NITRIC OXIDE PRODUCTION IN CELLS DERIVED FROM THE HUMAN JOINT

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SUMMARY

We have investigated the ability of cells derived from the human joint to generate nitric oxide (NO). Synovial fibroblasts, articular chondrocytes and osteoblasts were cultured from tissues of patients undergoing hip replacement surgery, and synovial fluid leucocytes were obtained from patients undergoing joint aspiration. There was little spontaneous generation of NO by any of the cells after culture, but synovial fibroblasts, articular chondrocytes and osteoblasts all produced large quantities of NO in response to a cytokine mix of interleukin (IL)-1β + tumour necrosis factor α (TNFa) + interferon (IFNγ). Reverse transcription–polymerase chain reaction (RT-PCR) analysis showed the presence of mRNA transcripts for the inducible isoform of NO synthase in cytokine-stimulated but not in unstimulated cells. In contrast, leucocytes from synovial fluid did not produce NO either spontaneously or after cytokine stimulation, and mRNA for inducible NO synthase (iNOS) was not detected in these cells even by nested PCR. There were significant differences in the regulation of NO production between chondrocytes and other cells. Only chondrocytes generated NO in response to IL-1β or TNFa alone, whereas synovial fibroblasts and osteoblasts required the presence of at least two cytokines to generate NO. Dexamethasone (10⁻⁴ M) had a small but significant inhibitory effect on NO production by chondrocytes, synovial fibroblasts and osteoblasts. Our results indicate that several cells within the human joint have the potential to generate NO in the presence of an appropriate pro-inflammatory cytokine stimulus, while leucocytes in synovial fluid are not a significant source of NO. The data support suggestions that NO is produced within the inflamed joint in diseases such as rheumatoid arthritis.

KEY WORDS: Nitric oxide, Inducible nitric oxide synthase, mRNA, Rheumatoid arthritis, Inflammatory joint disease, Synovial fibroblasts, Osteoblasts, Articular chondrocytes, Synovial leucocytes.

NITRIC oxide (NO) is a short-lived free radical with important roles in a number of biological processes [1, 2]. In mammalian vascular endothelium and nervous tissue, NO is produced by two distinct calcium/calmodulin-dependent forms of NO synthase, where it acts respectively as a mediator of smooth muscle relaxation and of neurotransmission via stimulation of cyclic guanosine monophosphate (cGMP) production [1]. Many other mammalian cells, including macrophages [3], neutrophils [4], lymphocytes [5], mesangial cells [6], hepatocytes [7] and bone marrow cells [8], can be induced to generate NO when exposed to bacterial endotoxin or inflammatory cytokines such as interleukin 1 (IL-1), tumour necrosis factor (TNF) and interferon gamma (IFNγ), through a third distinct NO synthase isoform. This cytokine-inducible NO synthase has been implicated in immunoregulation and host defence mechanisms [3, 5].

Recent evidence, much of which is derived from animal experiments, suggests that NO may act as a pro-inflammatory mediator in inflammatory joint disease. Inhibitors of NO synthesis have markedly altered the progression of joint destruction and inflammatory processes in animal models of arthritis [9–11]. In man, concentrations of the NO metabolite, nitrite, have been shown to be greater in serum and synovial fluid from patients with rheumatoid arthritis and osteoarthritis than in age- and sex-matched controls [12]. Since nitrite levels in synovial fluid in these patients were found to be greater than those in serum, it has been suggested that NO is produced within the inflamed joint. Experiments in vitro have identified chondrocytes in man [13] and rabbits [14], and synovial fibroblasts in rabbits [15], as sources of NO when stimulated with pro-inflammatory cytokines and bacterial endotoxin. We have also recently shown that human osteoblast-like cells produce NO in culture in response to cytokines [16].

Since there is little information on sources of NO production in human joint-derived cells, we have compared the ability of different cells derived from the joint to generate NO in vitro. We demonstrate differential regulation of NO production in human chondrocytes compared with osteoblasts or synovial fibroblasts, while showing that leucocytes from synovial fluid aspires do not produce NO either spontaneously or in response to cytokine stimulation.

