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Short Communication

Identification of Inducible Nitric Oxide Synthase in Human Macrophages Surrounding Loosened Hip Prostheses

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Exposure of rodent macrophages to certain cytokines and endotoxin results in the synthesis of inducible nitric oxide synthase (iNOS or NOS-II) leading to the production of large amounts of nitric oxide (NO). Cultures of human macrophages, in contrast, do not produce iNOS after cytokine stimulation, and their ability to act as a physiological source of NO remains questionable. Here we have used immunohistochemistry and in situ hybridization to demonstrate the presence of iNOS within human macrophages present in the interfacial membrane and pseudocapsule that surround failed prosthetic hip joints. Synovial tissue recovered from normal human joints did not express iNOS. Many of the iNOS-positive macrophages within the interfacial membrane had phagocytosed large amounts of polyethylene wear debris, suggesting a role for phagocytic stimuli in inducing iNOS in human macrophages. These findings additionally support a role for NO in modulating the localized bone resorption that accompanies the aseptic loosening of prosthetic joints. (Am J Pathol 1997, 150:1199–1206)

Nitric oxide (NO) is an important local mediator of mammalian physiology and pathophysiology (reviewed in Refs. 1–3). It is produced during the oxidation of L-arginine to L-citrulline by NO synthases (NOSs) present within various tissues,[4,5] including those of the musculoskeletal system.[6–10] Although cells containing constitutive isoforms of NOS produce only small amounts of NO in short bursts, cells in which the inducible isoform of NOS (iNOS or NOS-II) is expressed generate considerable amounts of NO for extended periods of time. Expression of iNOS has been linked to the pathophysiology of various inflammatory diseases, including arthritis,[11] diabetes,[12] and colitis.[13]

Studies with rodents have identified the macrophage as the major producer of large quantities of NO at sites of inflammatory disease. Cultures of rodent macrophages express high levels of iNOS, and thus produce copious amounts of NO, after treatment with endotoxin and cytokines.[14,15] However, despite extensive experimental effort, it has not been possible to provoke reproducibly the production of large amounts of NO by cultures of human macrophages. In fact, there has been considerable debate as to whether human macrophages are even capable of expressing iNOS.[16,17] In the present study we have used immunohistochemical and *in situ* hybridization techniques to determine whether cells present around loosened human hip prostheses express this enzyme.

Various types of hip replacement surgeries are performed for a variety of indications including arthritis, hip fracture, and the failure of previously in-

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serted prosthetic joints. By far the most common mode of failure of these implants is loosening in the absence of infection, a process known as aseptic loosening.[18,19] This process is associated with the formation of a tissue membrane, known as the interfacial membrane, at the junction of the prosthetic device with its surrounding bone.[19] The interfacial membrane is thought to be involved in periprosthetic osteolysis, which leads to premature failure of the surgical implants. After the surgical insertion of an artificial joint, the new joint space becomes encased in a tissue known as the pseudocapsule.

Human interfacial membranes contain large numbers of macrophages, many of which have phagocytosed wear debris.[19–22] Recent attention has been directed toward the presence of polyethylene wear particles eroded from the component of the prosthesis that is placed into the acetabulum of the hip.[23] Phagocytosis of wear particles is held responsible for the increased production of cytokines, such as interleukin (IL)-1, IL-6, and IL-8, matrix metalloproteinases, and eicosanoids, particularly prostaglandin E_2, by these membranes.[24,25] The combined actions of such mediators is thought to cause the localized osteolysis that accompanies aseptic loosening. Despite the popularity of this hypothesis, the key mediators have not been identified and aseptic loosening remains an important clinical problem.

In vitro experiments have suggested that the phagocytosis of particles can induce the synthesis of NO.[9] This raises the possibility that phagocytic cells within the periprosthetic membrane serve as a local source of NO that modulates local bone metabolism. The present study was designed to determine whether human iNOS could be identified in cells present within interfacial membranes and pseudocapsules recovered from prosthetic hips that had failed due to aseptic loosening.

