Inflamm. res. 50 (2001) 515–522
1023-3830/01/100515-08 $ 1.50+0.20/0

Inflammation Research

The cell specific temporal expression of nitric oxide synthase isoforms during Achilles tendon healing

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Received 20 January 2001; returned for revision 22 March 2001; accepted by W.B. van den Berg/M.J. Parnham 29 June 2001

Abstract. *Objective and design:* We have previously shown that nitric oxide synthase (NOS) activity is upregulated following tendon injury, and that this activity is important to Achilles tendon healing. The aim of this study was to identify the cellular distribution of nitric oxide synthase isoforms during tendon healing.
Material or subjects: Surgical division of the right Achilles tendon was performed in eighty-five male Sprague-Dawley rats. Healing Achilles tendons were harvested at 4, 7, 14 and 21 days following the surgery. The un-injured left Achilles tendons were used as controls. Using RNase protection assays, in situ hybridization and immunohistochemistry, mRNA and protein of NOS isoforms were evaluated.
Results: Minimal NOS expression was found in un-injured tendon. A cell specific temporal pattern for the mRNA and protein for all three NOS isoforms was found following injury to the Achilles tendon. iNOS was maximal on day 4 in macrophages and fibroblasts. eNOS was maximal on day 4 in endothelial cells and fibroblasts. bNOS expression gradually increased up to day 21 and was found only in fibroblasts.
Conclusions: These results suggest that all three nitric oxide synthase isoforms are expressed by fibroblasts in a coordinated temporal sequence during tendon healing. The sequential pattern of NOS expression in healing fibroblasts suggests that each NOS isoform may play a different role in the healing process and provides opportunities to modify tendon healing in the clinical setting.

Key words: Inducible nitric oxide synthase – Constitutive nitric oxide synthase – Nitric oxide – Tendon repair – Rat

Introduction

Tendon healing is a well-ordered and coordinated process involving inflammation, cell proliferation, matrix deposition, and tissue remodelling. After injury, new tissue generation starts with clot formation and is followed by granulation tissue formation. The latter process encompasses macrophage accumulation, fibroblast ingrowth, matrix formation, and angiogenesis. Inflammation and granulation tissue formation are driven by a complex mixture of growth factors, which are released coordinately into the area of injury. Besides these protein factors and mitogens, evidence is emerging for an important role of small diffusible molecules in wound repair. One of them is nitric oxide (NO), a free radical gas.

Nitric oxide (NO) is a short-lived free radical with biological functions in nervous, cardiovascular, immune systems, and tissue healing [1–6]. NO is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS). At least three isoforms of NOS have been identified. The neuronal isoform (bNOS, type I) is constitutively expressed in discrete neuronal populations, including a subpopulation of dorsal root ganglion neurons [7–9]. The endothelial isoform (eNOS, type III) is present in endothelial cells of blood vessels [10, 11], and the inducible isoform (iNOS, type II), is expressed in various cell types when activated, including macrophages, glial cells [12, 13] and chondrocytes [14].

NOS is upregulated following injury to tissues, and most evidence indicates that this increased local NO production promotes the normal healing process. For example, NOS inhibitors delay the healing of excisional skin wounds while provision of NO via donors accelerates skin wound healing [15, 16]. Mice deficient in iNOS exhibit impaired skin wound healing that is reversible by iNOS gene transfer [17]. The conditions associated with poor wound healing (e.g. diabetes and corticosteroid use) are also associated with reduced iNOS expression [6, 18]. It is likely that NO contributes to skin wound healing by promoting collagen synthesis [15, 19] and angiogenesis [20]. In the gastrointestinal tract, NO dilates blood vessels and increases mucosal blood flow [21, 22]. Inhibition of NOS activity delays gastric ulcer healing, whereas exogenous NO donors, reverse this effect [23, 24]. NO also has a role in tendon healing. In previous reports, we have presented evidence that NOS is induced during tendon healing and inhibition of NOS resulted in a significant reduction in cross-section area and failure load of healing Achilles tendon constructs [2]. Although it is now well established that many effects of tissue healing are mediated by the NOS pathway, relatively little is known about the isoforms of

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NOS expressed in tendon healing, and their cellular distribution.

Since different NOS isoforms may subserve different functions in tendon healing and since isoform selective inhibitors of NOS are currently being developed for therapeutic use, it is clearly important to define the patterns of expression for NOS isoforms in tendon healing. The purpose of the present investigation, therefore, was to examine the cellular distribution of NOS isoform expression. In this study, healing tendon was investigated at 4, 7, 14 and 21 days post injury, as these time points represent the inflammatory phase, granulation phase and remodelling phase for the healing process in this model.

