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Original Paper

Expression of cyclooxygenase isoforms in normal human skin and chronic venous ulcers

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Abstract

Chronic venous ulcers are an example of abnormal wound healing showing chronic inflammation which together with the underlying vascular pathology results in delayed healing. Prostaglandins are among the most important mediators of inflammation. They have proinflammatory effects, predominantly by affecting the vasculature. Cyclooxygenase (COX) is the rate-limiting enzyme in prostanoid synthesis. It is present in two isoforms: COX-1 (constitutive cyclooxygenase) which is produced in the body to maintain normal haemostatic functions, and COX-2 (inducible cyclooxygenase), which is induced during inflammation in response to cytokines. Using immunoenzymatic labelling and western blot analysis, this study has shown that both COX-1 and COX-2 were up-regulated in chronic venous leg ulcers by comparison with normal human skin. *De novo* appearance of COX-2 in chronic venous ulcers was demonstrated, which is not seen in normal human skin. The main cellular sources of both COX isoforms are macrophages and endothelial cells. COX-2 is also produced by mast cells and fibroblasts. A COX radioimmunoassay showed up-regulation of COX activity in chronic venous ulcers compared with normal skin ($p < 0.05$). Up-regulation of COX-1 in chronic venous leg ulcers could produce prostacyclin, which contributes to angiogenesis. Thus, inhibition of COX-1 by non-steroidal anti-inflammatory drugs (NSAIDs) could increase the local ischaemia and hypoxia associated with chronic venous ulcers. On the other hand, up-regulation of COX-2 is most likely responsible for the persistent inflammation in chronic venous leg ulcers. COX-2 selective inhibitors could therefore be effective in the treatment of chronic venous ulcers.

Keywords: cyclooxygenase; prostanoids; inflammation; wound healing; chronic venous leg ulcers; human skin; macrophages; nitric oxide; inducible enzymes; cytokines

Introduction

There are many theories regarding the pathogenesis of chronic venous ulcers, which we have recently reviewed [1]. These include pressure damage of capillaries with leakage of fibrinogen [2], leading to the formation of perivascular cuffs preventing normal oxygen exchange between capillary and tissue [3]; release of toxic metabolites, proteolytic enzymes, and toxic free radicals by the accumulated neutrophils (PMNs) [4]; and cytokine-mediated matrix cuff formation [5]. Emphasis has also been focused on the role of the endothelium in the regulation of vascular tone in the micro- and macro-vasculature [6,7]. The endothelium secretes both vasodilators, such as nitric oxide, prostacyclin, and endothelium-derived hyperpolarizing factor, and vasoconstrictors, such as prostaglandins and endothelin [8].

Cyclooxygenase (COX) is the rate-limiting enzyme in prostanoid synthesis. Prostanoids are involved in inflammation, thrombosis, and control of vascular tone, and they are classic targets for therapeutic intervention [9]. COX inhibition is the target of aspirin and a wide range of non-steroidal anti-inflammatory drugs (NSAIDs). COX is present in two isoforms, COX-1 and COX-2. COX-1, which is normally expressed in the body [10,11], is important for many physiological functions. These include the production of the thrombogenic thromboxane A_2 in platelets, antithrombogenic prostacyclin (PGI_2) in vascular endothelial cells, cytoprotective prostaglandins (PGs) in gastric mucosa, and vasoactive prostaglandin (PGE_2) for maintenance of renal blood flow [12,13]. The second isoform, COX-2, is not normally expressed in most cells [14–17] but is rapidly induced during inflammation by lipopolysaccharide (LPS) and some cytokines, such as interleukin IL-1α and IL-1β [17–19]. COX-2 reaction products are responsible for many of the cytotoxic effects of inflammation [20].

Persistence of inflammation is one of the major factors delaying healing in chronic venous leg ulcers. Inflammation is most prominent at the ulcer base and ulcer edge, where there is infiltration by inflammatory cells, mainly macrophages [21,22]. The recent trend to use COX-2 selective inhibitors in the treatment of

inflammation [23] led us to investigate the expression of both COX isoforms in chronic venous ulcers in comparison with normal human skin.