MATERIALS AND METHODS

Patients and tissues

Human femoral heads and synovial tissues from five patients with osteoarthritis and from four patients with rheumatoid arthritis were collected into sterile phosphate-buffered saline (PBS) during joint replacement surgery and transported to the laboratory within 2 h of operation.

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Collagenase (type I and type IV) and deoxyribonuclease I (DNase) were obtained from Sigma Chemical Co. (Poole, UK). Dulbecco's Minimal Essential Medium (DMEM), Basal Medium with Earle's salts (BME), Hanks Balanced Salt Solution (HBSS), fetal calf serum (FCS), L-glutamine, penicillin, streptomycin and trypsin were purchased from Gibco (Paisley). Tissue culture plastics (90 mm² flasks, 90 mm plates, 6- and 96-well plates) were purchased from Costar (Cambridge, MA, USA).

Human recombinant IL-1β, human recombinant TNFα and human recombinant IFNγ were obtained from Boehringer Mannheim (Lewes, UK). L-NAME, Monomethyl arginine (L-NMMA) and dexamethasone were obtained from Sigma Chemical Co. (Poole). Cytokines were diluted in culture medium immediately before use from concentrated stock solutions (prepared in culture medium with 5% heat-inactivated FCS and stored at −20°C in single-use aliquots). L-NMMA was added from a concentrated stock solution (5 x 10⁻⁴ M) prepared in PBS at −20°C. Dexamethasone was diluted from a stock solution in ethanol.

Osteoblasts

Osteoblasts were prepared and cultured as previously described [16]. Briefly, trabecular bone chips were washed in PBS and adherent cells were removed from the bone surface by digestion in collagenase. Chips were placed in 90 mm plates in DMEM supplemented with 10% FCS, glutamine (2 x 10⁻³ M), penicillin (50 IU/ml) and streptomycin (50 µg/ml), and the medium was changed twice weekly until a confluent layer of osteoblast-like cells was obtained (typically 3-5 weeks). These cells exhibited osteoblast characteristics at confluence, including increased alkaline phosphatase activity after 48-72 h of treatment with 1,25-dihydroxy-vitamin D₃ (10⁻⁴ M), and expressed type I collagen and osteocalcin mRNAs detected by reverse transcription-polymerase chain reaction (RT-PCR; data not shown).

Chondrocytes

Chondrocytes were isolated as described by Palmer et al. [13]. Cells were grown to confluence in BME supplemented with 10% FCS, glutamine (2 x 10⁻³ M), penicillin (50 IU/ml) and streptomycin (50 µg/ml), with medium changes every 3-4 days. At confluence, these cells exhibited a varied fibroblast morphology ranging from small round cells to large cells with multiple processes. Collagenase activity was detectable using zymography (data not shown) following culture in serum-free medium, in keeping with the description of these cells by Dayer et al. [17].

Synovial leucocytes

Synovial fluid was obtained from routine knee aspirations of eight patients into tubes containing 0.5 ml ethylenediaminetetraacetic acid (2 mg/ml) as anticoagulant. The cellular fraction was separated by centrifugation, washed thoroughly in PBS and placed in DMEM supplemented with 10% FCS, glutamine (2 x 10⁻³ M), penicillin (50 IU/ml) and streptomycin (50 µg/ml) at a density of 10⁶ cells/ml. Cells were used immediately after harvesting.

Nitric oxide production

Nitric oxide production was assessed by measurement of a stable NO metabolite, nitrite, in a microtitre plate adaptation of the Griess assay as previously described [18]. We have demonstrated elsewhere [16] that nitrite measured in the growth medium reflects a constant proportion of total NO production by cells in culture. Concentrations of nitrite in conditioned media were calculated with reference to standards of sodium nitrite in fresh culture medium.