Materials and Methods

Patients and Specimens

Eleven interfacial membranes from nine patients undergoing revision total hip arthroplasty for aseptic loosening were obtained from January to October, 1994, at our institution. All nine patients had exhibited aggressive bone resorption around the femoral implant. In addition to harvesting interfacial membranes, discrete samples of pseudocapsular tissue were also obtained from seven of the patients. Portions of these tissues were taken for aerobic and anaerobic culture; all cultures were negative. Patients comprised six females and three males ranging in age from 62 to 79 years with a mean age of 70.8 ± 6.23 (SD) years. The elapsed time to revision for all components involved ranged from 38 to 251 months with a mean of 99.9 ± 67.9 (SD) months. Normal synovial tissue was retrieved from undiseased joints of patients undergoing above-the-knee amputation and used as a negative control. All tissue specimens were placed in 2% paraformaldehyde/phosphate-buffered saline (PBS), pH 7.2, at 4°C for 1 hour followed by 30% sucrose/PBS, pH 7.2, at 4°C for 24 hours. The following day, tissue specimens were snap-frozen using Histofreeze and stored at −80°C to await staining.

Microscopic and Immunohistochemical Evaluation

Cryostat sections of tissue were stained with hematoxylin and eosin (H&E) for standard histological analysis and observed under polarized light microscopy to detect large polyethylene wear particles. Immunohistochemical techniques were used to identify macrophages and iNOS.

Frozen tissue was cut in 5-μm sections and placed on SuperFrost^{++} slides (Fisher Scientific, Pittsburgh, PA). The tissue was immediately rinsed in PBS, taking care to prevent the tissue from drying. The slides were then rinsed two more times in PBS and three times in PBS containing 0.5% bovine serum albumin, 0.15% glycine (buffer A). After this, any nonspecific binding was blocked by incubating the samples in goat serum (Sigma Chemical Co., St. Louis, MO) diluted 1:20 in buffer A for 1 hour. Next the specimens were rinsed three times in buffer A at 5-minute intervals.

Two different antibodies were used to detect iNOS. One of these, a monoclonal anti-mouse macrophage iNOS antibody, was obtained commercially (Transduction Laboratories, Lexington, KY). This antibody has been used previously to identify NOS in human cells.[26] A second antibody, NO53, was kindly supplied by Dr. Philip Davies of Merck Research Laboratories. NOS 53 is a rabbit polyclonal antibody raised against a synthetic peptide that contains the extreme carboxyl-terminal heptomer of human iNOS. This sequence is absent from human endothelial NOS (eNOS) and brain NOS (bNOS). The properties of this antibody and confirmation of its specificity for human iNOS have been published.[27]

The primary antibody incubation using anti-iNOS was done using 2 μg of antibody/ml of buffer A for 1 hour at room temperature. After being rinsed three times with buffer A, one of two paths was taken to

visualize the reaction. For the peroxidase result, the primary antibody was followed by incubation in biotinylated antibody for 1 hour, cold methanol for 30 minutes, and avidin-biotin complex for 45 minutes, and finally, the reaction product was shown using diaminobenzidine. The samples were then counterstained using toluidine blue and mounted using Permount (Fisher).

The fluorescent method utilized Cy3.18 (Jackson ImmunoResearch, West Grove, PA) at a dilution of 1:3000 to visualize the antibody binding sites. The fluorescently labeled specimens were then incubated with an anti-mouse macrophage antibody conjugated with fluorescein isothiocyanate (Accurate Chemical and Scientific Corp., Westbury, NY). This produces a dual-color image revealing the iNOS-positive and macrophage-positive cells. The specimens were rinsed and mounted in Gelvatol (Monsanto, St. Louis, MO).

In Situ *Hybridization*

The iNOS probe used was a 2.3-kb *Bam*HI fragment of the human hepatocyte iNOS cDNA clone. Both antisense and sense riboprobes were made using ^{35}S-labeled UTP, unlabeled CTP, ATP, and GTP, and the relevant polymerase for 2 hours at 37°C. The labeled RNA was precipitated in cold ethanol, dried, and resuspended in RNAse-free water.