Materials and methods

Specimens

For this study, 16 normal skin and 14 long-standing non-healing chronic venous ulcer biopsies were used, from patients with a mean age of 55 years. Patients with evidence of diabetes, diabetic ulcers, and clinical evidence of infection were excluded. No patients were taking NSAIDs or steroids or antibiotics. Skin biopsies were taken under local anaesthesia after written consent from the patients. The biopsies were taken to involve part of the surrounding skin, ulcer edge, and ulcer base. Half of the biopsies were placed immediately in liquid N_2 for biochemical analysis and half were snap-frozen in iso-propanol in a bath of liquid N_2 and then kept at $-80^{\circ}C$ until used for histological and immunohistological observations.

Immunoperoxidase staining

Snap-frozen biopsies were embedded in OCT and then frozen; 6 μm cryostat sections were cut and thaw-mounted onto poly-L-lysine-coated slides, air-dried, and stored at $-20^{\circ}C$ until used. Prior to immunolabelling, sections were fixed in acetone for 10 min and endogenous peroxidases were quenched by treatment with 0.5% H_2O_2 in methanol, with subsequent washing in Tris buffer saline (TBS) (0.7% Tris–HCl + 0.2% Tris base + 0.02% NaCl + 1% Triton X-100). Non-specific binding of IgG was blocked using normal goat serum diluted 1 : 50 in 0.1% bovine serum albumin in TBS for 30 min. The sections were incubated with 1 : 500 (for COX-1) or 1 : 1000 (for COX-2) diluted primary antibodies at 4°C overnight, washed, and incubated for a further 30 min with biotinylated secondary antibodies: goat anti-sheep for COX-1 and goat anti-rabbit for COX-2 (Vector Laboratories, Burlingame, USA) diluted 1 : 1000. Following a further 30-min incubation with Vectastain ABC kits, the substrate, diaminobenzidine tetrahydrochloride in distilled water (Sigma, Poole, UK), was added for 5–10 min. Positive cells were labelled brown. For the negative control, primary anti-serum was replaced with normal serum of the host species of the secondary antibody. Haematoxylin was used as a counterstain to show the nuclei. The antibodies used were polyclonal sheep anti-COX-1 and polyclonal rabbit anti-COX-2 (Cayman Chemicals, Ann Arbor, MI, USA). The slides were mounted using Pertex mounting media. Images were captured using a Spot digital camera (Diagnostic Instruments) connected to a DMRB microscope (Leica).