Materials and methods

Animal model

Eighty-five male rats of the Sprague-Dawley strain were used in the study. The animals were 11–12 weeks old, and their body weight ranged from 300 to 350 g. Two rats were housed per cage, and the rats received laboratory chow and water *ad libitum*. Anaesthesia was achieved by intraperitoneal injection of 80 mg/kg ketamine and 5 mg/kg xylazine. Surgical division of the right Achilles tendon was performed as previously outlined [2, 25, 26]. Briefly, the Achilles tendon was transected in an axial fashion 0.5cm from its calcaneal insertion. The tendinous portion of the plantaris was removed to prevent any possible action as an internal splint. No cast or dressings were applied and the animals were unrestricted during the healing phase. The animals were sacrificed by CO_2 inhalation and were utilized for western blot (n = 20 rats) and RNase protection assays (n = 25 rats), in situ hybridization (n = 20 rats) and immunohistochemistry (n = 20 rats). Specimens were harvested at 4, 7, 14 and 21 days following the surgery. The uninjured left Achilles tendons were used as controls. All procedures and protocols were approved by the Committee on Animal Research of the University of New South Wales, Sydney.

RNA extraction and RNase protection assay

Total RNA was extracted according to Trizol reagent protocol (Life Technologies, Inc, Cergy Pontoise, France). Purified RNA was dissolved in RNase-free water. RNA concentration and quality was assessed spectrophotometrically at wavelengths 260 and 280 nm.

Thirty micrograms of total RNA from injuried and uninjuried tendon were used for RNase protection assays. RNase protection assays were carried out as described [27]. Briefly, RNA samples were hybridized at 42 °C overnight with 100,000 cpm of the labelled antisense transcript. Hybrids were digested with RNase A/T1 mixture (dilution, 1:100, Ambion, Austin, TX, USA) for 30 minutes at 37 °C. Under these conditions, every single mismatch is recognized by the RNases. Protected fragments were separated on 5% (w/v) acrylamide/8 M urea gels. A probe for β-actin was included as a control for the amount of input total RNA and the recovery of protected probe fragments.

RNA probe synthesis

The rat bNOS clone was made from a 544 base pair (bp) *EcoRI-Kpn I* fragment of full-length rat nNOS cDNA clone (a kind gift from Dr. A.M. Snowman of Johns Hopkins University, Baltimore, MD). Bluescript II KS- (Strategene, La Jolla, CA. USA) plasmid was used for bNOS subclone. Both rat iNOS and eNOS clones were generated by polymerase chain reaction as previously described [28]. The amplified iNOS cDNA fragment corresponded to nucleotides 3191–3584 of the published sequence (Genebank accession #U03699). The amplified eNOS cDNA fragment corresponded to nucleotides 1–691 of the published sequence (Genebank accession #U02534). pGET-Easy plasmid (Promega, Madison, WI, USA) was used for both iNOS and eNOS subclone. Antisense and sense cRNA probes were synthesized by in vitro transcription with the relevant RNA polymerases (Boehringer-Mannheim, Mannheim, Germany). RNA probes labelled with ^{32}P-UTP were used for RNase protection assays. RNA probes labelled with digoxigenin-UTP were used for in situ hybridization.

In situ hybridization

Paraffin-embedded sections of 5 µm thickness were deparaffinized in xylene and dehydrated through graded ethanol concentrations, then treated with proteinase K (1 µg/ml), fixed with 4% (w/v) paraformaldehyde in PBS (pH 7.4), and acetylated in 0.25% (v/v) acetic anhydrate containing 0.1 M triethanolamine. The sections were then covered with hybridization solution [50% deionized formamide, 1× Denhardt's solution, 10% (w/v) Dextran sulphate and 0.5% (w/v) sodium dodecyl sulphate (SDS), 200 µg/ml salmon sperm DNA, 5 × SSC (sodium chloride, trisodium, citrate), 50 µg/ ml sodium pyrophosphate and 1mM levamisole] and prehybridized for 1h at 55 °C. The digoxigenin-UTP labeled antisense or sense probes were applied at a concentration of 5ng/µl to the hybridization solution, and the sections were then hybridized for 12–18 h at 55 °C. After hybridization, the sections were washed in 2× SSC then in 0.2 × SSC, all for three times for 10 minutes each at 55 °C. The final wash was in 0.1 × SSC for 10 minutes while the ambient temperature dropped from 55 °C to room temperature. The immunologic detection of digoxigenin-labeled transcripts was performed according to the manufacturer's protocol (Boehringer-Mannheim, Mannheim, Germany). Briefly, sections were incubated with alkaline phosphate conjugated with anti-digoxigenin polycolonal sera diluted 1:250 for 2 h at room temperature. The hybridization products were visualized with 5-bromo-4-chloro-3-iodolyl-phosphate (BCIP) and nitroblue tetrazolium chloride (NBT). Finally, the sections were counterstained lightly with Mayer's hematoxyline and mounted.

