Expression of cyclooxygenase 1 and 2 by human gastric endothelial cells

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Abstract

Background—Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit cyclooxygenase (COX) and cause gastric ulceration. NSAIDs also impair granulation tissue angiogenesis and healing of established gastric ulcers in humans. The mechanism whereby NSAIDs inhibit granulation tissue angiogenesis is unknown but may involve inhibition of either or both COX isoforms (COX-1 and COX-2).

Aims—To investigate COX expression by human gastric endothelial (HuGE) cells during angiogenesis in vitro.

Methods—COX-1 and COX-2 expression by HuGE cells was investigated by western blot analysis, indirect immunofluorescence, reverse transcriptase polymerase chain reaction, and measurement of prostaglandin E\(_2\) synthase activity.

Results—Under normal culture conditions (30% serum), HuGE cells expressed COX-1 and low levels of COX-2. COX-2 expression was induced in HuGE cells in both angiogenesis models. Prostaglandin E\(_2\) production by HuGE cells increased significantly by a selective COX-2 inhibitor (NS-398).

Conclusion—Angiogenesis by HuGE cells in vitro was associated with induction of functional COX-2 expression. A selective COX-2 inhibitor significantly decreased HuGE cell angiogenesis on basement membrane matrix. Extrapolation of these data to human gastric ulcer angiogenesis in vivo suggests that selective COX-2 inhibitors could delay gastric ulcer healing to the same extent as traditional NSAIDs which are non-selective COX inhibitors.

Keywords: angiogenesis; cyclooxygenase; endothelial cell; gastric mucosa; gastric ulcer; prostaglandins

Prostaglandins (PGs) are believed to play an important role in maintenance of human gastric mucosal homoeostasis. Inhibition of endogenous gastric mucosal PG synthesis by non-steroidal anti-inflammatory drugs (NSAIDs) is thought to underlie the ulcerogenic activity of such drugs and administration of exogenous PGs protects gastric mucosa from a variety of insults in animal models.

Misoprostol (a PGE\(_1\) analogue) prevents NSAID associated peptic ulceration and its complications in humans. PGs are synthesised from arachidonic acid by cyclooxygenase (COX) via the unstable prostaglandin intermediate PGH\(_2\), which, in turn, is converted into other PG species such as PGE, by the action of specific PG synthases, for example, PGE synthase. Two isoforms of COX are known to exist, namely a constitutive isoform, COX-1, and a mitogen inducible isoform, COX-2. Current dogma is that COX-1 is responsible for PG synthesis in normal gastric mucosa in order to maintain mucosal homoeostasis and that COX-2 is expressed by normal gastric mucosa at low levels, with induction of expression during ulcer healing or following endotoxin exposure. Iseki has reported the localisation of COX-1 and COX-2 to mucous cells of normal rat gastric mucosa but these data have not been confirmed by others who have been unable to detect COX-2 immunoreactivity in normal rat stomach. There are no published studies of COX localisation in human gastric mucosa.

Gastric microvascular endothelial cells are a possible important source of human gastric mucosal PGs. Capillary endothelial cells are a major site of PGE production in canine fundic mucosa and COX-1 has been localised to endothelium of submucosal blood vessels in rat gastric mucosa. Furthermore, cultured human gastric endothelial (HuGE) cells synthesise PGE and PGI\(_2\) in vitro. It is established that human umbilical vein endothelial cells (HUVECs) express COX-1 constitutively and COX-2 following induction by factors such as interleukin 1 and phorbol ester in vitro. However, data from endothelial cells derived from large vessels such as the umbilical vein cannot necessarily be extrapolated to microvascular endothelial cells such as HuGE cells because endothelial cell function, for example, PG synthesis and cytokine production varies depending on the blood vessel(s) of origin. Therefore, we investigated COX-1 and COX-2 expression by HuGE cells in vitro.