Triple immunofluorescence

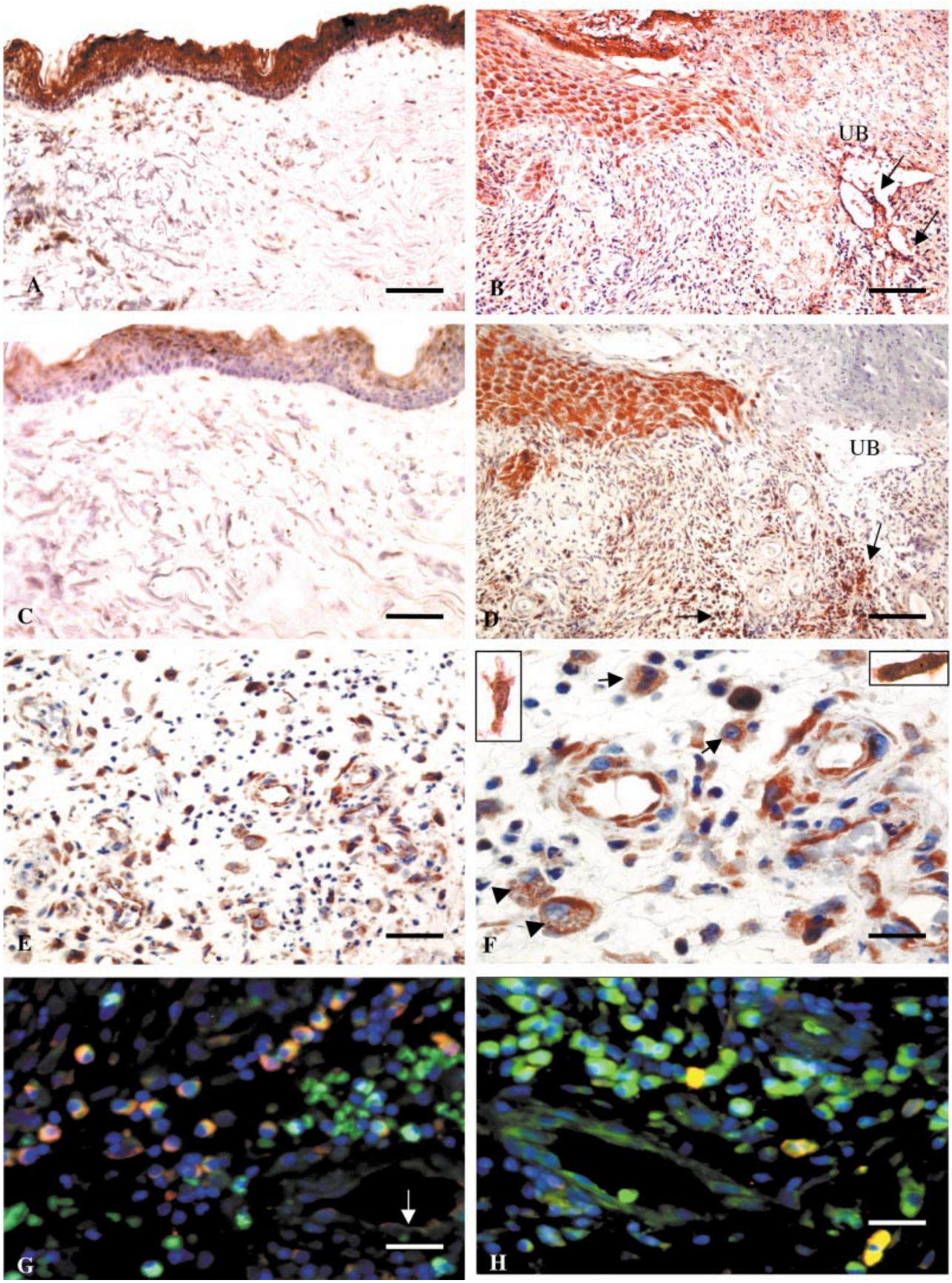
Sections were prepared and incubated with antibody to COX-2 as mentioned above and then washed and incubated for a further 30 min with a TRITC-conjugated goat anti-rabbit secondary antibody diluted 1 : 200 in TBS. Sections were incubated with CD68 antibody (Dako), a macrophage marker (1 : 1000), at 4°C overnight. Then they were washed and incubated for a further 30 min with a biotinylated secondary antibody (1 : 500) and then with streptavidin–fluorescein (Amersham Pharmacia Biotech, Buckinghamshire, UK), 1 : 100, in TBS for 1 h at room temperature. For co-localization of COX-2 and COX-1, the same protocol was used, but FITC-conjugated secondary antibody (1 : 100) was used to label COX-2 and TRITC-conjugated secondary antibody was used to label COX-1. Hoechst No. 33258 (bis-benzimide fluorescent DNA stain that intercalates in A-T regions of DNA) was used as a nuclear counterstain. A stock solution of Hoechst 33258 was prepared (500 μg/ml) in distilled water and kept at 4°C; from the stock, a 1 : 1000 dilution was used for 15 min at room temperature to counterstain the nuclei.

Homogenization

For each of the seven patients, normal skin or the base of the ulcers was homogenized in a mixture of protease inhibitors (10 μg/ml leupeptin, 10 μg/ml pepastatin A, and 100 μg/ml phenylmethylsulphonyl fluoride) in 50 mM Tris–HCl, pH 7.5. After centrifugation for 15 min at 2000 *g*, the supernatants of the tissue extracts were used for western blot analysis and measurement of enzyme activities. The protein concentration in the supernatant was measured by the Bradford assay [24], using bovine serum albumin as a standard. Biopsies used were taken from seven different patients.

