DISTRIBUTION OF CYCLOOXYGENASE ISOFORMS IN MURINE CHRONIC GRANULOMATOUS INFLAMMATION.
IMPLICATIONS FOR FUTURE ANTI-INFLAMMATORY THERAPY

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SUMMARY

Inhibition of the enzyme cyclooxygenase (COX) is the basis for the mechanism of action of non-steroidal anti-inflammatory drugs (NSAIDs). COX exists as a constitutive (COX-1) and a mitogen-inducible (COX-2) isoform. The relative contribution of COX-1 and COX-2 to inflammation is unknown. This study investigated COX activity and the distribution of COX-1 and COX-2 during the development of a murine air pouch model of chronic granulomatous inflammation. COX activity progressively rose and was maximal at day 14. Of the COX metabolites measured, PGE₂ was the greatest >6-keto PGF₁₀, >TXB₂, >PGF₂α. By day 7, COX-2-labelled fibroblast- and macrophage-like cells were observed and their number and distribution increased with time. At all time points, endothelial cells of venules in the loose connective tissue of the dermis showed immunoreactivity for COX-2. After day 14, labelling of capillaries in the granuloma was also observed. This study is the first to show that COX-2 is the predominant COX isoform in all stages of the inflammatory response. These results suggest that selective inhibition of COX-2 may prove more beneficial, with fewer gastric and renal side-effects, than existing NSAID therapy for the treatment of chronic inflammatory diseases.

KEY WORDS—cyclooxygenase; chronic inflammation

INTRODUCTION

Eicosanoids are mediators involved in a multitude of diverse physiological and pathophysiological processes, particularly inflammation. They are formed by the action of cyclooxygenase (COX) on membrane-derived arachidonic acid.¹ The inhibition of COX is the basis for the mechanism of action of the non-steroidal anti-inflammatory drugs (NSAIDs)². It is well established that COX from various tissues is differentially inhibited by NSAIDs, both in vitro³ and in vivo⁴. A possible explanation for these observations may be attributed to the recent finding that two isoforms of COX exist, a constitutive COX (COX-1) and a mitogen-inducible COX (COX-2). The COX-1 isoform encodes a 2·8 kb mRNA and the primary structure of the enzyme has been determined from the cDNA of sheep⁵,⁶ mice⁷, and humans⁸. The COX-2 isoform encodes a 4 kb mRNA and has been reported in Rous sarcoma virus-transformed chick embryo fibroblasts⁹ and phorbol myristate acetate-stimulated 3T3 fibroblasts¹⁰ (for a review of mitogen-inducible COX see ref. 11).

We have recently demonstrated that NSAIDs can selectively inhibit either COX-1 or COX-2.¹² Thus, depending on the predominant isoform of COX in various tissues and pathologies, NSAIDs may have differential inhibitory effects. It is essential, therefore, that the relative contribution of
COX-1 and COX-2 in inflammation be determined. This study has investigated the biochemical activity of COX and the distribution of COX-1 and COX-2 during the acute, chronic, and resolving phases of the inflammatory response in a murine air pouch model of granulomatous inflammation.

MATERIALS AND METHODS

Female Tuck original mice (Tuck and son Ltd., Rayleigh, Essex, U.K.) weighing 30 ± 2 g were used in these studies. In the first set of experiments, the time course of granuloma development, as assessed by tissue dry weight, was determined. The second group of studies evaluated the biochemical activity of COX during the formation of the granulomatous tissue. The temporal and cellular distributions of COX-1 and COX-2 were localized in the third group.

Formation of the murine air pouch

The mice were anaesthetized with 0.5 ml Hypnol/Hynovel intra peritoneally (1 part Hypnorm, 1 part Hynovel, 2 parts distilled water). Three millilitres of air was injected into the dorsal subcutaneous tissue at day 0, followed 24 h later by injection of 0.5 ml of 0.1 per cent v/v croton oil in Freund's complete adjuvant [5 mg/ml Mycobacterium tuberculosis strain C, DT, and PN (Central Veterinary Laboratory, Weybridge, Surrey, U.K.) in Freund's incomplete adjuvant]. The animals were killed by ether overdose at 6, 12, and 24 h and at 3, 5, 7, 14, and 21 days after injection of the irritant. The tissues were prepared as detailed below.

Assessment of granulomatous tissue dry weight

Prior to 3 days, no extractable granuloma had developed; samples were therefore taken at successive intervals from the first time point at which a granuloma could be dissected, i.e., 3, 5, 7, 14, and 21 days. The granulomatous tissue lining the air pouch cavity was dissected and the dry weight obtained.