Hybridization was performed as follows. Frozen sections were cut, as in the immunohistochemistry section, fixed in 2% paraformaldehyde in PBS (10 minutes), permeabilized in 2% paraformaldehyde in PBS containing 0.1% Triton X-100 (10 minutes), washed twice in PBS, digested with proteinase K (10 μg/ml for 5 minutes), washed in PBS containing 1% glycine, and acetylated. After dehydration through graded alcohols, the sections were hybridized overnight at 42°C in ^{35}S-labeled, iNOS-specific riboprobe (controls were generally the sense strand of the probe and a no probe control). Sections were then washed twice in 50% formamide/2X standard saline citrate (SSC; RNA wash solution 1) for 15 minutes at 50°C and nonspecific probe binding digested in RNAse for 30 minutes at 37°C. After additional washes in RNA wash solution 1 and 2X SSC, sections were dehydrated in a graded series of alcohols (30, 70, 90, and 100%), dipped in NTB2 emulsion (1:1 dilution in water), and dried. After a 2-week incubation, during which time the slides were kept in light-tight boxes at 4°C, slides were developed in D19 (Kodak, Rochester, NY) for 2 minutes, fixed, and washed. The sections were then counterstained in 0.1% toluidine blue in water, dehydrated, and mounted in Permount (Fisher).

Image Analysis

To determine co-localization of either polarized light and dark-field images in conjunction with bright-field morphology or to co-localize the iNOS reaction product with polarized light, digital images were collected using a three-chip color camera. To collect the polarized light image, shown as red in the displayed image, an intermediate red filter was placed after the second polarizer. In the case of the *in situ* hybridization signal, a green filter was used. The bright-field images showing either synovium or pseudocapsule morphology (see Figure 3, A and B) or the localization of iNOS protein (see Figure 3C) were collected using standard bright-field illumination. To present the different images simultaneously, images were superposed by addition of Optimas (Optimas, Seattle, WA) a PC-based image collection and analysis package.

Results

Typical morphologies of interfacial membranes and pseudocapsules are seen in Figure 1, A and D. In each case, there is abundant fibrous tissue present; in the pseudocapsule, this fibrous tissue appears somewhat laminar in structure.

In both cases, an inflammatory infiltrate is present as well as numerous fibroblasts. When samples are viewed under polarized light, it is possible to detect birefringent inclusions typical of polyethylene wear debris (Figure 1, B and E).

The same sections labeled for iNOS mRNA, detected autoradiographically and visualized using dark-field microscopy, show large areas of iNOS-positive cells (Figure 1, C and F). Comparison of Figure 1, B with C and E with F, reveals areas of tissue that are positive both for the presence of wear debris and iNOS mRNA and areas that are not. In general, the larger particles (eg, that arrowed in Figure 1, B and C) are not associated with iNOS message. When signal is coincident, (eg, Figure 1, E and F, arrows) it is generally seen with smaller wear particles that are of sizes that permit phagocytosis. These observations suggest that phagocytosis of the wear debris is, in many cases, associated with the expression of iNOS.

Higher-magnification images of the results obtained by *in situ* hybridization are shown in Figure 2. Interfacial membrane is shown in Figure 2, A and B,