Western blot analysis

The protein concentrations in all the samples were equalized. Tissue homogenates were mixed with gel loading buffer (Tris–HCl, 50 mM; SDS, 10%; glycerol, 10%; 2-mercaptoethanol, 10%; bromophenol blue, 2 mg/ml) in a ratio of 1 : 1 and then boiled for 8 min. Forty microlitres of each sample was loaded in each well on a 10% SDS-polyacrylamide mini-gel using the Laemmli buffer system. The proteins were transferred to 0.45 μm nitrocellulose membranes using a transblotting apparatus (Biorad). The transfer was performed overnight using transfer buffer (1600 ml of distilled water, 400 ml of methanol, 30 g of glycine and 6 g of Tris-base). Non-specific IgG binding was blocked by incubation with 5% dried milk protein for 30 min and then the membrane was incubated with primary antibodies (diluted 1 : 1000) overnight at 4°C. The bands were detected using an amplified alkaline phosphatase kit (Sigma, Poole, UK) and developed with 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (Sigma, Poole, UK) as a chromogen. Broad range pre-stained rainbow markers (Biorad Laboratories, Hemel Hempstead, UK) were used for molecular weight determinations.



Measurement of cyclooxygenase activity

This was a modification of the method of Vane *et al.* [25]. The principle underlying this immunological quantification method is a competition between unlabelled (standard or sample) and labelled molecules of a PGE_2 compound for the binding sites of an anti-PGE_2 anti-serum. The more unlabelled molecules present in the sample, the more labelled molecules will be displaced from the antibody binding sites. The extent of this replacement is then quantified. One hundred microlitres of tissue extract was mixed with 450 µl of 30 µM arachidonic acid (AA) diluted in phosphate-buffered saline (PBS). AA is metabolized by COX in the sample when warmed at 37°C for 30 min. The samples were boiled for 10 min to stop the reaction and centrifuged at 10 000 *g* for 15 min. One hundred microlitres of either the samples or the standard was mixed with 500 µl of diluted anti-serum. The tubes were vortexed and incubated at 100 µl for 30 min and then 100 µl of diluted radioactive PGE_2 was added and the tubes were vortexed and incubated for 1 h at 4°C. Two hundred microlitres of cold dextran charcoal suspension was added to each tube and the tubes were vortexed and incubated on ice for 10 min. The tubes were centrifuged at 2000 *g* for 10 min at 4°C and the supernatant was removed from each tube and added to scintillation tubes containing 4 ml of scintillation fluid and the radioactivity was counted using a scintillation counter. The PGE_2 concentration in the sample was worked out from the PGE_2 standard curve and the results were further standardized according to the protein concentration. Results are expressed as ng PGE_2 per mg protein.

Results

Immunolocalization of COX-1

Immunoperoxidase staining showed that COX-1 was present in both the epidermis and the dermis, both in normal human skin and in chronic venous ulcers (Figures 1A and 1B). In normal skin, COX-1 was mainly present in vascular endothelial cells and macrophage-like cells (Figures 2A and 2B). In chronic venous ulcer biopsies, dense immunoreactivity for COX-1 was seen at the ulcer base and edge (Figure 1B). Higher magnification showed that large numbers of blood vessels and inflammatory cells in chronic venous leg ulcers showed dense immunoreactivity for COX-1 (Figures 2C and 2D). This staining was absent from the negative control in which the primary antibody was omitted (data not shown).

Immunolocalization of COX-2

COX-2 was hardly visible in normal human skin, with little seen in the basal layer of the epidermis and in the dermis (Figure 1C). In chronic venous ulcer tissues, COX-2 showed dense immunoreactivity in both the epidermal and the dermal cells at the ulcer edge and ulcer base (Figure 1D). In the chronic venous leg ulcers, COX-2 was localized to blood vessels and inflammatory cells (Figures 1E, 2E, and 2F). At higher magnification, COX-2 immunoreactivity was present in macrophages, fibroblasts, and mast cells (Figure 1F). This staining was absent from the negative control in which the primary antibody was omitted (data not shown).

Triple immunofluorescence

Triple immunofluorescence showed that most of the COX-2 immunoreactivity was present in macrophages (Figure 1G) and that while a large number of inflammatory cells expressed COX-2, only a few expressed COX-1 (Figure 1H).

Western blot analysis of COX-1 and COX-2

Western blot analysis was used to investigate the protein levels of each isoform, both in normal skin and in chronic venous ulcers (Figure 3). COX-1 was expressed both in normal skin and in chronic venous ulcers. There was an increase in COX-1 protein levels in chronic venous ulcers by comparison with normal human skin.