Measurement of COX activity

Samples of granulomatous tissue were taken from the ventral region of the air pouch at 3, 5, 7, 14, and 21 days, for the measurement of COX activity in the chronic and resolving phases of inflammation. Tissues were immediately homogenized at 4°C in Tris buffer (50 mM, pH 7.4) containing phenylmethylsulphonyl fluoride (1 mM), pepstatin A (1.5 mM), and leupeptin (0.2 mM). The protein concentration in the homogenates was measured using the Bradford assay. Homogenates were incubated at 37°C for 30 min in the presence of 30 µM arachidonic acid. The samples were boiled and centrifuged at 10,000 g for 30 min. The concentrations of prostaglandin E₂ (PGE₂), 6-keto PGF₁α, thromboxane (TXB₂), and PGF₂α present in the supernatant were measured by radioimmunoassay (radio isotopes and eicosanoid antibodies were from Amersham International plc, Amersham, U.K.). The results are expressed as ng eicosanoid/mg protein per 30 min.

Antibodies

The antibodies used for the radioimmunoassay were polyclonal rabbit anti-PGE₂, polyclonal rabbit 6-keto PGF₁α, polyclonal rabbit anti-TXB₂, and polyclonal rabbit anti-PGF₂α and were obtained from Sigma, Poole, U.K. The PGE₂ antibody does not discriminate between PGE₂ and PGE₁, has a detection limit of 15 pg PGE₂, and has the following cross-reactivity to other eicosanoids: >10 per cent PGE₁, 20 per cent PGA₁; 33 per cent PGA₂; 21 per cent PGD₂. The antibody for 6-keto-PGF₁α has a detection limit of 10 pg 6-keto-PGF₁α and has the following cross-reactivity: 22 per cent PGE₂; 16 per cent PGF₂; and >10 per cent for all other eicosanoids. The antibody for TXB₂ has >0.5 per cent cross-reactivity to other eicosanoids and has a detection limit of 5 pg. The antibody for PGF₂α has a detection limit of 5 pg, is 100 per cent cross-reactive with PGF₁α, and >0.01 per cent with other eicosanoids.

Two sources of antibodies to COX-2, both polyclonal rabbit anti-murine, were used in this study. The first antibody (a gift from Professor K. Wu, Houston, TX, U.S.A.) was raised to the 17 amino acid peptide Cys–Tyr–Ser–His–Ser–Arg–Leu–Asp–Asp–Asp–Asn–Pro–Thr–Val–Leu–Ile–Lys, which corresponds to the carboxyl terminal unique to the COX-2 protein, showing no cross-reactivity to COX-1. Similarly, the second COX-2 antibody (Cayman Chemical Co., Ann Arbor, MI, U.S.A.) cross-reacted with COX-2 from many sources but not COX-1.
The COX-1 antibody, a polyclonal rabbit anti-ovine (a gift from Professor K. Wu, Houston, TX, U.S.A.) was raised to the whole COX-1 protein and showed approximately 10 per cent cross-reactivity to COX-2. However, used at an appropriate dilution, this antibody recognized a band of approximately 70 kD by Western blot analysis in bovine aortic endothelial cells (COX-1 specific) that was not recognized by antibodies to COX-2. In contrast, COX-2 protein was recognized by both COX-2 antibodies in extracts of lipopolysaccharide-induced J774.2 macrophages.12

**Immunocytochemistry**

The skin was shaved and full thickness biopsies of control skin and of air pouch skin were taken at 6, 12, and 24 h and at 3, 5, 7, 14, and 21 days after injection of the irritant. Tissue samples from each time point were snap-frozen in n-hexane in a bath of liquid nitrogen. Ten-micrometer cryostat sections were cut through the skin and granulomatous tissue, thaw-mounted on poly-L-lysine coated slides, and air-dried. Prior to immunolabelling, the sections were fixed in either acetone for 10 min or 4 per cent paraformaldehyde in 0·1 M phosphate buffer for 1 h. Endogenous peroxidases were quenched with 0·3 per cent H2O2 in methanol and sections washed with 0·1 per cent Triton X100 in phosphate-buffered saline. Non-specific binding of IgGs was blocked using normal goat serum 1:50 in 0·1 per cent essentially globulin-free bovine serum albumin. The sections were incubated with polyclonal rabbit anti-ovine COX-1 (1:300) or polyclonal rabbit anti-murine COX-2 (1:400) overnight at 4°C, washed, and incubated for a further 30 min with biotinylated goat anti-rabbit secondary antibody. Following a further 30 min incubation with Vectastain ABC horseradish peroxidase (Vector Laboratories, Peterborough, U.K.), the substrate (0·05 per cent 3,3-diaminobenzidine tetrahydrochloride in Tris buffer) was added for the appropriate time period (5–10 min). This resulted in positive immunoreactivity labelling brown. Primary antiserum was replaced with normal serum, as a negative control.