Experimental cultures

Osteoblasts, synovial fibroblasts and chondrocytes were harvested from confluent cultures by incubation with trypsin. In most experiments, cells were plated out at a density of 0.5 x 10⁶ cells/well in 96-well tissue culture plates in 200 µl culture medium and allowed to adhere for 24 h. Synovial leucocytes were plated out at a density of 1 x 10⁶ cells/well and studied without pre-incubation. In all experiments, at least four replicates for each treatment were set up. Cytokines and drugs were added to experiments in fresh culture medium, and all experimental wells were adjusted to a final volume of 250 µl. Control experiments contained medium alone or an equivalent amount of vehicle in medium where appropriate. Cells were incubated for 48 h at 37°C, at which time experiments were terminated. Plates were centrifuged for 5 min at 800 g, and 200 µl of the medium from each well were transferred to a fresh microtitre plate and assayed for nitrate.

Molecular biology

RT-PCR was performed with total RNA from some cultures to identify which isofrom of NO synthase was expressed. In these experiments, cultures were set up in duplicate in 6-well plates at a density of 3 x 10⁶ cells/well in 3 ml culture medium. Synovial fibroblasts, osteoblasts and chondrocytes were allowed to adhere for 24 h prior to incubation with cytokines and other reagents for a further 24 h. Synovial leucocytes were
used immediately after harvesting or following a 24 h incubation in the presence or absence of cytokines. Aliquots (200 μl) of conditioned media were assayed for nitrite and the remaining medium was aspirated. Total RNA was extracted from cells by the method of Chomczynski and Sacchi [19], and quantified by spectrometry. RT-PCR was performed as previously described for 35 cycles using oligonucleotide primers recognizing motifs in mammalian NO synthase isoforms which are conserved across species [16]. The identities of products from PCR were confirmed by direct sequencing on an ABI373 sequencer. Nucleotide sequences were compared with those held in the GenBank database [20].

**Statistical analysis**

Results shown in the figures are taken from typical representative experiments, and displayed as means ± s.d. for four replicates. Individual experiments were analysed by one-way ANOVA, and Tukey's test or Dunnett's test were employed where appropriate to determine significance between treatments.

**RESULTS**

Basal and cytokine-stimulated NO production by synovial fibroblasts, osteoblasts and chondrocytes from typical experiments are shown in Fig. 1. None of the cultured cells derived from human joints

![Graph A](https://example.com/graph_a.png)

![Graph B](https://example.com/graph_b.png)

**Fig. 1.**—Nitric oxide production in cells derived from the human joint in response to (A) single cytokines and (B) combinations of cytokines. Nitric oxide was measured as nitrite accumulation in culture medium over 48 h from cells incubated either without cytokines (Control), or with IL-1β 10 U/ml (IL-1), TNFα 25 ng/ml (TNF) or IFNγ 100 U/ml (IFN) either alone or combined. Culture medium incubated for 48 h in the absence of cells is shown (Medium). *P < 0.05 compared to Control. Results are taken from typical experiments and are means ± s.d. (n = 4 replicates).
spontaneously generated NO, measured as nitrite released into culture medium. The amount of nitrite measured in unstimulated experiments was not significantly different from that measured in culture medium incubated over the same period of time in the absence of cells (Fig. 1A) and was typically <1.5 nmol/10^6 cells over 48 h.

There was little or no production of NO by synovial fibroblasts or osteoblasts in response to any of IL-1β, TNFa or IFNγ at the concentrations shown (Fig. 1A), or at higher concentrations up to 100 U/ml IL1-β, 100 ng/ml TNFα or 1000 U/ml IFNγ (data not shown). In contrast, chondrocytes generated small but significant quantities of NO in response to IL-1β or TNFα alone, but not IFNγ (Fig. 1A). NO production by chondrocytes in response to these cytokines was dose dependent (data not shown).

Synovial fibroblasts, osteoblasts and chondrocytes all generated significant quantities of NO in response to combinations of two cytokines (Fig. 1B), but chondrocytes and synovial fibroblasts generally produced more NO than osteoblasts for any cytokine combination.

