Regulation of stromal cell cyclooxygenase-2 in the Apc\textsuperscript{Min/+} mouse model of intestinal tumorigenesis

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Cyclooxygenase-2 (Cox-2) is expressed predominantly by stromal cells in intestinal adenomas from the Apc\textsuperscript{Min/+} mouse model of familial adenomatous polyposis. We investigated the mechanistic basis of stromal cell Cox-2 expression in Apc\textsuperscript{Min/+} mouse adenomas, as well as Cox-2 expression and activity in histologically normal (HN) Apc\textsuperscript{Min/+} mouse intestine, in order to gain further insights into regulation of Cox-2 as a potential chemoprevention target. Upregulation of Cox-2 in intestinal tumours is not an intrinsic feature of Apc\textsuperscript{Min/+} macrophages as bone marrow-derived Apc\textsuperscript{Min/+} macrophages did not exhibit an abnormality in Cox-2 expression or activity. Intestinal permeability to lactulose or mannitol was similar in Apc\textsuperscript{Min/+} mice and wild-type littersmates, implying that macrophage activation by luminal antigen is unlikely to explain stromal cell Cox-2 induction. Moreover, stromal cells exhibited differential expression of Cox-2 and inducible nitric oxide synthase, suggesting ‘alternative’ (M2) rather than ‘classical’ (M1) macrophage activation. Flow cytometric sorting of isolated stromal mononuclear cells (SMNCs), on the basis of M-lysozyme and specific macrophage marker expression, demonstrated that macrophages, neutrophils and non-myelomonocytic cells all contributed to lamina propria prostaglandin (PG) E\textsubscript{2} synthesis. However, the majority of PGE\textsubscript{2} synthesis by macrophages was via a Cox-2-dependent pathway compared with predominant Cox-1-derived PGE\textsubscript{2} production by non-myelomonocytic cells. SMNCs from HN Apc\textsuperscript{Min/+} intestinal mucosa exhibited similar levels of Cox-2 mRNA and protein, but produced more Cox-2-derived PGE\textsubscript{2} than wild-type cells at 70 days of age. There was an age-dependent decline in PGE\textsubscript{2} synthesis by Apc\textsuperscript{Min/+} SMNCs, despite tumour progression. These data suggest that other Cox-2-independent factors also control PGE\textsubscript{2} levels during Apc\textsuperscript{Min/+} mouse intestinal tumorigenesis. Regulation of macrophage Cox-2 expression and other steps in PGE\textsubscript{2} synthesis (e.g. PGE synthase) are valid targets for novel chemoprevention strategies that could minimize or avoid systemic COX-2 inhibition.

Introduction

Experiments involving genetic deletion or pharmacological inhibition of the inducible isoform of cyclooxygenase (Cox), Cox-2 have demonstrated that this enzyme plays an important role in intestinal tumorigenesis in mouse models of familial adenomatous polyposis (FAP), such as the Apc\textsuperscript{Min/+} mouse (1–4). These models are relevant to the study of the early stages of human colorectal carcinogenesis (5).

COX-2 is recognized as a potential human colorectal cancer (CRC) chemoprevention target (6). However, recent clinical observations have cast doubt on the vascular safety profile of long-term systemic administration of coxib selective COX-2 inhibitors in humans (7). An alternative CRC chemoprevention strategy that would minimize systemic toxicity is inhibition of COX-2 expression and/or activity in a cell- and tissue-specific manner, which requires detailed understanding of the mechanism(s) that regulate intestinal COX-2 expression.

Several independent studies have demonstrated that stromal cells are the predominant source of Cox-2 in mouse and human intestinal adenomas (1–2,8–16). In addition, it has been reported that stromal cell Cox-2 expression and levels of prostaglandin (PG) E\textsubscript{2} (the predominant COX-derived product in intestinal mucosa) are increased in histologically normal (HN) Apc\textsuperscript{Min/+} mouse intestinal mucosa (9,17,18) and HN mucosa from CRC patients (18). However, the mechanistic basis of stromal cell Cox-2 induction in intestinal adenomas and the extent of Cox-2 expression or activity in HN intestinal mucosa during different stages of intestinal tumorigenesis have not been addressed previously.