**Statistics**

Results are expressed as the mean ± standard error of the mean (SEM) for at least n=4–10 separate animals for each time point.

**RESULTS**

**Granulomatous tissue dry mass**

At day 3, the dry weight of the granulomatous tissue was 47·7 ± 3·8 mg. This increased to 66·9 ± 3·6 mg at day 5 and reached a peak dry weight at day 7 (120·9 ± 4·4 mg). The dry weight of the tissue fell to 76·8 ± 3·3 mg at day 14 and by day 21 had fallen to 69·6 ± 6·5 mg (Fig. 1).

**Assessment of COX activity**

Homogenates of granulomatous tissue contained increasing levels of COX activity, reaching a peak at day 14 for all the eicosanoids measured. The generation of PGE2 was proportionally greater than for 6-keto PGF1α>TXB2>PGF2α, with a maximum at day 14 of 56·3 ± 5·2, 42·1 ± 4·5, 15·1 ± 2·7, and 7 ± 0·8 ng/mg protein per 30 min, respectively (Fig. 2).

**Histology**

At day 3, the granulomatous tissue comprised a dense collection of cells, predominantly polymorphonuclear neutrophils (PMNs). Smaller numbers of macrophages and lymphocytes were also observed, with occasionally fibroblasts and capillaries, surrounded by a fibrinaceous extracellular
matrix (ECM). Numerous PMNs and macrophages were present in the loose connective tissue of the dermis and occasional degranulated mast cells were seen. By day 21, there was an approximately three-fold increase in granulomatous tissue, with noticeable regional variations, in terms of both cell populations and ECM composition. The region of granulomatous tissue juxtaposed to the skeletal muscle was highly vascularized with capillaries and venules within a fibrotic matrix. Inferior to this area was a region of active fibrogenesis with a high collagen content, when stained with Van Gieson (data not shown). Underlying this region were fibroblasts surrounded by closely packed inflammatory cells, mainly macrophages. The region adjacent to the pouch cavity was essentially the same, in terms of cell populations and ECM composition, as the granulomatous tissue described at day 3. Small numbers of eosinophils and mast cells, some degranulated, were observed at all time points, widely distributed throughout the granulomatous tissue.

**Immunolocalization of COX-1 and COX-2**

In normal skin samples, no COX-1 or COX-2 immunoreactivity was observed. At all time points, the smooth muscle of arterioles in the dermis showed low-level labelling for COX-1 (data not shown). Nerve bundles en passage, but not innervating the dermis and granulomatous tissue, also showed low levels of immunoreactivity for COX-1. The specific localization to nerves was confirmed using serial sections labelled with the pan neuronal marker, protein gene product 9.5. Cells in the granulomatous tissue showed no COX-1 labelling at any time point. Although the COX-1 antibody used has 10 per cent cross-reactivity to COX-2, the immunolocalization profile obtained with the two antibodies was distinct and can therefore be ascribed to specific recognition of their respective antigens.

Nerve bundles traversing the dermis showed low-level labelling for COX-2 at all time points. At day 3, with the exception of an occasional macrophage-like cell, all cellular elements of the granulomatous tissue were negative for COX-2. By day 7, fibroblast- and macrophage-like cells, intensely labelled for COX-2, could be discerned in the connective tissue of the dermis and the region of granulomatous tissue juxtaposed to the skeletal muscle. The cytoplasm of a minority of these cells appeared granular. The number of COX-2 immunopositive cells increased with time and by days 14–21 they were increasingly associated with areas of active fibrogenesis in the mid-region of the granulomatous tissue (Figs 3a and 3b). A number of these cells had a granular cytoplasm as shown in Fig 3d. At all time points, endothelial cells of venules in the loose connective tissue of the dermis showed low-level immunoreactivity for COX-2. Post day 14, labelling of endothelial cells of capillaries was also detected in the most fibrotic part of the granulomatous tissue juxtaposed to the skeletal muscle (Fig. 3c). Endothelial cells of newly forming blood vessels showed no immunoreactivity for COX-2 at any time point. The fibroblast- and macrophage-like cells immunopositive for COX-2 formed a small proportion of the total number of these cells within the granulomatous tissue. In Fig 3e, an immunopositive macrophage and fibroblast but an unlabelled PMN are shown. Lymphocytes, PMNs, epidermal cells, eosinophils, and mast cells were negative for COX-1 and COX-2 at all time points.