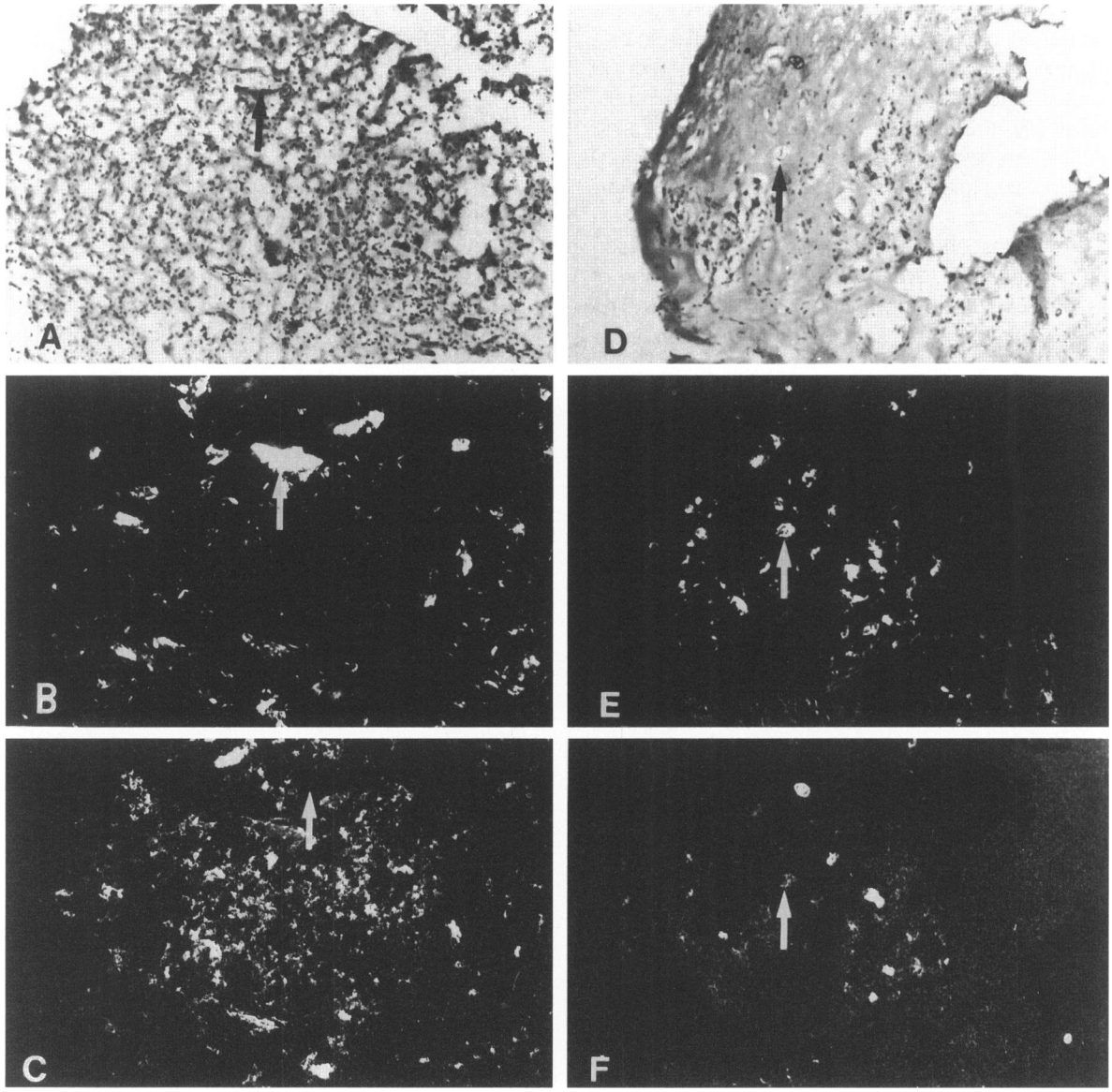

Figure 1. *Consecutive sections of interfacial membrane* (left panels) *and pseudocapsules* (right panels) *stained with H&E* (A *and* D), *viewed under polarized light* (B *and* E), *or subjected to* in situ *hybridization for iNOS mRNA and viewed in dark-field microscopy* (C *and* F). *Magnification,* ×*50. The* arrow *in* A *points to a large, extracellular polyethylene particle, visualized by polarized light microscopy in* B (white arrow), *which is not associated with iNOS mRNA expression* (white arrow *in* C). *The* arrow *in* D *points to a smaller, intracellular polyethylene particle, visualized by polarized light microscopy in* E (white arrow), *which is associated with iNOS mRNA expression* (white arrow *in* F).

and pseudocapsule is shown in Figure 2, C and D (A and C show dark-field image and B and D show bright-field images). The arrows point to areas of high iNOS mRNA expression.

The degree of superposition of iNOS expression and phagocytosis of particles is seen in Figure 3, A and B. In these images, the wear particles have been pseudocolored red and the iNOS mRNA-positive cells are green. When the signals are superimposed and an orange/yellow signal is seen, phagocytosis of wear particles by iNOS-positive cells has occurred.

In the interfacial membrane (Figure 3A), the polyethylene particles (red) are nearly all associated with some degree of yellow, suggesting that they are associated with iNOS expression. Larger particles are colored yellow only around the edges, probably because the size and optical opacity of the particles precludes observation of the iNOS signal. Large numbers of cells expressing iNOS message without evidence of polyethylene debris are also seen (Figure 3A).