Therefore, we studied stromal cell Cox-2 expression and function in adenomas and HN intestinal mucosa at different stages of intestinal tumorigenesis in Apc\textsuperscript{Min/+} mice, in order to gain further insights into mechanisms regulating intestinal stromal cell Cox-2 expression and PGE\textsubscript{2} synthesis during tumour growth.

Materials and methods

Animals

All mice were kept under specific pathogen-free conditions in isolators at 19–23°C on a 12 h light–dark cycle, according to Institutional and UK Home Office guidelines. Animals had free access to food (Complete Maintenance Diet, B&K Universal, Hull, UK) and water.

Mice of C57BL/6 Apc\textsuperscript{Min/+} type were obtained from The Jackson Laboratory, Ann Arbor, MI, USA. The Apc\textsuperscript{Min/+} mouse colony was maintained by mating male Apc\textsuperscript{Min/+} mice with wild-type C57BL/6J females. Genotyping for Apc was by ARMS-PCR as described (19). Apc\textsuperscript{Min/+} mice were studied at 30–37 days (termed D30); 54–61 days (D60); 69–74 days (D70); 93–97 days (D90); 107–117 days (D110); or 135–148 days (D140). Comparative experiments were always performed using age-matched wild-type littersmates. Generation of C57BL/6 \times 129/Sv Lys-EGFP-ki (M-lysozyme-enhanced green fluorescent protein knock-in) mice has been described previously (20). Genotype analysis was performed by PCR as described previously (20). Mice of Lys-EGFP-ki type were crossed with Apc\textsuperscript{Min/+} mice and the resultant Lys-EGFP-ki \times Apc\textsuperscript{Min/+} mice and Lys-EGFP-ki wild-type littersmates were compared at D70. All Lys-EGFP-ki animals used in this study were heterozygous for the ‘knock-in’ allele (ki\textsuperscript{+}). There was no difference in small

Abbreviations: BMDM, Bone marrow-derived macrophages; CRC, colorectal cancer; COX, cyclooxygenase; FAP, familial adenomatous polyposis; HN, histologically normal; Hprt, hypoxanthine-guanine phosphoribosyl transferase; IFN, interferon; Lys-EGFP-ki, M-lysozyme-enhanced green fluorescent protein knock-in mice; Nos2, nitric oxide synthase 2; PG, prostaglandin; SI, small intestine; SMNCs, stromal mononuclear cells.
Stromal cell Cox-2 in intestinal tumorigenesis

Intestine (SI) or colonic adenoma multiplicity in heterozygous Lys-EGFP-Ki67-/- x ApcMin-/- mice compared with C57BL/6 x 129/Sv ApcMin-/- mice with wild-type M-Isoyoze alleles (data not shown).

Measurement of intestinal permeability

Mice were acclimatized in wire-bottomed metabolic cages for 3 days with free access to food and water. Animals were fasted overnight before administration of 13.3 mg lactulose and 10.1 mg mannnitol (both Sigma, Poole, UK) in 200 μl water by oral gavage. Urine produced during the 6 h test period was collected in 20 μl 20% (v/v) chlorohexidine (21). Urinary lactulose and mannnitol were measured by enzymatic methods as described (22,23). Data are presented as the ratio of the percentage administered dose of lactulose excreted in the urine to the percentage of the administered dose of mannnitol in the same urine collection (L/M ratio).

Immunohistochemistry for Cox-2

Goat polyclonal anti-Cox-2 antibody and goat IgG control (both Santa Cruz Biotechnology, Santa Cruz, CA) were labelled with AlexaFluo 488 using a protein labelling kit (Molecular Probes, Invitrogen, Paisley, UK) according to the manufacturer’s instructions. Frozen sections (5–7 μm thick) of intestine were fixed in 100% methanol at −20°C for 15 min, air dried and then rehydrated in Tris-buffered saline [0.02% (v/v)] Tween 20 (TBS-T) followed by blocking with TBS-T containing 10% (v/v) goat serum (DakoCytomation, Ely, UK) for 1 h at 20°C. Sections were then labelled for 1 h at 20°C with anti-Cox-2 or control antibody (1:100 dilution in TBS-T containing 10% goat serum), washed 3 times for 15 min each with TBS-T and then mounted with 4', 6-diamidino-2-phenylindole (DAPI)-incorporated Vectashield (Vector Laboratories, Burlingame, CA). Labelling specificity was confirmed by pre-absorption of tagged antibody with protein Ki-1-negative for 4 h at 20°C, prior to incubation with sections.