It has recently been shown that healing of acetic acid induced gastric ulcers in mice is associated with induction of COX-2 expression and that COX-2 is localised to...
endothelial cells in granulation tissue of cryoprobe induced gastric ulcers in rats.\(^5\) NSAIDs (which are COX inhibitors) reduce gastric ulcer granulation tissue angiogenesis\(^2\) and delay gastric ulcer healing in humans\(^1\) and animals.\(^2\) Therefore, we investigated HuGE cell COX expression and the effect of selective COX-2 inhibition during angiogenesis in vitro.

### Methods

**ISOLATION AND CULTURE OF HUMAN GASTRIC ENDOTHELIAL (HuGE) CELLS**

Human gastric endothelial (HuGE) cells were isolated from normal organ donor stomachs as described previously.\(^1\) Cells were routinely cultured on 1% gelatin in Medium 199 containing 1 mM l-glutamine, 90 µg/ml heparin, 100 U/ml penicillin G, 100 µg/ml streptomycin, 250 ng/ml amphotericin B (all GIBCO BRL, Paisley, UK), 40 µg/ml endothelial cell growth supplement (ECGS; Advanced Protein Products, Brierley Hill, UK), and 30% fetal calf serum at 37\(^\circ\)C in 5% CO\(_2\). The endothelial nature of cell monolayers was confirmed regularly by indirect immunofluorescence for PECAM-1.\(^1\) HuGE cells were used up to passage 18.

HUVECs were isolated according to the method of Jaffe et al\(^5\) and cultured as above except that fetal calf serum was added to the culture medium at 20% vol/vol.

**IN VITRO ANGIOGENESIS MODELS**

**Plating on basement membrane matrix (Matrigel)**

Cells were plated onto a thin layer of basement membrane matrix (Matrigel, Becton Dickinson, Bedford, Massachusetts, USA)\(^2\) in six well plates at 2 \(\times\) 10\(^5\) cells/well in Medium 199 containing 1 mM l-glutamine, 90 µg/ml heparin, and 30% fetal calf serum and incubated at 37\(^\circ\)C in 5% CO\(_2\) for up to 24 hours. At four or 24 hours cells were removed from basement membrane matrix by incubation with 50 U/ml dispase (Becton Dickinson) for 60 minutes at 37\(^\circ\)C. Dispase activity was neutralised by dilution (\(\times\) 5) in Hank's balanced salt solution without calcium and magnesium (GIBCO BRL) and cells were collected by centrifugation. The number of viable cells obtained at the end of experiments was counted using a haemocytometer and exclusion of 0.2% Trypan blue (Sigma, Poole, UK).

Quantification of the degree of tubular structure formation by HuGE cells at four and 24 hours was performed by counting the number of complete circles produced by interlinking, tubular HuGE cells. Photomicrographs of each quadrant of individual wells were obtained using an Olympus OK2 inverted microscope and an Olympus OM-1\(_x\) camera with 200 ASA photographic film. A grid was superimposed on the photomicrographs using a custom made lens. The number of complete circles of interlinking, tubular HuGE cells with any part within the grid was counted in each quadrant by two independent observers blinded to the origin of the photomicrograph. The mean number of circles counted by both observers in each quadrant was used for further analysis.

**Phorbol ester stimulation**

Alternatively, cells were incubated with 1 µM phorbol 12,13-dibutyrate (PdBu; Sigma) in 1% gelatin coated wells for up to 24 hours under the same conditions. Cell suspensions were obtained using 0.5% trypsin solution (GIBCO BRL) and viable cells counted as above.

**REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR) AMPLIFICATION OF COX-1 AND COX-2 mRNA**

Total RNA was extracted from cells using a guanidinium isothiocyanate-phenol:chloroform extraction technique\(^2\) followed by precipitation of RNA with isopropanol (Micro RNA Isolation Kit, Stratagene, La Jolla, California, USA). A 5 µg aliquot of total RNA was reverse transcribed with 100 ng/µl oligo(dT)\(_{12-18}\) primers (Pharmacia Biotech Ltd, St Albans, UK), 200 nmol dNTPs, 40 U ribonuclease inhibitor, and 50 U MuLV reverse transcriptase (StrataScript RT-PCR kit, Stratagene). The reaction mix was incubated at 37\(^\circ\)C for 60 minutes and then 90\(^\circ\)C for five minutes. PCR was performed using specific primers (COX-1: forward, 5'-GAGTCTTTTCACGATGAGC-3', reverse, 5'-ACCTGGTACTTGAGTTTCCCA-3'; COX-2: forward, 5'-TGAAACCCACCTCCAAACACAG-3', reverse, 5'-TCATGCGCAGAGAGGAAAG-3') at 20 µM in the presence of 0.8 mM dNTPs and 1.5 mM MgCl\(_2\) in a Trio-Thermoblock TB-1 thermocycler (Biometra, Gottingen, Germany). A 5 µl aliquot of first strand product was heated to 91\(^\circ\)C for five minutes and 2.5 U DNA polymerase was added at the annealing temperature of 54\(^\circ\)C. PCR was carried out for 30 cycles as follows: 95\(^\circ\)C for 90 seconds, 54\(^\circ\)C for 90 seconds, 72\(^\circ\)C for 90 seconds. Finally, the reaction was heated to 54\(^\circ\)C for two minutes and then 72\(^\circ\)C for three minutes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (Stratagene)\(^14\) were used as a control at 1 µM. PCR products were separated on 1.2% agarose gels and stained with 0.5 µg/ml ethidium bromide. The size of PCR products was assessed by comparison with 2 µg \(\phi\)X174/Hae III digest markers (Promega, Madison, Wisconsin, USA). To ensure specificity of anti-COX antibodies for their COX isoforms, western blotting was carried out using 500 ng of ram seminal vesicle COX-1 and sheep placental COX-2 (both Cayman Chemical Co.) using standard methodology as described previously.\(^2\) Following overnight incubation with 1/500 dilutions of COX-1 and COX-2 immunoglo-
antibodies at 4°C, membranes were incubated for 30 minutes at room temperature with biotinylated goat antirabbit IgG and developed using an ABC-peroxidase technique (both VECTASTAIN Elite ABC kit, Vector Laboratories, Burlingame, California, USA).

HuGE cells were washed with phosphate buffered saline (PBS) and removed using a cell scraper (Becton Dickinson). Cells were pelleted by low speed centrifugation and 50 µl lysis buffer (10 mM PBS, 0.1% Triton-X-100, 0.01% EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), and 0.03% leupeptin) was added before boiling for three minutes. Cell lysates were centrifuged at 10 000 g for 10 minutes and the supernatant obtained. Western blot analysis of equal amounts of total protein (30 µg) was carried out as above.

INDIRECT IMMUNOFLUORESCENCE CYTOCHEMISTRY FOR COX-1 AND COX-2
Cells grown on 1% gelatin coated coverslips were fixed in 100% methanol at −30°C and indirect immunofluorescence studies performed as described previously. COX-1 and COX-2 antibodies were used at 1/50 dilutions. The goat antirabbit immunoglobulin-fluorescein isothiocyanate conjugate (Sigma) was used at a 1/20 dilution. Omission of the primary antibody and incubation with either normal rabbit serum (Sigma) or rabbit immunoglobulin (Dako, High Wycombe, UK) at the same concentration as the respective primary antibody were used as controls.

COX INHIBITORS USED
The selective COX-2 inhibitor N-[2-cyclohexyloxy-4-nitrophenyl] methanesulphonamide (NS-398) was obtained from Cayman Chemical Co. SC-560, a selective COX-1 inhibitor, was a kind gift from Searle Research and Development (St Louis, Missouri, USA).