For negative controls, in situ hybridization with a sense probe was performed. Sections pretreated with 100 μg/ml RNase A before incubation with the labeled probe were also used as negative controls to evaluate probe specificity.

Immunohistochemistry

The longitudinally orientated paraffin-embedded sections were cut to 5 μm thickness. Sections were deparaffinized in xylene and dehydrated through graded ethanol concentrations. After the antigen retrieval, the sections were blocked by 3% (v/v) H_2O_2, followed by 10% (w/v) non-fat dry milk, then incubated with one of the following NOS antibodies (dilution, 1:50 to 1:300, Tansduction Laboratories, Lexington, KY. USA): polyclonal anti-bNOS, monoclonal anti-eNOS, or monoclonal anti-iNOS. After unbound primary antibodies were washed off with PBS, the sections were incubated with biotinylated anti-IgG serum [LSAB + kit (DAKO)], followed with streptavidin peroxidase. Antigenic sites were visualized using diaminobenzidine (DAB) as the chromagen. Slides were then counterstained with Mayer's hematoxyline, dehydrated, cleared, and mounted with mounting medium. A similar protocol was used for negative control sections except that anti-NOS antibody was replaced by mouse or rabbit IgG.

Double-labeling immunohistochemistry for phenotypic markers

To determine whether NOS-expressing cells were of fibroblastic or monocyte/macrophage lineage cells, sections of healing tendon on days 4 and 7 after injury were subjected to double-immunofluorescence staining, using a monoclonal mouse anti-rat ED-1 (Serotec Ltd., Oxford, UK) as a monocyte/macrophage phenotypic marker [30] and using monoclonal mouse anti-rat prolyl 4-hydroxylase (β) (Fuji Chemical Industries Ltd., Toyama, Japan) as a marker for fibroblasts [31].

After blocking with 10% (w/v) nonfat dry milk and goat anti-rat IgG (dilution, 1:50, Zymed Laboratories Inc., San Francisco, CA. USA), the longitudinally orientated sections were incubated with anti-ED-1 antibodies for 1 h, incubated with goat anti-mouse IgG HRP conjugate and developed for 1–3 minutes in DAB. Tissue sections were then thoroughly washed in PBS and re-blocked with goat anti-rat IgG, and then incubated with anti-rat prolyl 4-hydroxylase (β) antibody for another 1 h, followed with biotinylated anti-mouse IgG serum and streptavidin-alkaline phosphatase. For the antigenic site of fibroblast, AP fast read reagent was used as the chromagen.

Statistical analysis

All values in the text and figures are expressed as mean ± SE of *n* observations. Statistical analysis between experimental groups was performed using unpaired two-tailed Student's *t* tests and analysis of variance (ANOVA). Statistical analysis between the right surgically divided and the left uninjured Achilles tendon were performed using paired two-tailed Student's *t* tests. Significance was accepted at the 5% level.

Results

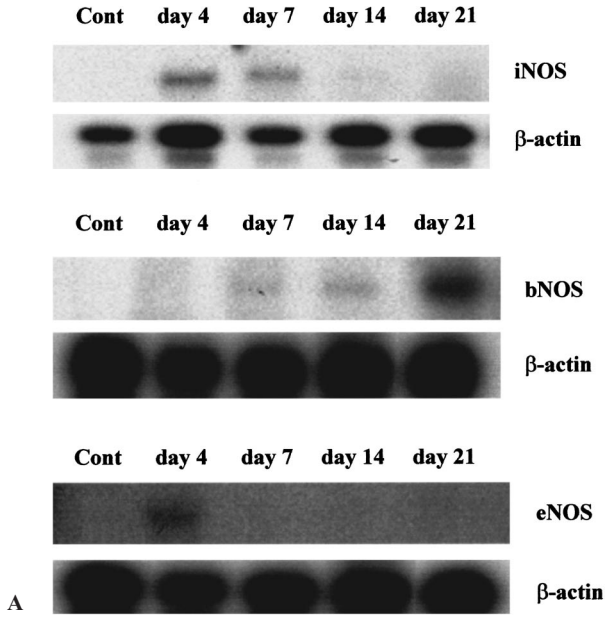
NOS isoforms mRNA are highly induced during tendon healing