**DISCUSSION**

It was proposed by Needleman et al. that increased COX expression is the key factor in driving the increase in PG production at an
Fig. 3—Distribution of COX-2 immunolabelling in paraformaldehyde-fixed (a, b, and d) and acetone-fixed (c and e) granulomatous tissues at various times of development. The specific localization of COX-2 in fibroblast- and macrophage-like cells (arrow) in a 21-day granulomatous tissue are shown in a and at higher magnification in b. The majority of these cells, some of which had a granular cytoplasm (d), were located in areas of active fibrogenesis in the mid-region of the granulomatous tissue. Low-level labelling was associated with endothelial cells of capillaries in the most fibrotic area, juxtaposed to the skeletal muscle, of a 14-day granulomatous tissue (c). In e, a COX-2 immunopositive macrophage (*) and fibroblast (arrow-head), but negatively labelled PMN (*), are illustrated. Magnification: (a) × 100; (b) × 200, (c, d, e) × 500
inflammatory site. The present studies were designed to assess the relative contribution of COX-1 and COX-2 in all stages of the inflammatory response, using the murine chronic granulomatous tissue air pouch model. We have shown previously, in this model, that there is an initial peak of COX activity at 24 h, followed by a second peak at 14 days. Here, these studies have been extended to demonstrate that during the chronic and resolving stages of inflammation, PGE$_2$ is the major COX metabolite $>6$-keto PGF$_{1a}$$>$$TXB_2$$>$$PGF_{2a}$. Furthermore, the cellular sources of this activity have been identified.

At day 3, when the predominant cell types are the PMN and macrophage, no cells within the granulomatous tissue showed immunoreactivity for COX-2, with the exception of the occasional macrophage-like cell. However, small numbers of macrophage- and fibroblast-like cells positively labelled for COX-2 were first seen at day 5, their numbers increasing and reaching a peak at 14–21 days. The level of immunolabelling corresponds to the rise in COX activity. The findings of this study are similar to those of Sano et al., who reported that fibroblast-like cells, blood vessels, and mononuclear cells in rheumatoid arthritic pannus tissue exhibited immunoreactivity for COX. However, the antibody used by Sano et al. did not distinguish between COX-1 and COX-2. In the present study, COX-2 immunoreactivity, at later time points, was observed in only a small proportion of endothelial cells, macrophage-like cells, and fibroblast-like cells. This finding is not surprising, considering that in normal tissues only 15 percent of cells showed COX immunoreactivity, again using a non-specific COX antibody. COX-2 labelling of endothelial cells of mature blood vessels in the granulomatous tissue may be a facet of maturity, as endothelial cells of newly forming blood vessels were negative. Monocytes/macrophages may exhibit the same phenomenon. For example, as monocytes in vitro mature into macrophages, their elaboration of PGs increases.

This study has also shown COX-1 and COX-2 immunoreactivity to be associated with nerves. COX-2 expression has been demonstrated previously in central neurones. The finding that COX-2 was observed in peripheral nerves may be due to their passage through a chronically inflamed area. No innervation of the granulomatous tissue was seen at any time point.

Although eicosanoid production is ubiquitous throughout the body, the specific product and subsequent effect may vary due to cell-specific isomerases and reductases. For example, the potent platelet aggregatory factor TXA$_2$ is the major arachidonic acid metabolite of platelets. Endothelial cells from large vessels produce PGI$_2$ (prostacyclin), whereas in contrast, capillary endothelial cells produce mainly PGF$_{2a}$. PMNs produce primarily the 5-lipoxygenase product leukotriene B$_4$ (LTB$_4$), which stimulates PMN migration, adherence, and degranulation, whilst the major COX product of mast cells is PGD$_2$ and PGE$_2$ in fibroblasts and macrophages. Hence, as cell types can differentially produce arachidonic acid metabolites, their individual levels may fluctuate during inflammation depending on the predominant cell. This was demonstrated in acute inflammation by Simmons et al., using the subcutaneous sponge implant, where it was found that TXB$_2$, LTB$_4$, and PGE$_2$ were present in the early exudate, whereas after 24 h only PGE$_2$ remained.