HUVECs were used in preliminary experiments to confirm that the concentrations of SC-560 and NS-398 used in HuGE cell experiments selectively inhibited COX isoforms. PGE2 synthesis by unstimulated HUVECs and by HUVECs after incubation with 1 µM PdBu was used as a measure of COX-1 and COX-2 activity respectively as described previously.

Figure 1 Western blot analysis of (A) cyclooxygenase (COX) 1 and COX-2 standards (500 ng) and (B) human gastric endothelial (HuGE) cell homogenates (50 µg total protein) at four and 24 hours with or without 1 µM phorbol 12,13-dibutyrate (PdBu). COX-2 was not detectable in basal HuGE cells but there was induction of COX-2 expression by PdBu at four and 24 hours compared with control cells.

Figure 2 Inhibition of human umbilical vein endothelial cell (HUVEC) prostaglandin (PG) E2 synthesis by selective cyclooxygenase (COX) inhibitors. PGE2 levels were measured at 24 hours in medium conditioned by (A) basal HUVECs (COX-1 activity) or (B) HUVECs stimulated by 1 µM phorbol 12,13-dibutyrate (PdBu) (COX-2 activity). Differing concentrations of SC-560 or NS-398 were added with fresh medium at the start of experiments. Control experiments were performed in the absence of COX inhibitor. Data shown are the mean of duplicate experiments for each drug concentration.
MEASUREMENT OF PGE2 SYNTHESIS BY HuGE CELLS

Aliquots (200 µl) of cell culture medium were removed at zero, four, and 24 hours, centrifuged at 2000 g for two minutes, and then assayed using a commercially available PGE2 competitive ELISA (Amersham, Little Chalfont, UK).

Results

SPECIFICITY OF THE COX ANTIBODIES

Western blot analysis of COX standards confirmed the specificity of the COX antibodies for their isoforms (fig 1A). There was slight cross reactivity of the COX-2 antibody with COX-1 protein.

BASAL COX EXPRESSION BY MONOLAYERS OF HuGE CELLS

HuGE cells expressed COX-1 mRNA and protein constitutively when cultured on 1% gelatin (figs 1B, 3, and 4). Under basal conditions, HuGE cells did not express COX-2 protein detectable by western analysis (fig 1B) although COX-2 mRNA was detected by RT-PCR (fig 3) and some specific cytoplasmic staining for COX-2 was shown by indirect immunofluorescence (fig 4).

EFFECT OF PLATING HuGE CELLS ON MATRIGEL: TUBE FORMATION AND COX EXPRESSION

HuGE cells formed circles of interlinking, tubular structures within two to four hours of plating onto basement membrane matrix (fig 5), which persisted at 24 hours. This has been described previously by us using HuGE cells and others with HUVECs. HuGE cell number and viability remained unchanged at 24 hours (2 × 10^5 cells/well; greater than 95% viability). Expression of COX-2 mRNA (and to
a lesser extent COX-1) by HuGE cells was increased at four and 24 hours after plating onto basement membrane matrix compared with cells replated onto 1% gelatin (fig 3). The amount of basement membrane matrix required to harvest sufficient HuGE cells for western blot analysis of COX protein precluded this experiment but increased functional COX-2 protein expression was confirmed by increased PGE2 production which was significantly inhibited by NS-398 but not SC-560 at four hours (fig 6). By contrast, NS-398 did not significantly inhibit PGE2 production by HuGE cells on basement membrane matrix at 24 hours (fig 6).

**HuGE CELL MORPHOLOGY AND COX EXPRESSION FOLLOWING PHORBOL ESTER STIMULATION**

There was significant non-specific staining of basement membrane matrix by COX-1 and COX-2 antibodies. Therefore, the phenotypic changes in HuGE cells induced by phorbol ester, namely intracellular vacuole formation and appearance of long cellular processes (fig 4), were used as an alternative model of angiogenesis for western blot analysis and indirect immunofluorescence studies. Addition of 1 µM PdBu induced COX-2 protein expression by HuGE cells at four and 24 hours (fig 1B) but there was no change in immunoreactive COX-1 protein expression. Indirect immunofluorescence studies carried out on HuGE cells after four hours incubation with PdBu showed that increased COX-2 staining remained cytoplasmic but with additional strong perinuclear staining (fig 4).