To determine a possible role of NOS isoforms in Achilles tendon healing, we first analyzed the time course of mRNA expression for the NOS isoforms during this healing process. We isolated total RNA from healing tissue at different intervals after tendon injury and performed RNase protection assays. This time-course study indicated that the mRNA for all three NOS isoforms were significantly increased in the healing tendon (Fig. 1). iNOS mRNA was significantly increased 4 days post injury (3.7 fold of control values, $p < 0.05$) and then decreased to near non-injured control values by 14 days post injury. The increase in eNOS mRNA occurred later, compared to iNOS. eNOS mRNA levels were lower at 4 days, increased at day 7 (2.4 fold of control value, $p < 0.05$), and then returned to basal levels by 21 days post injury. bNOS mRNA was also upregulated in healing tendon. BNOS mRNA exhibited a slow steady rise with a maximal signal 21 days post injury (4.2 fold of control, $P < 0.05$). In contrast, uninjured tendon had a very low amount of mRNA for all three NOS isoforms.

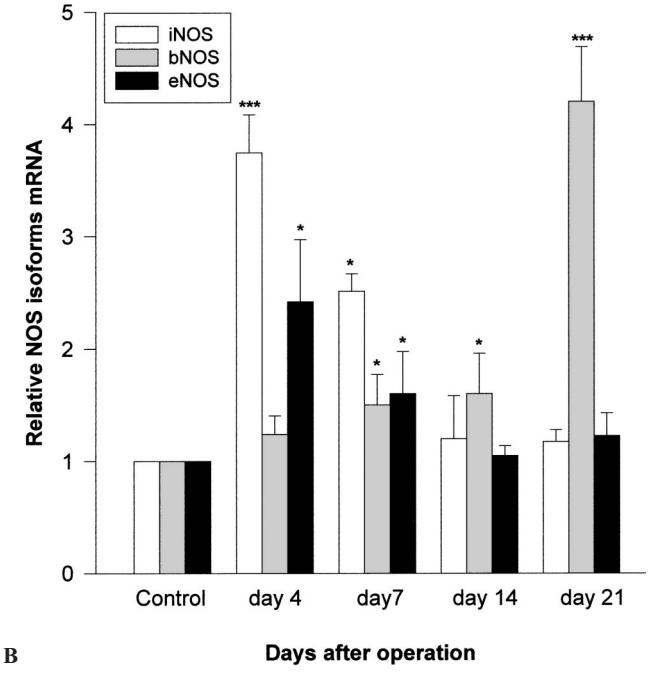
NOS isoforms were expressed in a cell-type specific manner

Monocytes and fibroblasts are the two major cell types in healing tendon tissue. We used serial sections stained for NOS isoform and monoclonal antibodies directed against cell lineage-specific markers, to evaluate the cellular distribution of NOS isoform mRNA and protein expression. The results were consistent with the patterns of mRNA and protein expression in tendon healing tissue noted using RNase protection assay (RPA) and immunoblotting (49).

iNOS mRNA and protein expression were simultaneously detected in serial sections of Achilles tendon day 4 post injury (Fig. 2). At this time point, there was maximal expression of iNOS mRNA and protein. Comparisons with serial sections stained for cell-type-specific markers suggested that



A



B

Fig. 1. RNase protection assay for the temporal expression of mRNA for three nitric oxide synthase (NOS) isoforms in healing rat Achilles tendon. **A**: representative Rnase protection assay from cRNA probes for rat iNOS, bNOS and eNOS that were hybridized overnight to 30 µg of total RNA prepared from healing tendon tissue. Lane 1: mRNA from normal tendon. Lane 2–5: mRNA from healing tendon day 4, 7, 14, and 21 post injury. **B**: densitometric values for NOS isoform mRNA (normalized to β-actin mRNA and to control values), determined in three separate experiments. Values are means ± SE. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ vs. controls using unpaired two-way Student's *t* tests.