In situ hybridization

Specific localization of Cox-2 and nitric oxide synthase 2 (Nos2) mRNA was determined by in situ hybridization using unhydroydised riboprobes generated from linearized plasmid sense and antisense murine Cox-2 and Nos2 probes templates (Cayman Chemical, Ann Arbor, MI) using [35S]-UTP (~800 Ci/ mmol; Amersham Pharmacia, Amersham, UK). An antisense β-actin probe was used to test for the presence of hybridizable mRNA in the tissues studied. Sections of 4 μm were cut from paraffin-embedded blocks of intestinal tissue onto silane-treated slides under RNase-limited conditions. The methods for pre-treatment, hybridization, washing and dipping of slides in Ilford K5 for autoradiography were as described (24). Sections were counterstained by Giemsa’s method and examined under conventional or reflected light dark-field conditions using an Olympus BH2 microscope with epi-illumination.

Isolation and culture of bone marrow-derived macrophages

Bone marrow-derived macrophages (BMDM) were differentiated in vitro in the presence of macrophage colony-stimulating factor for 7 days as described previously (25,26). BMDM were stimulated for 18 h with 100 U/ml interferon (IFN)-γ and 1 μg/ml lipopolysaccharide (LPS; both Sigma). Semi-quantitative RT-PCR for Cox-2 and Hypoxanthine-guanine phosphoribosyl transferase (Hprt) mRNA, as well as western blot analysis of Cox-2 and β-actin protein, was performed as described (25).

Isolation of stromal mononuclear cells

Stromal mononuclear cells (SMNCs) were isolated from mouse SI by the method of Newberry et al. (27), with the following modifications: the whole SI was cut into ~50 pieces, which were incubated sequentially in three changes of collagenase/dispase digestion medium for 30 min at 37°C. After each incubation period, isolated SMNCs were collected by filtration through 70 μm cell mesh (Porex, Warrington, UK) and maintained at 37°C in Iscove’s Modified Eagle’s Medium containing 10% (v/v) foetal calf serum (FCS), 1 x non-essential amino acid solution, 1 mM sodium pyruvate, 0.075% (w/v) sodium bicarbonate, 50 IU/ml penicillin, 50 μg/ml streptomycin, 0.5 mg/ml gentamicin (all from Invitrogen) and 10% (v/v) -l-cell conditioned medium (26), termed SMNC culture medium, before pooling. The total number of viable SMNCs was determined using a haemocytometer and exclusion of 0.4% (v/v) trypan blue (Sigma).

Immunofluorescence for Cox-2 on SMNCs

Immunofluorescence for Cox-2 was performed on cytospins of SMNCs fixed with 4% (v/v) paraformaldehyde in PBS for 15 min at 20°C. After blocking with 15% (v/v) donkey serum in PBS for 30 min at 20°C, slides were incubated with a 1:40 dilution of affinity-purified rabbit polyclonal anti-mouse Cox-2 IgG (Cayman Chemical) in blocking solution for 60 min at 20°C. Following washes (4x 5 min), the secondary antibody [fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG from Chemicon, CA; 6 μg/ml] in blocking solution was added for 30 min at 20°C. Controls included omission of the primary antibody and pre-absorption of the primary antibody with a 10-fold molar excess of its cognate peptide (Cayman Chemical) for 2 h at 20°C. The percentage number of Cox-2-positive SMNCs was calculated by fluorescence and phase-contrast microscopy of at least 1000 cells in each case.