Western blot analysis for COX-2 in normal human

Figure 1. The localization of COX-1 and COX-2 using immunoperoxidase staining is shown in normal human skin and chronic venous leg ulcers. (A) COX-1 was present in both epidermis and dermis of normal skin. Some sites which showed COX-1 immunoreactivity were magnified to show the details in Figure 2A. (B) In chronic venous ulcers, COX-1 was present in both epidermis and dermis. It was mainly localized to blood vessels (arrows) in the ulcer base (UB). (C) Faint staining for COX-2 was present both in the epidermis and in the dermis of normal skin. (D) In chronic venous leg ulcers, dense staining for COX-2 was present both in the epidermis and in the dermis. In the ulcer base, COX-2 was localized to large numbers of inflammatory cells (arrows). (E) Large numbers of blood vessels and inflammatory cells showing dense COX-2 immunoreactivity were present in chronic venous ulcers. (F) Enlargement of some COX-2-positive cells seen in E, showing dense COX-2 immunoreactivity in endothelial cells, macrophage-like cells (arrows), mast cells (arrow-heads), and fibroblast-like cells (insets). (G) Co-localization of COX-2 in macrophages. COX-2 was localized by using a specific primary antibody and secondary TRITC-labelled antibody (red). Macrophages were localized using CD68 and then secondary biotinylated antibody followed by streptavidin–fluorescein (green). Hoechst 33258 was used as a nuclear counterstain (blue). Large numbers of macrophages were seen; some expressed COX-2 (orange) and some did not (green). COX-2 was also seen in vascular endothelial cells (arrow). (H) Co-localization of COX-2 and COX-1. COX-2 was localized by using a specific primary antibody and secondary FITC-labelled antibody (green). COX-1 was localized using a specific COX-1 antibody and TRITC-conjugated secondary antibody (red). Hoechst No. 33258 was used as a nuclear counterstain (blue). While a large number of cells showed expression of COX-2, only a few showed expression of COX-1 along with COX-2 (yellow cells). COX-2 was also seen in vascular endothelial cells. Scale bars = (A, C) 400, (B, D) 200, (E) 100, and (F–H) 50 µm