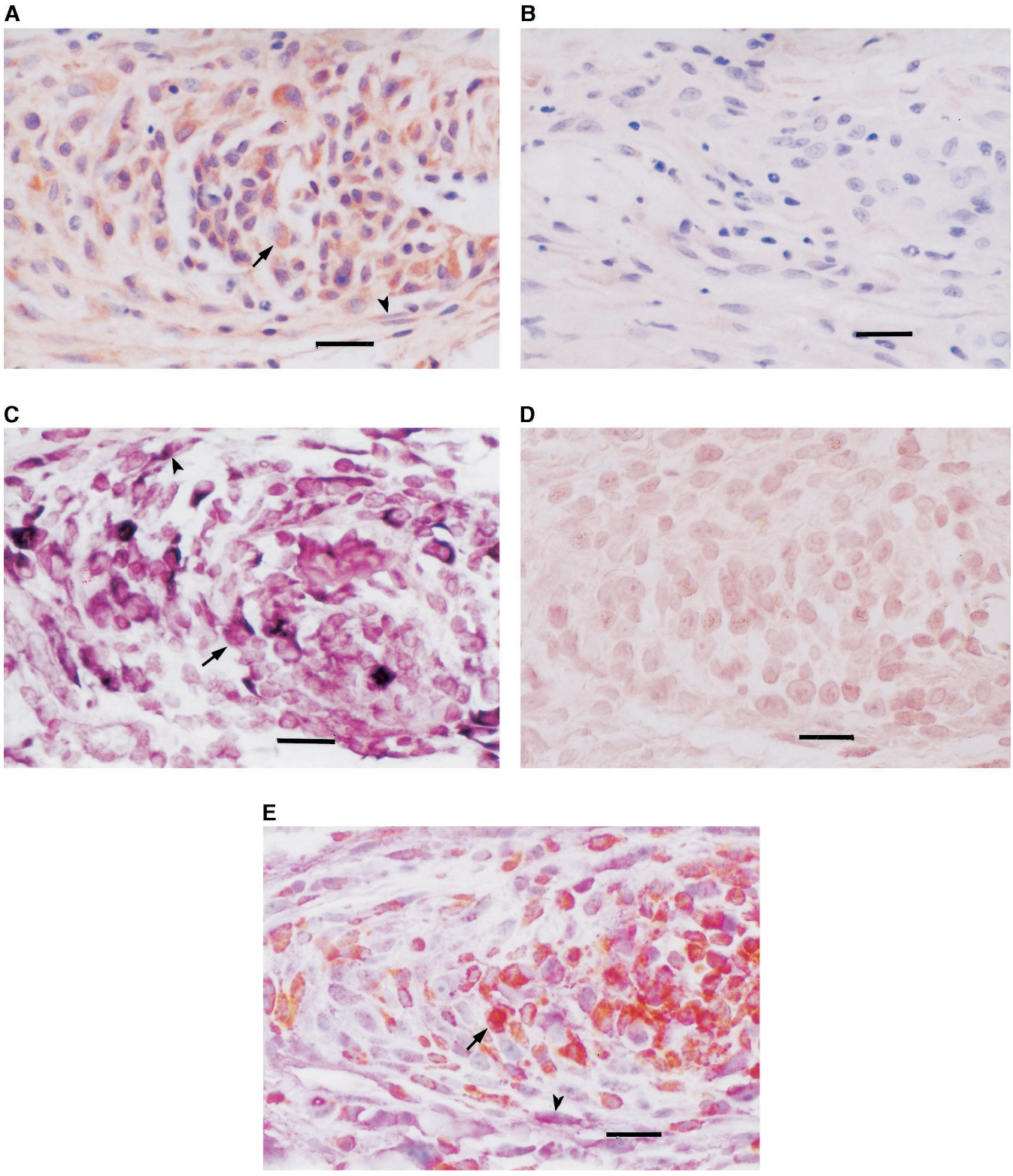


Fig. 2. iNOS expression in healing rat Achilles tendon day 4 post injury. Immunohistochemistry revealed: (**A**) iNOS-immunoreactivity was localized in cells morphologically idenftified as macrophages (arrows), and fibroblasts (arrowheads). (**B**) There was no iNOS immunoreactivity in the serial section which used rabbit IgG (negative control); In situ hybridization revealed. (**C**) iNOS mRNA in both macrophage like cells (arrows) and fibroblast like cells (arrowheads). (**D**) There was no signal detected in a serial section hybridized with sense probe (negative control). (**E**) immunohistochemical double staining with rat macrophage (arrows) and fibroblast (arrowheads) specific cell markers, in a serial section, to confirm that both fibroblasts and macrophages expressed iNOS. Black scale bar represents 20 µm.

