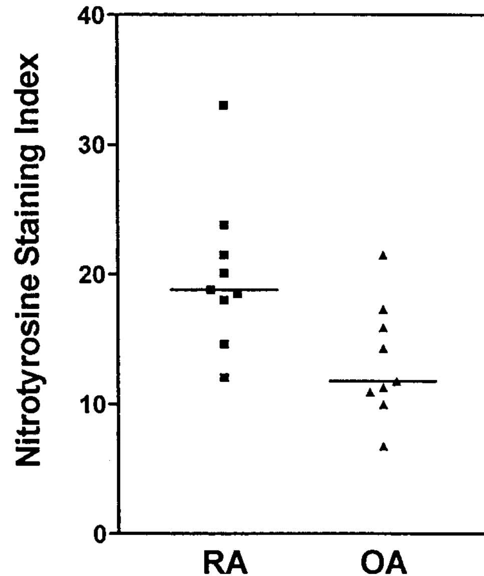

Figure 4. Nitrotyrosine staining index of RA and OA synovial tissue. Image analysis of stained sections is described in Materials and Methods. Each symbol represents tissues from a single patient; bar represents the median for each disease group.

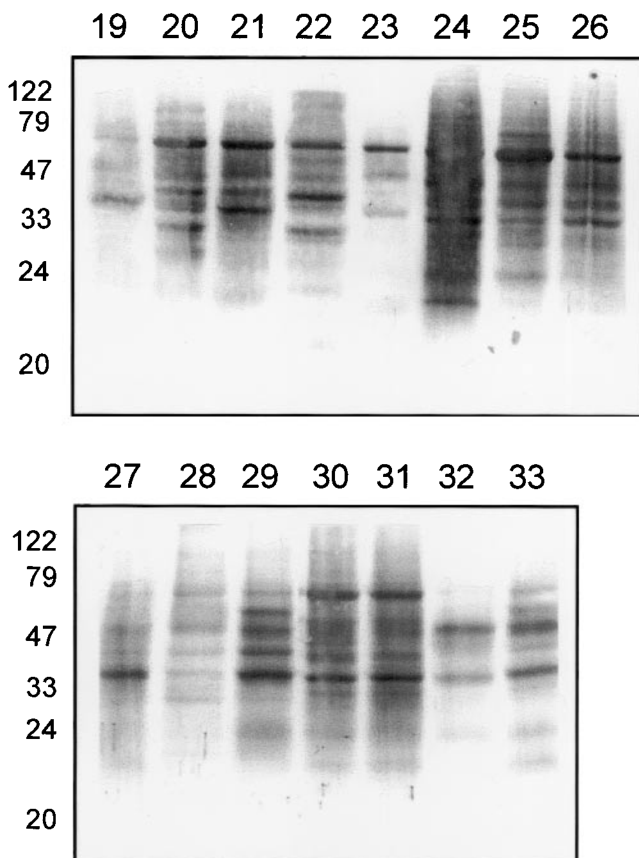

Figure 5. Western blot analysis of protein nitrotyrosine in homogenates of synovial tissue from 3 RA and 11 OA patients and one SLE patient. Numbers above the lanes refer to patients shown in Table 1.

potentially clinically useful biomarkers of longterm exposure to RNOS.

We attempted to correlate our biochemical and histological findings with clinical disease variables. The nitrotyrosine staining index was very statistically different for RA and OA synovia. There was no correlation with age. The sample size was too small to investigate a correlation with disease duration, number of lymphoid aggregates, polymorphonuclear cells, degree of necrosis, fibrin deposits (data not shown), ESR, or CRP. Recently, CRP was found to correlate positively with the number of iNOS-positive mononuclear cells in the SF of patients with RA[38]. While further studies will be needed to assess their significance in disease progression, our findings add to the evidence that oxidative and nitrative damage to synovial proteins is common in the arthritic joint. Our findings also strengthen the possibility that some of the current therapies for RA exert their effects by decreasing oxidative and nitrative damage. If proven to be true, this could lead to the design of a new class of therapeutic agents.

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