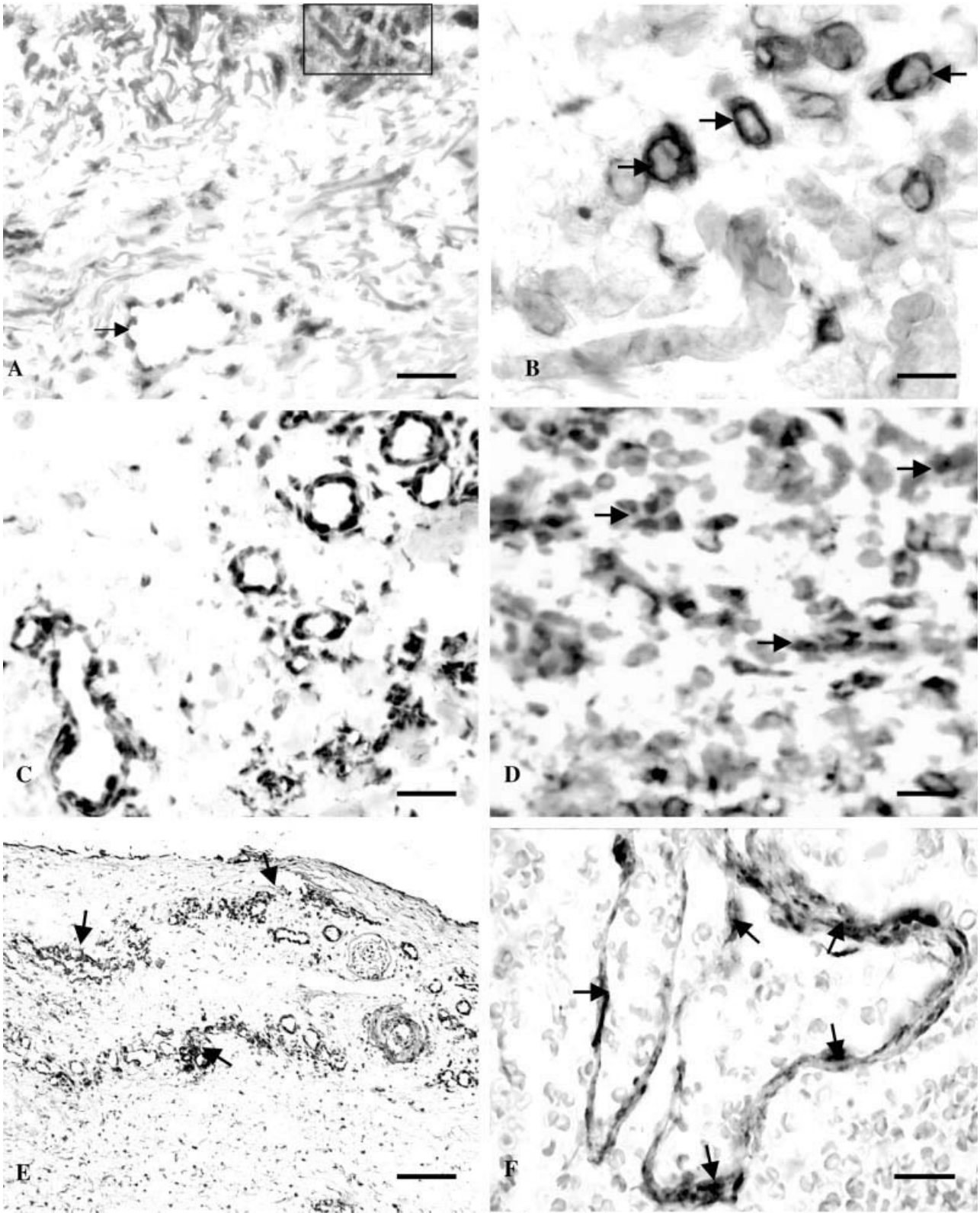


Figure 2. (A) Higher magnification showed COX-1 in the vascular endothelial cells (arrow) and some nearby inflammatory cells. (B) Enlargement of the stained cells seen in A (rectangle) showing dense perinuclear localization of COX-1 in macrophage-like cells (arrows). (C) COX-1 was present in vascular endothelial cells of a large number of vessels seen in chronic venous ulcers. (D) Large numbers of inflammatory cells in chronic venous ulcers showed dense perinuclear localization of COX-1 (arrows). (E) Chronic venous ulcer showed large numbers of blood vessels in the ulcer base. COX-2 immunoreactivity was present in vascular endothelial cells and in the perivascular inflammatory cell infiltrate (arrows). (F) COX-2 was localized in vascular endothelial cells (arrows) of two large vessels seen in the ulcer base within an area containing many red blood cells. Scale bars = (A) 100, (B) 10, (C, D) 50, (E) 200, and (F) 20 μm