In the pseudocapsule (Figure 3B), the association between particles and iNOS expression is more ob-

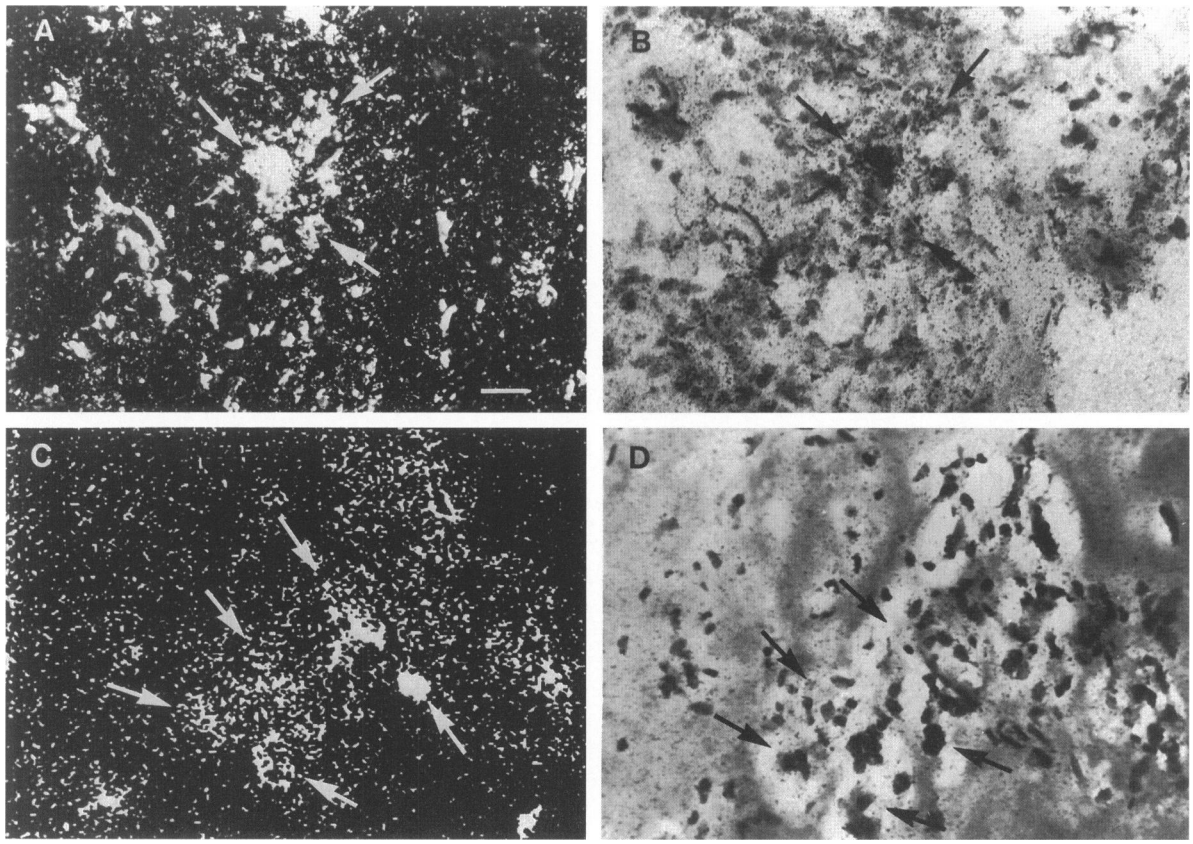

Figure 2. *Higher-magnification images of iNOS mRNA expression, identified by* in situ *hybridization.* A *and* B: *Interfacial membrane.* C *and* D: *Pseudocapsule.* A *and* C *are dark-field images.* B *and* D *are bright-field images.* Arrows *indicate areas of particularly intense iNOS expression. Bar, 20 μm.*

vious, as practically all the signal is yellow. This may reflect the observation that the polyethylene particles tend to be smaller, and thus more easily internalized, in the pseudocapsule than in the interfacial membrane (eg, Figure 1B).