**EFFECT OF NS-398 ON HuGE CELL ANGIOGENESIS IN VITRO**

Having shown induction of functional COX-2 expression by HuGE cells in two in vitro models of angiogenesis, we investigated the effect of the selective COX-2 inhibitor, NS-398 on tubular structure formation by HuGE cells on
basement membrane matrix using a quantitative assay (fig 5). The majority of tubular structure formation by HuGE cells occurred during the first four hours after plating onto basement membrane matrix with little increase in the number of circles of interlinking, tubular structures up to 24 hours (fig 7). NS-398 (3 × 10⁻⁷ M) significantly inhibited formation of circles of interlinking, tubular structures by HuGE cells at four hours compared with control conditions (p=0.05, Mann-Whitney U test; figs 5 and 7). The effect of NS-398 on tubular structure formation by HuGE cells was less pronounced at 24 hours (fig 7). NS-398 treatment did not effect HuGE cell number or viability up to 24 hours.

Discussion

We have shown that unstimulated HuGE cells express COX-1 and low levels of COX-2 in vitro. HuGE cells required the presence of 30% fetal calf serum for continued growth and did not tolerate serum free conditions or 0.5% serum so we cannot rule out slight induction of COX-2 by serum factors which has been shown in HUVECs.32 Low level COX-2 expression by unstimulated HUVECs has been previously reported using RT-PCR15 but Morita et al were unable to detect COX-2 in HUVECs and bovine aortic endothelial cells grown in the presence of 10% serum by indirect immunofluorescence.16

It has recently been reported that human intestinal microvascular endothelial cells express inducible cytokines such as interleukin 8 constitutively.15 With the caveat discussed above, our data suggest that a similar situation exists for COX-2 as well as for COX-1 in HuGE cells. Currently, there are no published data on endothelial cell COX expression in the gastrointestinal tract except for a report by Mikkelsen et al who showed immunoreactivity in endothelial cells throughout the human intestine using a non-specific anti-COX antibody which probably recognised both COX isoforms.33 Even though basal PG production by HuGE cells in vitro is low, the vast number of HuGE cells comprising the dense mucosal capillary network of the stomach in humans may mean that endothelial cells are an important source of human gastric mucosal PGs in vivo as has been shown in canine gastric mucosa.12 Constitutive COX-2 expression by gastric endothelial cells may explain the ability of a specific COX-2 inhibitor to abrogate the protective effect of 20% ethanol on subsequent 70% ethanol induced gastric injury in rats.18

We have shown that HuGE cell angiogenesis is associated with induction of functional COX-2 expression using basement membrane matrix and phorbol ester models. Basement membrane matrix is well established as a model of angiogenesis. The capillary like network produced one to two hours after plating of endothelial cells onto basement membrane matrix consists of tubular structures which contain a lumen surrounded by endothelial cells.39 Phorbol esters are angiogenic in vivo and in vitro.40 The morphological changes induced by phorbol esters are dependent on the type of gel on which the endothelial cells are plated. Formation of long cell processes by bovine microvascular endothelial cells and HUVECs on plastic after incubation with phorbol ester similar to that seen by HuGE cells has been reported previously.41 Intra-cellular vacuole formation, which is believed to be an early stage of lumen development, has previously been described in human foreskin capillary endothelial cells.42 Induced COX-2 protein in HuGE cells was predominantly perinuclear, which is similar to the localisation previously reported in HUVECs.19