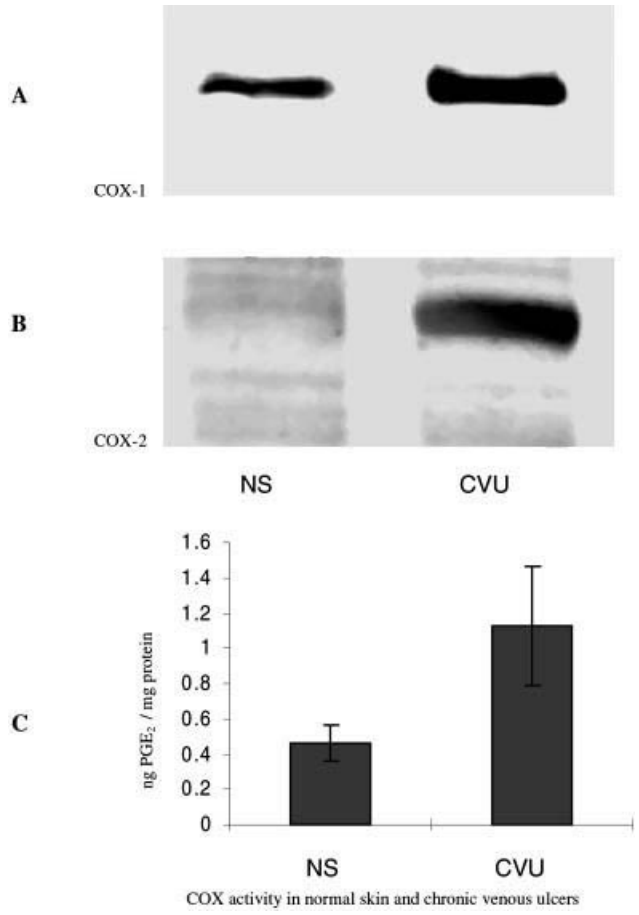


Figure 3. Western blot analysis for COX-1 and COX-2 both in normal skin (NS) and in chronic venous ulcer (CVU). An equal amount of protein from both normal skin and chronic venous ulcer biopsies was loaded in each lane. The proteins were transferred to a blotting membrane and then stained using COX-1 and COX-2 antibodies. Both COX-1 and COX-2 bands were detected at approximate molecular weights 70 kD. (A) The COX-1 band was present both in the normal skin and in the chronic venous ulcer biopsies, but it was denser and thicker in the latter. (B) Chronic venous ulcer biopsies showed expression of COX-2 as indicated by the appearance of a band at approximate molecular weight 70 kD. This was not present in the lane containing normal skin. (C) The activity of COX in normal human skin and chronic venous ulcer biopsies was measured as the ability of the homogenized tissues to metabolize arachidonic acid into prostaglandin E_2. There was a significant increase of COX-2 activity in chronic venous ulcer biopsies ($p < 0.05$) when compared with normal human skin. In both cases, the number of samples was 7

skin and chronic venous ulcer tissues showed a high level of COX-2 expression in chronic venous ulcers, as seen by the thick dense band. This was different from normal skin, which did not express COX-2.

COX activity in normal skin and chronic venous ulcers

Analysing the activity of COX in normal human skin and in chronic venous ulcers using a COX radioimmunoassay showed significant ($p < 0.05$) up-regulation of COX activity in chronic venous ulcer tissues when compared with normal human skin (Figure 3). This assay measures the total COX activity without discrimination between the two isoforms. However, data from the immunostaining and western blot analysis suggest that most of this COX activity would have been due to COX-2.

Discussion

Using immunocytochemistry, we have shown a wide distribution of COX isoforms in chronic venous ulcers, in particular the inducible isoform COX-2. COX-2 was present in inflammatory cells characteristic of chronic inflammation, such as macrophages and mast cells and also in endothelial cells, fibroblasts, and keratinocytes. Western blot analysis confirmed the immunocytochemistry since it showed *de novo* appearance of COX-2 and an increase in the level of COX-1 in chronic venous ulcers. The COX radioimmunoassay revealed that COX activity was significantly up-regulated in chronic venous ulcers.

In a number of animal models of inflammation, COX-2 has been found to be up-regulated [26,27]. In this study, we have shown that both COX-1 and COX-2 are up-regulated in chronic venous ulcers, but COX-2 was still the predominant isoform. Up-regulation of COX-1 could be due to the increase in the number of sites which expressed it, such as blood vessels and macrophages. Recently, several studies have shown that COX-1 may have a role during inflammation [28,29]. Thus, in chronic venous ulcers, both COX-1 and COX-2 seem to operate and both may have a role in the development and maintenance of the pathology.

Keratinocytes at the ulcer edge strongly express both COX-1 and COX-2. Prostaglandin (PG) production by keratinocytes is known to be growth promoting for the epidermis [30]. Healing of partial-thickness burns takes three times longer in essential fatty acid-deficient rats, which are devoid of fatty acid precursors for PG synthesis [31]. Thus, the synthesis of PGs by the epidermis may be enhanced in wound healing and disease states where epidermal continuity is disrupted, such as in chronic venous ulcers.