Synovial fibroblasts, osteoblasts and chondrocytes produced substantial quantities of NO when stimulated with a combination of pro-inflammatory cytokines (Fig. 1B) consisting of IL-1β (10 U/ml) + TNFα (25 ng/ml) + IFNγ (100 U/ml). In contrast, synovial leucocytes failed to generate NO in response to individual cytokines or to the combination of all three cytokines (data not shown). NO production by synovial fibroblasts, osteoblasts and chondrocytes occurred at similar rates, ranging from ~5 to 20 nmol of nitrite/10^6 cells over 48 h. Production of NO by synovial fibroblasts, osteoblasts and chondrocytes was significantly inhibited to <35% of cytokine-stimulated levels in all experiments in the presence of the competitive inhibitor of NO synthase, L-NMMA, at a concentration of 10^{-4} M (Fig. 2). Dexamethasone at a concentration of 10^{-6} M produced a small but significant inhibition of cytokine-stimulated NO production by synovial fibroblasts, osteoblasts and chondrocytes (Fig. 2). The mean inhibition ranged from 8 to 28% with synovial fibroblasts (three experiments), from 15 to 35% with osteoblasts (four experiments), and from 5 to 18% with chondrocytes (three experiments).

We characterized the type of NO synthase present in cell cultures by RT-PCR using degenerate oligonucleotide primers which generate characteristic products for inducible and constitutive NO synthase isoforms [16]. A 490 bp product was detected in the cytokine-stimulated cultures of osteoblasts, synovial fibroblasts and chondrocytes which produced NO. This corresponded to the predicted product size for inducible NO synthase (iNOS). When directly sequenced, all the PCR products were identical to the published sequence for human iNOS [21]. There was no evidence of a 610 bp product corresponding to neuronal or endothelial isoforms of NO synthase. In unstimulated cultures of osteoblasts, synovial fibro-

**DISCUSSION**

Recent studies have indicated that NO may be an important mediator in inflammatory joint disease, since competitive inhibitors of NO synthase suppress the inflammation and tissue damage in animal models of arthritis [9–11]. A number of sources of NO within the inflamed joint have been suggested, including chondrocytes [13, 14] and synovial fibroblasts [15], but little is known about the relative contribution of these and other cells to NO production in man. The aim of this study was to identify sources of NO in the human joint.

The data presented in this paper demonstrate that a variety of cells within the human joint have the potential to generate NO when stimulated with inflammatory cytokines which are known to be present in the inflamed joint in diseases such as rheumatoid arthritis. Using primary cultures of cells we have shown that osteoblasts, synovial fibroblasts and chondrocytes generated little or no NO spontaneously after growing in culture, but these cells produced substantial quantities of NO in the presence of...
stimulated cultures was confirmed by RT-PCR using degenerate oligonucleotide primers. Products for β2-microglobulin (β2M) are shown as a control for RT-PCR. NO synthase mRNA expression of iNOS in human articular chondrocytes (3 × 10⁶ cells) were incubated for 24 h in the absence (Control) or presence of IL-1β (10 U/ml) + TNFα (25 ng/ml) + IFNγ (100 U/ml), or with cytokines + dexamethasone (DEX; 10⁻³ M). Inducible NO synthase mRNA expression in stimulated cultures was confirmed by RT-PCR using degenerate oligonucleotide primers. Products for β2-microglobulin (β2M) are shown as a control for RT-PCR.

Fig. 3.—Effects of cytokines and dexamethasone on inducible NO synthase mRNA expression of iNOS in human articular chondrocytes. Chondrocytes (3 × 10⁶ cells) were incubated for 24 h in the absence (Control) or presence of IL-1β (10 U/ml) + TNFα (25 ng/ml) + IFNγ (100 U/ml), or with cytokines + dexamethasone (DEX; 10⁻³ M). Inducible NO synthase mRNA expression in stimulated cultures was confirmed by RT-PCR using degenerate oligonucleotide primers. Products for β2-microglobulin (β2M) are shown as a control for RT-PCR.

a combined cytokine stimulus consisting of IL-1β + TNFα + IFNγ. As expected, NO production was inhibited in all three cell types by L-NMMA, a competitive inhibitor of the NO synthases. Analysis of the nucleotide sequences of PCR products, amplified using degenerate NO synthase oligonucleotide primers, indicated that mRNA for inducible rather than endothelial or neuronal NO synthase was expressed in all three cell types which produced NO in response to the cocktail of pro-inflammatory cytokines.