These data were confirmed by immunohistochemistry. Both primary antibodies used in this work gave the same result. To avoid duplication, only the result with the Transduction Laboratories antibody is shown in Figure 3. In Figure 3C, wear debris is again visualized by polarized light and pseudocolored red, and then superimposed on the immunoperoxidase image. In this, many cells are seen to show positive staining for iNOS, and the small wear particles have been internalized by iNOS-positive cells. However, the large particles at the top, right-hand side of the image remain extracellular. Again, there are iNOS-positive cells that lack visible wear particles.

Histological examination of the tissue sections fails to identify the cells responsible for phagocytosis of polyethylene wear particles and iNOS expression. To determine whether macrophages were involved, double-immunofluorescent labeling with antibodies specific for macrophages (Figure 3D) and iNOS (Figure 3E) was performed. This analysis detected cells, with a macrophage morphology, in which the labels were co-localized (Figure 3F). Quantitation showed that 59 ± 5% (mean ± SE; n = 11) of the macrophages within the interfacial membranes and 52 ± 8% (mean ± SE; n = 7) of the macrophages within pseudocapsule stained positive for iNOS. No staining of endothelium was seen, confirming antibody specificity. No staining for iNOS by *in situ* hybridization or immunohistochemistry was seen in normal human synovium (data not shown).

Discussion

These data confirm iNOS expression by human macrophages *in vivo* and they raise the possibility that NO is involved in aseptic loosening. They further suggest phagocytosis as a previously unrecognized inducer of iNOS in these cells, although additional work is required to determine whether this is actually so.