LPS, inflammatory cytokines, and nitric oxide (NO) could be the main inducers for COX-2 in chronic venous ulcers. COX-2 is rapidly induced during inflammation by IL-1α, IL-1β, and TGFβ1 [17–19,32]. NO activates COX-1 and COX-2, resulting in enhanced production of PGs [33]. Recently, we have shown up-regulation of NOS both in diabetic foot ulcers [34] and in chronic venous ulcers [35]; and in acute incisional wounds, we have shown that iNOS and COX-2 are predominantly expressed during the inflammatory phase of wound healing (unpublished observation). Thus, in chronic venous ulcers, iNOS could induce COX-2 and both pathways might operate together to amplify the inflammatory response, which is achieved

by a synergic interaction between NO and PGs on blood flow and microvascular permeability [36].

The induction of COX-2 in chronic venous ulcers implies a role for this isoform in inflammation. Elevated levels of PGs have been found in numerous human inflammatory conditions including rheumatoid arthritis, osteoarthritis, psoriasis, allergic eczema, gout, and ulcerative colitis [37]. In a number of animal models of inflammation, PGE_2 was found to be the major metabolite of arachidonic acid [38]. The production of PGE_2 at sites of injury is pro-inflammatory [39]. In the murine air pouch model of chronic granulomatous inflammation, COX activity rose progressively in the chronic phase of the inflammatory response. COX activity was 2–3 times greater than in the acute stage and this profile of activity was mirrored by COX-2 protein levels [26]. A number of COX metabolites have been measured in this model and PGE_2 was always proportionally greater at all time points measured. Thus, in chronic venous ulcers, pro-inflammatory PGE_2 could be the main COX-2 product.

Although expression of COX-2 has been considered an important determinant of the cytotoxicity associated with inflammation [20,40], the factors responsible for the cytotoxicity of COX-2 have not been fully defined. There are several possibilities by which COX-2 could produce tissue damage in chronic venous ulcers: the production of pro-inflammatory prostanoids which mediate tissue damage [20]; the production of reactive oxygen species by the peroxidase step of the COX reaction, in which PGG_2 is converted to PGH_2 [41], with these free radicals mediating tissue damage [42]; and the induction of apoptosis, where COX-2 could contribute to cell death by inducing apoptosis [43].

Up-regulation of COX-1 in chronic venous leg ulcers may have a role in the pathogenesis of this disease. Prostacyclin is the main PG product from the endothelial cells [44] and it is mainly produced by COX-1 [29,45]. Recently, prostacyclin has been found to enhance wound healing through promoting angiogenesis and enhancing blood flow [46]. VEGF, which is a potent angiogenic factor contributing to angiogenesis in chronic venous ulcers [47], induces prostacyclin release [48]. Thus, VEGF could produce its angiogenic effect in chronic venous ulcers through inducing prostacyclin release. PG synthesis in microvascular endothelial cells may play a role in maintaining vessel patency by inhibiting intramural platelet aggregation and by the production of prolonged vasodilatory effects [44]. Thus, up-regulation of COX-1 and the subsequent release of prostacyclin in chronic venous ulcers may represent an endogenous defence mechanism against thrombosis and endothelial damage; it could also contribute to the angiogenesis associated with chronic venous leg ulcers and its inhibition could affect local blood flow.

It is well established that COX is the target for the therapeutic effects of NSAIDs and it is equally well documented that NSAIDs have varying degrees of efficacy [23]. COX-1 is important for many physiological functions in the body, such as the production of the thrombogenic thromboxane A_2 in platelets, anti-thrombogenic PGI_2 in vascular endothelial cells, cytoprotective PGs in gastric mucosa, and vasoactive PGs for the maintenance of renal blood flow [13]. Hence, the removal of these cytoprotective effects may account for the gastric and renal side-effects associated with the use of NSAIDs. Moreover, in chronic venous ulcers, COX-1 could also be important for controlling angiogenesis, maintaining blood vessel patency, and enhancing local blood flow; inhibition of this isoform could increase the local hypoxia and ischaemia associated with the disease. From this study, it is clear that the main isoform involved in inflammation in the chronic venous ulcer is COX-2 and PGE_2 may be the main product of this enzyme. Thus, selective inhibition of COX-2 might be beneficial in selectively reducing PGE_2 production in chronic venous ulcers and overcoming its pro-inflammatory effects. This would allow treatment of the inflammation associated with chronic venous ulcers without affecting other PGs produced, either locally or systemically, and thus without affecting the local vascularity of the ulcer and without renal and gastric side-effects.

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