Eicosanoid production is induced by a variety of agents including bradykinin, histamine, arginine vasopressin, angiotensin II, and thrombin. Cytokines including platelet-derived growth factor (PDGF), interleukin-1 (IL-1), epidermal growth factor (EGF), basic fibroblast growth factor, transforming growth factor beta (TGF/β), and tumour necrosis factor alpha (TNFα) also induce eicosanoid production in a number of cell types by increasing COX expression. Previously, we have shown that the levels of IL-1α, IL-1β, PDGF, EGF, and TNFa were greatest at the early stages of granulomatous tissue formation. All of these cytokines, either alone or acting in synergy, are thus possible candidates for the early induction of COX. However, immunoreactive levels of PDGF, EGF, IL-1α, IL-1β, and TNFα declined rapidly, whereas TGF/β levels increased, reaching a peak at 14 days, coincident with the peak of COX activity and COX-2 protein. The increased COX activity seen at day 14 may therefore be due to the presence of high levels of immunoreactive TGF/β. In most human tissues, COX-1 and COX-2 mRNAs are constitutively expressed. However, COX-2 may be selectively increased on exposure to cytokines. Thus, the observation that immunoreactive COX-2 was only detected in a small sub-population of cells may be a reflection of exposure to those cells to a particular cytokine milieu or may be an aspect of maturity.
The peak of COX activity at 24 h\textsuperscript{15} is in keeping with the known pro-inflammatory action of COX metabolites in acute inflammation. However, the second larger peak of COX activity at 14 days is inconsistent with a pro-inflammatory role for the eicosanoids, as the inflammation within the granulomatous tissue starts to resolve after day 7 (see Fig. 1). The generation of PGE\textsubscript{2} at sites of injury is pro-inflammatory by virtue of its ability to cause vasodilatation\textsuperscript{41} and to potentiate pain.\textsuperscript{42} However, PGE\textsubscript{2} also has anti-inflammatory properties, as it inhibits cytokine production\textsuperscript{43,44} and proliferation of mesangial cells,\textsuperscript{45} fibroblasts,\textsuperscript{46} macrophages,\textsuperscript{47} and lymphocytes.\textsuperscript{48} In vivo, PGE\textsubscript{2} will suppress adjuvant-induced arthriti\textsuperscript{49,50} and the injection of PGE\textsubscript{1} into the rat air pouch inhibits PMN influx and exudate volume.\textsuperscript{51} PGI\textsubscript{2} (as measured by the stable degradation product 6-keto PGF\textsubscript{1a}) was the second major metabolite of COX in comparison with TXB\textsubscript{2} and PGF\textsubscript{2a}. PGI\textsubscript{2} has anti-inflammatory actions similar to those of PGE\textsubscript{2}. It inhibits platelet aggregation,\textsuperscript{52} PMN adherence,\textsuperscript{53} and lymphocyte proliferation.\textsuperscript{48} In the chronic and resolving stages of the murine chronic granulomatous tissue air pouch model, the predominant COX metabolites were PGE\textsubscript{2} and PGI\textsubscript{2}, which have profound anti-inflammatory actions. Therefore, as the inflammation progresses, due to time-dependent influx of different cell types, the production of eicosanoids and their ultimate effects on the inflammatory response may change. Thus, in acute inflammation, platelets and PMNs predominantly produce the pro-inflammatory eicosanoids TXA\textsubscript{2} and LTBA\textsubscript{4}.\textsuperscript{20,23} The subsequent transition to chronic inflammation, with the accompanying influx of macrophages, fibroblasts, and endothelial cells which produce primarily PGE\textsubscript{2} and PGI\textsubscript{2}, may switch to anti-inflammatory action. Cell-specific COX-2-derived eicosanoid production may therefore lead to selective modulation of the inflammatory process, depending on the major cell type.

This study is the first to show the cellular distribution of COX isoforms in a model of inflammation and has demonstrated that COX-2 is the predominant isofrom in all stages of the inflammatory process. Selective inhibition of COX-2 may therefore prove more beneficial, with fewer gastric and renal side-effects, than existing NSAIDs for the treatment of inflammatory diseases. However, the finding of high levels of COX-2 activity at 14 days, a time when the inflammation has started to resolve, may indicate that selective antagonism of COX-2 in the chronic and resolving phases of the inflammatory response may inhibit the immunomodulatory and anti-inflammatory properties of PGE\textsubscript{2} and PGI\textsubscript{2}.

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