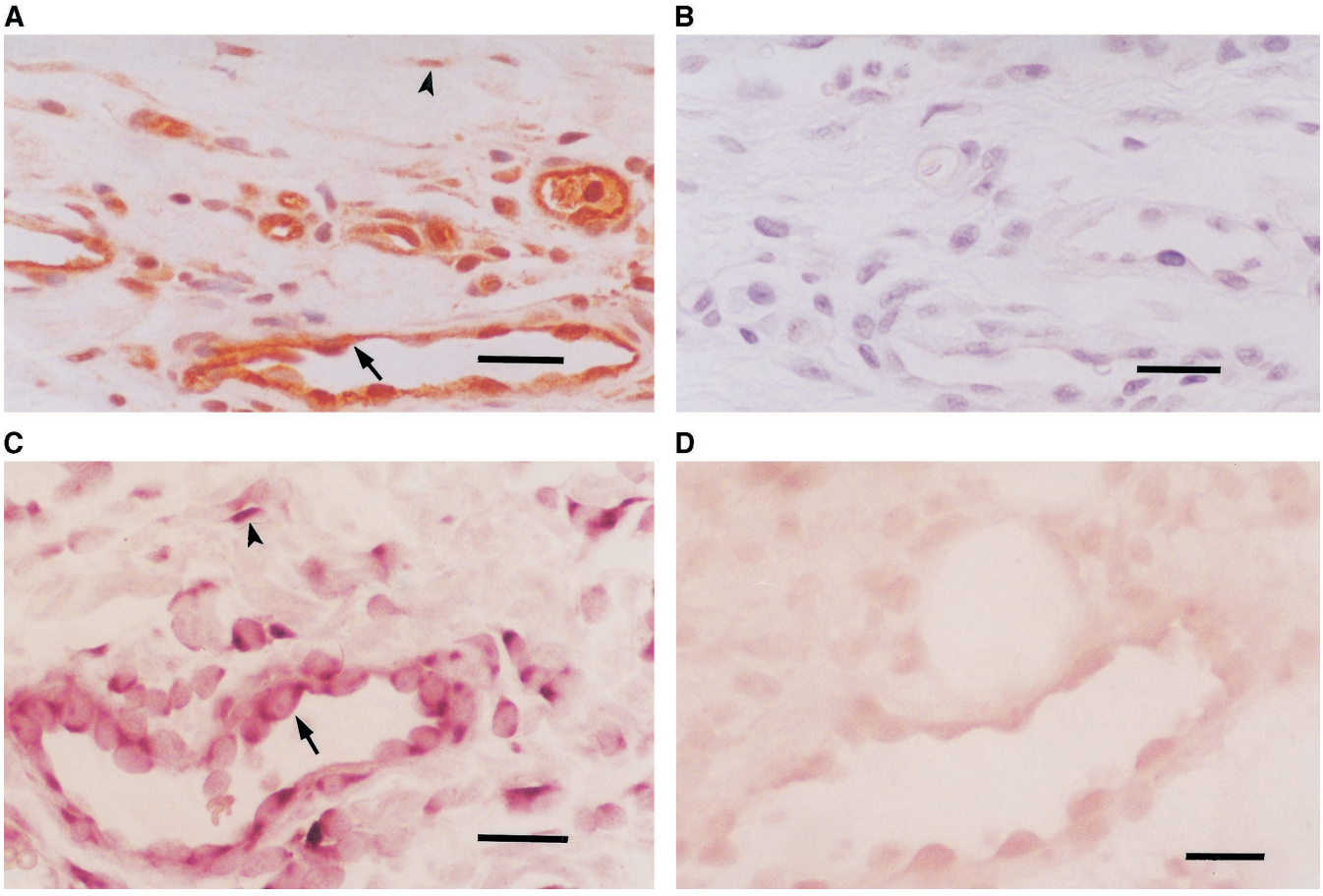


Fig. 3. eNOS expression in healing rat Achilles tendon day 7 post injury. Immunohistochemistry revealed: (**A**) eNOS protein was present mainly in the vascular endothelium (arrows) and a few of the fibroblasts (arrowheads). (**B**) There was no immunoreactivity in the serial section in which rabbit IgG was used (negative control). In situ hybridization revealed: (**C**) eNOS mRNA was present in the vascular endothelium (arrows) and fibroblasts (arrowheads); (**D**) There was no signal detected in a serial section hybridized with eNOS sense probe (negative control). Black scale bar represents 20 μm.

most iNOS protein expressing cells were of the macrophage likeage. Healing tendon fibroblasts also expressed iNOS, however the intensity of staining of iNOS was stronger among macrophages. Endothelial cells were negative or faintly stained for iNOS. When the primary antibodies were replaced by non-immune mouse IgG, very little or no staining was present in the cells in tendon healing tissue. In situ hybridization demonstrated a similar cell expression pattern of iNOS mRNA as that of iNOS protein found during immunohistochemistry staining. No signal was evident in segments hybridized to sense probe.

Sections of healing Achilles tendon day 7 post injury were used to evaluate the cellular distribution of eNOS, as this time point represented maximal eNOS mRNA and protein expression. We found that eNOS was located mainly in the endothelial cells and in a few fibroblast-like cells in the healing tendon tissue (Fig. 3).

For bNOS localization, sections of day 21 samples were used for immunohistochemistry and in situ hybridization. At this time point, bNOS protein and mRNA were abundantly expressed (Fig. 4). Fibroblasts were the major cells of healing tissue at day 21 post Achilles tendon division. At this time point, fibroblasts were easily identified as longitudinal cells within a collagenous matrix. We found that bNOS was expressed exclusively in the fibroblasts.