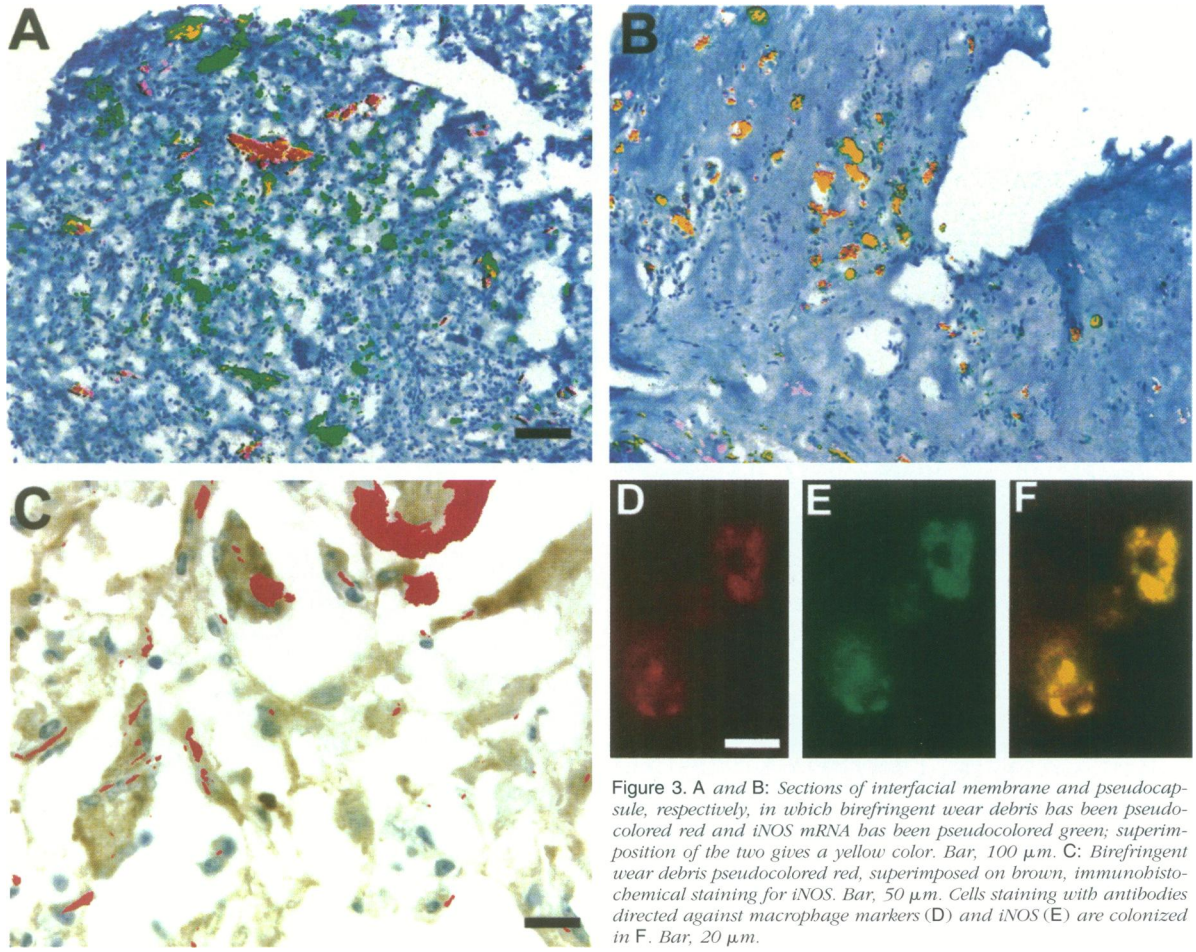

Figure 3. A *and* B: *Sections of interfacial membrane and pseudocapsule, respectively, in which birefringent wear debris has been pseudocolored red and iNOS mRNA has been pseudocolored green; superimposition of the two gives a yellow color. Bar, 100 μm.* C: *Birefringent wear debris pseudocolored red, superimposed on brown, immunohistochemical staining for iNOS. Bar, 50 μm. Cells staining with antibodies directed against macrophage markers* (D) *and iNOS* (E) *are colonized in* F. *Bar, 20 μm.*

Although the unequivocal identification of iNOS in human tissue samples can sometimes prove problematic, agreement between the results obtained with the multiple different probes used in the present work permits a certain degree of confidence on this matter. The monoclonal anti-mouse iNOS used in the present study recognizes human NOS but in Western blotting appears to cross-react with nNOS.[27] However, the polyclonal antibody raised against a heptomer that is unique to human iNOS has no such ambiguity.[27] The fact that these two antibodies gave identical results, and that no staining of vascular endothelium (eNOS) or nervous tissue (nNOS) was seen, strongly indicates that iNOS was indeed being expressed in interfacial membrane and pseudocapsule. Independent confirmation of this conclusion is provided by the *in situ* hybridization analysis.

The literature contains only three other reports of iNOS expression by human macrophages *in vivo*.[28–30] In two of these studies,[28,29] lung tissue was used, whereas the third used rheumatoid synovium.[30] These findings, taken with ours, encourage the conclusion that human macrophages have the ability to produce iNOS *in vivo* and that the failure to reproduce this consistently in cell culture reflects a failure to arrive at the appropriate *in vitro* conditions.

Although the addition of soluble cytokines to cultures of unactivated human macrophages has generally failed to induce iNOS,[16,17] data suggest that infection with pathogens,[31–33] cross-linking of CD69,[34] and direct contact with tumor cells[35] may be effective. Our observation that many of the macrophages that stained positively for iNOS also contained internalized polyethylene particles suggests that phagocytosis should also be considered as a pathophysiological inducer of iNOS. However, the identification of iNOS within macrophages that did not contain particles indicates either a response to soluble factors or, perhaps, the presence of particles that were too small to see. The latter explanation is quite possible, as Maloney et al[23] have used electron microscopy to measure the sizes of polyethylene wear particles in periprosthetic membranes. Their analysis provided a mean size of 0.5 μm, which

means that most of the particles are too small to be seen by optical microscopy. In addition, polarized light microscopy would not reveal metallic wear particles that might be present. Experiments conducted in this laboratory have demonstrated that the addition of particles to cultures of rabbit synovial fibroblasts[9] and the rat macrophage cell line J774[36] induces NO production, but there are no data concerning human macrophages.

Phagocytosis of wear particles has been implicated as a pathophysiological factor in a number of orthopedic conditions including osteoarthritis[37,38] and adverse reactions that occur in response to artificial ligaments[39,40] and prosthetic joints.[22–25,41] The present data suggest that phagocytosis may promote the induction of iNOS and thus NO biosynthesis. Although NO has been already implicated in arthritis,[10,11] it has not been previously considered a factor in aseptic loosening. As NO has profound effects upon bone remodeling,[8,42,43] it has the potential to modulate the osteolysis that occurs during the aseptic loosening of prosthetic joints. The identification of iNOS within interfacial membranes opens novel possibilities for pharmacological intervention in this distressing condition.

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