Induction of COX-2 expression in HUVECs via protein kinase C (PKC) activation is well recognised.17 The formation of tubular structures by HUVECs on basement membrane matrix is also dependent on activation of PKC.13 It seems likely that basement membrane matrix may also, at least in part, induce COX-2 expression and angiogenesis by HuGE cells via this pathway. In our experiments, phorbol ester and basement membrane matrix both caused similar morphological changes and both induced COX-2 expression. Identifying which components of basement membrane promote angiogenesis and COX expression and elucidating the signalling pathways involved are important because of the central involvement of angiogenesis in ulcer healing and the adverse impact of NSAIDs on both these processes.43

Having shown induction of functional COX-2 expression by HuGE cells during angiogenesis in vitro, we confirmed a causal role...
for COX-2 during HuGE cell angiogenesis on basement membrane matrix using a quantitative tubular structure formation assay similar to those described previously. \(^4\) of NS-398, at a concentration which selectively inhibited endothelial cell COX-2 function, significantly attenuated HuGE cell tubular structure formation at four hours. At 24 hours, the effect of NS-398 on tubular structure formation and PGE, production by HuGE cells was less pronounced. A possible explanation for the relative lack of effect of selective COX-2 inhibition at 24 hours, despite continuing upregulation of COX-2 mRNA by HuGE cells on basement membrane matrix, is lack of sufficient arachidonic acid substrate for COX-2 function. It has been shown that COX-1 and COX-2 utilise different pools of arachidonic acid\(^6\) and it is possible that, at 24 hours, there is a lack of arachidonic acid available for COX-2 because of pool exhaustion or diversion to COX-1.

Tsujii et al have previously reported the effect of coculture of colorectal cancer cells with HUVECs on tubular structure and COX expression by HUVECs plated on thick type I collagen gels.\(^7\) Tubular structure formation by HUVECs was associated with induction of COX-1 and COX-2 expression. Importantly, it was shown that a COX-1 (but not COX-2) antisense oligonucleotide inhibited angiogenesis in this model of tumour angiogenesis.\(^8\) The differences between the study of Tsujii et al\(^7\) and the results reported here could be explained by endothelial cell specific differences in COX expression or the possibility that different substrates induce tubular structure formation by endothelial cells via different pathways which involve either or both COX isoforms. Otherwise there are few existing reports of the effect of COX inhibition by NSAIDs and exogenous PGs on angiogenesis. Several NSAIDs have been shown to reduce angiogenesis and growth of tumours in rats.\(^9\) Conversely, PGE, and PGE, promote angiogenesis in vivo.\(^10,11\) Decreased granulation tissue vascularity has been reported in experimental gastric ulcers in rats administered NSAIDs\(^8\) and in NSAID associated gastric ulcers in humans.\(^12\) However, these studies do not necessarily imply a direct role for COX expression in gastric ulcer angiogenesis as NSAIDs could be acting directly on angiogenesis via a non-COX dependent mechanism or indirectly by altering expression of angiogenic factors by other granulation tissue cells such as macrophages and fibroblasts.

In summary, the data presented in this paper show that, under normal culture conditions, HuGE cells express both COX-1 and COX-2 in vitro. We have also shown that induction of COX-2 expression is associated with angiogenesis by HuGE cells in two in vitro models and that COX-2 plays a role in HuGE cell angiogenesis on basement membrane matrix.

The role of each COX isoform in human gastric ulcer granulation tissue angiogenesis in vivo remains to be elucidated. It has been shown that the selective COX-2 inhibitor, L-745,337, reduces granulation tissue vascularity and delays healing of cryoprobe induced gastric ulcers in rats to the same extent as the non-selective COX inhibitors, indomethacin and diclofenac.\(^1\) In the light of our data showing the role of the COX-2 pathway in angiogenesis by HuGE cells in vitro, further studies are warranted to investigate whether selective COX-2 inhibitors impair granulation tissue angiogenesis and delay gastric ulcer healing in humans.

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