Flow cytometric sorting of SMNCs

SMNCs were incubated in the presence of 20 U/ml DNase I (Sigma) in SMNC culture medium for 30 min at 37°C and then washed in 10% FCS in PBS for 5 min at 37°C. Pelleted cells were blocked with 10% (v/v) mouse serum in PBS for 30 min at 20°C followed by incubation with a mix of rat anti-mouse macrophage-specific antibodies [ER-HR3, 0.5 μg/ml (28); F4/80, 10 μg/ml (29); MOMA-1, 0.5 μg/ml (29)], termed Mac-mix, or 10 μg/ml pre-immune rat IgG (Sigma) in SMNC culture medium for 30 min at 20°C. Following a wash in 10% (v/v) FCS in PBS, cells were incubated with 4 μg/ml phycocyanin (PE)-conjugated goat anti-rat antibody (Sero tec, Oxford, UK) in SMNC culture medium containing 10% (v/v) mouse serum for 30 min at 20°C. Finally, cells were washed in 10% (v/v) FCS in PBS containing 0.5 μg/ml propidium iodide (PI; Sigma) for 5 min at 20°C and then kept on ice in SMNC culture medium.

Cell sorting was performed on 5 × 10⁴ SMNCs using a FACS Vantage (Becton Dickinson, NJ, USA) and Cell Quest software (3000 events/s). Gates were set to exclude PI-positive cells and cells with low forward scatter (deemed non-viable), and sort EGFP-positive cells (fluorescence level greater than that obtained by <0.1% of wild-type SMNCs) and macrophage marker-positive cells (compared with non-specific labelling with control rat IgG). Cell collection tubes were changed every 15 min and sorted cells were kept on ice until they were re-suspended in fresh SMNC culture medium.

Measurement of PGE2 production by SMNCs

Total SMNCs and sorted cell sub-populations based on EGFP- and Mac-mix-positivity were cultured for 24 h in SMNC culture medium at 37°C in the presence of 5% CO2. The selective Cox-2 inhibitor SC236 was a kind gift from Professor LaManna, Skokie, IL [1 mM stock solution in dimethyl sulphoxide (DMSO)]. Cell-free conditioned medium was obtained and the number and the percentage viability of combined floating and adherent cells was determined by a haemocytometer and trypan blue [0.4% (v/v) in PBS] exclusion. Levels of PGE2 were measured using a PGE2 enzyme immunoassay (Amersham Pharmacia).

Results

Upregulation of Cox-2 in stromal cells in adenomas is not an intrinsic property of ApcMin-/- mouse macrophages

We, and others, have previously identified the predominant Cox-2-expressing stromal cell type in ApcMin-/- mouse intest-ine as the macrophage (9,18). Induction of Cox-2 expression in intestinal macrophages of ApcMin-/- mice could represent an intrinsic property of macrophages with a ApcMin-/- genotype. If this was the case, Cox-2 up-regulation should occur in other macrophage populations from the ApcMin-/- mouse compared with wild-type cells. Therefore, we compared Cox-2 expression and function in in vitro differentiated BMDM from ApcMin-/- mice and wild-type animals. RT–PCR and western blot analysis demonstrated that treatment with IFNγ and LPS for 16 h induced Cox-2 mRNA and protein expression in both ApcMin-/- and wild-type BMDM (Figure 1A and B). The presence of the ApcMin allele did not alter Cox-2 mRNA or protein levels in either un-stimulated or IFNγ/LPS-stimulated BMDM (Figure 1A and B). There was also no significant difference in PGE2 expression by IFNγ/LPS-stimulated ApcMin-/- BMDM compared with wild-type BMDM (Figure 1C). Similar findings were obtained from a comparison of ApcMin-/- and wild-type mouse peritoneal macrophages (data not shown). These data confirm that there is no significant intrinsic abnormality of Cox-2 expression by ApcMin-/- mouse macrophages and, in turn, suggest that a local stimulus may be responsible for upregulation of Cox-2 in ApcMin-/- mouse intestinal macrophages.
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H2O denotes a negative control in which cDNA template was substituted

Columns (bars) denote the mean (±SEM) value from three individual

Fig. 1. Cox-2 expression and activity by in vitro differentiated BMDM.