No positive signals for any of the three NOS isoforms were found in uninjured left tendon using in situ hybridization and immunohistochemistry.

Discussion

In the present study, an increased NOS expression was demonstrated at both protein and mRNA levels during Achilles tendon healing in macrophages and fibroblasts as well as in the vascular endothelial cells. All three NOS isozymes were expressed in a temporal manner in fibroblasts at the healing tendon.

We have previously found that NO synthase activity was induced during the early phases of tendon healing in rats and that systemic inhibition of this enzyme reduced the magnitude of the healing response [2]. These results imply that NO

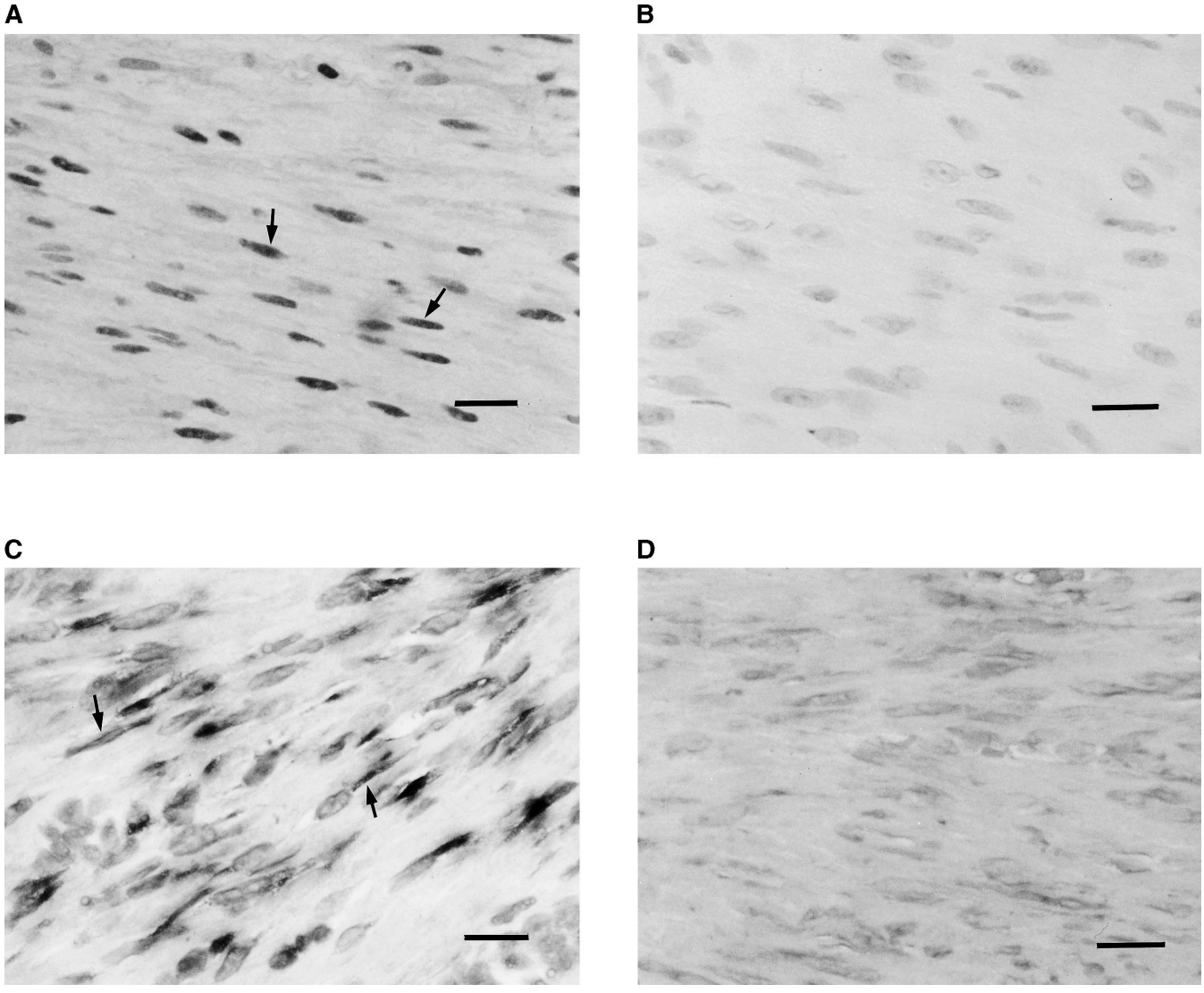


Fig. 4. bNOS expression in healing rat Achilles tendon day 21 post injury. Immunohistochemistry (**A**) and in situ hibridization (**C**) revealed the signals for bNOS protein and mRNA were in fibroblasts (arrows). There was no bNOS immunoreactivity in the negative controls (**B**); or when hybridized with the sense RNA probe (**D**). Black scale bar represents 20 μm.

produced by the three NOS isozymes has important physiological roles in tendon healing.