Combinations of any two cytokines induced modest but significant quantities of NO in osteoblasts, synovial fibroblasts and chondrocytes. In contrast, only chondrocytes generated significant quantities of NO in response to either IL-1β or TNFα alone. The results of our experiments support previous studies using a variety of human and animal cells, which demonstrate that single cytokines are less effective inducers of NO than combinations of cytokines [1, 2, 6–8, 16].

We have confirmed previous observations that human chondrocytes generate NO in response to single cytokine stimuli [13], while other cells such as osteoblasts and synovial fibroblasts require multiple cytokine stimuli to generate substantial quantities of NO. This observation suggests that chondrocytes must either utilize an alternative cytokine signaling pathway to that used by osteoblasts or synovial fibroblasts, or that they may invoke different regulators in the transcriptional activation or post-transcriptional processing of the inducible NO synthase gene.

In contrast to the tissue-derived cells, the synovial fluid-derived cells failed to produce NO either spontaneously or in response to stimulation by pro-inflammatory cytokines. This observation was strengthened by our failure to detect mRNA for iNOS in freshly isolated cells, even in a nested RT-PCR. These cells are largely (~85%) polymorphonuclear leucocytes, and the remainder of the cells are lymphocytes with a small proportion (1–2%) of macrophages. In rodents, phagocytic cells such as macrophages generate NO abundantly in response to cytokines and lipopolysaccharide [3]. However, our results are consistent with other reports that NO production is not readily induced by cytokines in human phagocytic cells [22].

NO has a short half-life and its actions are thought to be confined to within a few cells' distance of its source of production [23]. Farrell et al. [12] suggest that NO is generated within the joint in inflammatory diseases such as rheumatoid arthritis. The role of NO in inflammatory joint diseases is not clear. NO may contribute to inflammation through its vasodilatory actions. It can react with superoxide radicals to form peroxynitrite, which is highly toxic and decomposes readily to form an oxidant with hydroxyl radical reactivity and nitrogen dioxide [24]. Recent evidence indicates that NO is involved in specific processes within skeletal tissues such as bone and cartilage. We have demonstrated that NO contributes to IL-1-induced bone resorption in mouse calvariae [25], while Taskiran et al. [26] have shown that NO mediates suppression of proteoglycan synthesis by IL-1 in rabbit articular cartilage explants.

It is not known which cells within the joint generate NO in vivo. In these experiments, we were unable to conclude whether any of the cells studied were actively producing NO at the time of biopsy, since the procedures used to isolate the cells from joint-derived tissues involved digestion with enzymes derived from bacteria, which potentially contain contaminating products that may in themselves induce NO synthase activity. Nonetheless, our results confirm that there are a number of potential cellular sources of NO. Our studies have demonstrated differences in the regulation of NO production between cells, but it is difficult to speculate on the relative importance of these cells in the production of NO in vivo since induction of NO synthase in any cell will depend not only on which stimulatory signals are present in the local extracellular milieu, but also on the presence or absence of inhibitory factors. To answer the question of which cells produce NO in vivo, we are currently investigating the expression of NO synthase directly in joint-derived tissues by immunohistochemistry and in situ hybridization.

We have also demonstrated that NO production in human joint-derived cells is relatively insensitive to inhibition by dexamethasone. Palmer et al. [13] previously showed that dexamethasone at 10⁻³ or
10⁻⁴ M inhibited NO production in human articular chondrocytes to a small degree. Similarly, in our experiments, the inhibition of NO production with dexamethasone (10⁻⁴ M) was not extensive in synovial fibroblasts or chondrocytes, and only reached >30% in some experiments with osteoblasts. This contrasts with the situation in vascular endothelial cells where inducible NO production is almost abolished by dexamethasone [27]. These results may have implications for the treatment of diseases such as rheumatoid arthritis where glucocorticoids are widely used to suppress inflammation, and to reduce joint erosions [28]. Inhibitors of NO synthesis have recently been used successfully to prevent or reverse joint destruction in animal models of arthritis [9-11], and the development of novel therapeutic strategies involving suppression of NO production may prove to be beneficial in inflammatory joint diseases in the future.

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