(A) RT–PCR for Cox-2 and Hprt mRNA from total RNA isolated from
unstimulated (-) or IFNγ/LPS-stimulated (+) BMDM from age-matched
wild-type (WT) and ApcMin/+ (Min) mice (D110). A representative gel is
shown above a corresponding histogram of the mean (± SEM)
densitometric Cox-2/Hprt band ratios from three separate pairs of mice.
H2O denotes a negative control in which cDNA template was substituted
for water. The figures on the right denote individual amplicon sizes in base
pairs. (B) Western blot analysis of Cox-2 and β-actin protein from
unstimulated (-) or IFNγ/LPS-stimulated (+) BMDM from age-matched
wild-type (WT) and ApcMin/+ (Min) mice (D110). The size of the bands is
noted on the right. (C) PGE2 synthesis over 18 h by unstimulated (-) or
IFNγ/LPS-stimulated (+) BMDM from age-matched wild-type (WT) and
ApcMin/+ (Min) mice (D110). The number of viable bone marrow cells
seeded per well was counted. The amount of PGE2 (ng) is quoted per 107
cells after subtraction of background levels of PGE2 in the culture medium.
Columns (bars) denote the mean (± SEM) value from three individual
experiments.

Intestinal permeability is similar in ApcMin/+ mice at different stages of intestinal tumorigenesis to age-matched wild-type animals

We noted that Cox-2 immunoreactivity was most intense in stromal cells located in superficial areas of ApcMin/+ mouse SI and colonic adenomas (Figure 2A and B), often just below eroded luminal epithelium. One hypothesis is that upregulation of Cox-2 in stromal macrophages, just below luminal epithelium, occurs following antigenic stimulation, secondary to increased epithelial permeability. Therefore, we measured paracellular transport of lactulose, expressed as a ratio to mannitol excretion (used as a control for variations in intestinal surface area and renal function), as a measure of SI permeability (30). Percentage lactulose and mannitol recoveries and L/M ratio values were consistent with previous rodent SI permeability experiments (Figure 3; 21,30). There was a small age-dependent increase in percentage lactulose and mannitol recovery in both wild-type and ApcMin/+ mice (Figure 3). However, there was no significant difference between the L/M ratio of wild-type and ApcMin/+ mice at any age, even when adenomas are evident macroscopically. This suggests that increased permeability of epithelium in intestinal adenomas does not explain Cox-2 induction in tumour-associated superficial stromal cells.

Differential expression of Cox-2 and Nos2 in tumour stroma

If Cox-2 upregulation in tumour-associated ApcMin/+ mouse macrophages was due to activation by luminal contents, including bacterial endotoxin such as LPS, crossing ‘leaky’ epithelium, one might expect parallel upregulation of Cox-2 and Nos2, which characteristically occurs upon ‘classical’ activation of macrophages by IFNγ and/or LPS in vitro (31,32). Therefore, we performed in situ hybridization for Cox-2 and Nos2 mRNA on adjacent sections of ApcMin/+ mouse SI adenomas in order to determine whether these two immediate early genes are co-expressed by tumour-associated macrophages in ApcMin/+ mouse neoplasms. Although Cox-2 transcripts were present in superficial stromal tissue within SI adenomas (Figure 4A, B and H), Nos2 mRNA was not present in the equivalent area of adjacent sections of the same tissue (Figure 4C, D and G). However, Nos2 protein levels and nitrate production are increased by IFNγ and LPS treatment of ApcMin/+ mouse BMDM (data not shown), confirming that lack of Nos2 expression is not an inherent property of ApcMin/+ mouse macrophages. Taken together, the observations that there is no change in L/M permeability ratio in ApcMin/+ mice and that Cox-2-expressing stromal cells do not express Nos2 suggest that stromal cell Cox-2 expression in ApcMin/+ mouse intestine is not due to ‘classical’ macrophage activation by intestinal luminal content.

Stromal cell Cox-2 expression and function at different stages of ApcMin/+ mouse intestinal tumorigenesis