In the present data, one of the most interesting findings was that fibroblasts expressed all three NOS isoforms during tendon healing. The reason for this is not clear. For iNOS, it can be speculated that following tendon injury, the fibroblasts were stimulated by a complex composition of inflammatory cytokines and growth factors [32, 33] to express iNOS. This higher level of NO might play a role in maintaining the blood supply to the healing site and in controlling bacterial infection during the early stages of wound healing [34]. Moreover, the slightly higher NO level inside and adjacent to the fibroblasts might protect the cells from damage caused by oxygen-free radicals released by activated infiltrating leukocytes such as neutrophils and macrophages, because NO can neutralize both oxygen and hydroxyl radicals [35]. NO released from fibroblasts by the constitutive nitric oxide synthases (bNOS and eNOS) may have regulatory roles in tendon healing. NO generated within a cell also has the potential to modify the biological activities of adjacent cells because of the ease with which this gaseous molecule can pass through cell membranes [36]. Mathematical evidence suggests that NO generated from a point source may be active over distances up to and beyond 0.2 mm within a few seconds [37]. The range of NO action is governed by the rate of NO formation that, in turn, is based on the number of cells producing NO and the type of NOS isoform present [38]. All these findings imply that expression of eNOS in fibroblasts may be beneficial for tendon healing by augmenting granulation tissue blood flow and angiogenesis at the injury site.

NO synthesized by bNOS is thought to act in the central nervous system as a retrograde messenger in certain synapses, where it is possibly involved in potentiation and memory [3,39]. Recent studies have also shown that NO biosynthesis in excitable tissues is not restricted to neurons. bNOS has

been identified in skeletal muscle [40, 41] and bronchial epithelial cells [42, 43]. The work presented here is the first time that bNOS has been found in healing wounds and in fibroblasts. The roles of bNOS expression in healing tendon are yet to be determined. However, it is likely that its major role occurs after the inflammatory phase and during the remodelling phase as this is when bNOS was most highly expressed.

Fibroblasts are the major cell population in healing tendon tissue. These cells may be important in maintaining an adequate NO concentration in tissue. Historically, the fibroblast has been considered to be a rather inert collagen synthesizing cell. Recent data from other laboratories has shown that fibroblasts can be activated to produce cytokines and chemokines [44, 45]. Rodent fibroblasts have been shown to produce NO on stimulation with cytokines and lipopolysaccharide [46]. Human dermal fibroblasts also have been shown to express both constitutive and inducible NO synthase isoforms [47]. In the present study, we found fibroblasts were the only type of cell, which had the capability to express all three NOS isoforms during the phases of tendon healing.

Overuse activity has been implicated as an etiologic factor in injury to the Achilles tendon. Since we used the contralateral uninjured tendon as control, it is possible these control tendons were potentially overused during this experiment. However, we found no evidence for inflammatory or degenerative processes in histologic examination of these control tendons , and their gross morphologic characteristics were normal.

In this study the temporal expression of the NOS isoform mRNA synthesis detected by RNase protection assay, a semiquantative method, corresponded well with our previous competitive RT-PCR results, and also was consistent with the intensity of the immunoreactivity for the corresponding NOS isoform proteins [49]. This result further confirmed that in this model the regulation of NOS isoform production mainly occurred at a transcriptional level.

It is interesting to note that the temporal expression pattern of nitric oxide synthase isoforms in healing tendon is very similar to that of healing bone [48–50] – yet the cells that express the NOS isoforms are different in the two healing tissues. In both tissues, macrophages and endothelial cells express iNOS and eNOS respectively. In healing tendon, fibroblasts express iNOS, eNOS and bNOS in a sequential fashion, while in healing bone chondrocytes express iNOS, eNOS and bNOS in a sequential fashion [51].

We have previously shown that inhibition of NOS is detrimental to healing tendon [2] and to healing bone [48, 52], and that addition of NO may enhance fracture healing [48]. Our data here show that all three NOS isoforms are strongly induced in specific cells within healing tendon in a temporal fashion that is unique to each isoform. This information may be important in designing strategies to enhance or inhibit tendon healing.

Acknowledgements. The authors wish to thank St George Private Hospital/Health Care of Australia and St George Hospital/South East Health for financial support.

Parts of this work were presented at the 46th Annual Meeting of the Orthopaedic Research Society, Orlando, Florida, March 12–15, 2000.

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