Previous studies of stromal cell Cox-2 expression in the ApcMin/+ mouse model have used immunohistochemistry alone to demonstrate Cox-2 protein localization (9,18). In this study, we isolated SMNCs from the SI in order to combine cell phenotype analysis by flow cytometry with quantitation of ex vivo stromal cell Cox-2 function (using PGE2 synthesis as a ‘read-out’). Newberry et al. (27) have previously demonstrated that Cox-2 levels and activity in isolated intestinal stromal cells accurately reflect mucosal Cox-2 expression and function in mouse intestine in vivo using a similar isolation technique (33). Initially, we analysed the percentage number of Cox-2-positive cells in mouse neoplasms. Although Cox-2 transcripts were present in superficial stromal tissue within SI adenomas (Figure 4A, B and H), Nos2 mRNA was not present in the equivalent area of adjacent sections of the same tissue (Figure 4C, D and G). However, Nos2 protein levels and nitrate production are increased by IFNγ and LPS treatment of ApcMin/+ mouse BMDM (data not shown), confirming that lack of Nos2 expression is not an inherent property of ApcMin/+ macrophages. Taken together, the observations that there is no change in L/M permeability ratio in ApcMin/+ mice and that Cox-2-expressing stromal cells do not express Nos2 suggest that stromal cell Cox-2 expression in ApcMin/+ mouse intestine is not due to ‘classical’ macrophage activation by intestinal luminal content.
and D), thus providing further evidence that our \textit{ex vivo} observations on isolated cells were relevant to mucosal Cox-2 expression \textit{in vivo}.

In keeping with the lack of any significant difference in the number of Cox-2 positive SMNCs between Apc\textsuperscript{Min/+} and wild-type littermates, there was no significant difference in Cox-2 mRNA levels (relative to \textit{Hprt} mRNA levels) in freshly isolated SMNCs from D70 Apc\textsuperscript{Min/+} and wild-type mice, as measured by real time RT–PCR (data not shown). However, significant differences in \textit{ex vivo} Cox-2-dependent PGE\textsubscript{2} synthesis by cultured SMNCs from Apc\textsuperscript{Min/+} and wild-type mice at different ages were apparent. We confirmed that the selective COX-2 inhibitor SC236 decreased PGE\textsubscript{2} production by wild-type D70 SMNCs in a concentration-dependent manner, with maximal inhibition (\(\approx 90\%\)) occurring at a minimum concentration of 1 \(\mu\text{M}\) SC236, without evidence of non-specific cell toxicity (Figure 6A). We then studied \textit{ex vivo} PGE\textsubscript{2} synthesis by SMNCs from Apc\textsuperscript{Min/+} and wild-type mice at different ages in the absence or presence of 1 \(\mu\text{M}\) SC236 (Figure 6B). SMNCs from D30 mice (when only ‘nascent’ micro-adenomas are present) demonstrated low level PGE\textsubscript{2} synthesis, which was not inhibited by the selective COX-2 inhibitor SC236 implying predominant Cox-1-dependent PGE\textsubscript{2} production (Figure 6B). However, by 70 days of age (at which stage, adenomas have increased in size and are now visible macroscopically), SMNCs produced 10–30-fold higher PGE\textsubscript{2} levels due almost exclusively to an increase in Cox-2-dependent PGE\textsubscript{2} synthesis (Figure 6B). At D70, Apc\textsuperscript{Min/+} SMNCs produced 2–3-fold higher PGE\textsubscript{2} levels than

![Fig. 2. Immunofluorescence for Cox-2 on Apc\textsuperscript{Min/+} and wild-type mouse SI. Frozen sections were probed with a directly conjugated goat anti-Cox-2 antibody in order to minimize non-specific signal related to the use of secondary anti-goat immunoglobulin. Nuclei are stained with DAPI. Prior incubation of the tagged antibody with protein G-sepharose resulted in a complete loss of fluorescent signal, thus confirming the specificity of the technique (data not shown). (A) Strong fluorescence of Cox-2-positive stromal cells (white arrows) in superficial areas of a Apc\textsuperscript{Min/+} mouse SI adenoma (D110). Less intense fluorescent signal is also present in stromal cells in deeper areas of the adenoma (yellow arrows). There is no Cox-2 immunoreactivity in epithelial cells. 200\times magnification. A similar distribution of Cox-2 protein in superficial tumour-associated stromal cells has previously been noted (9) using the rabbit polyclonal anti-Cox-2 antibody used in the experiments on isolated SMNCs (see Figure 5). (B) Higher power view of Cox-2-positive stromal cells (arrows) just below superficial epithelium (asterisk) of an Apc\textsuperscript{Min/+} mouse SI adenoma. 400\times magnification. (C) HN SI from an Apc\textsuperscript{Min/+} mouse (D110). Cox-2-positive stromal cells are present in normal villi (arrows). There is also faint immunoreactivity of muscle cells. 200\times magnification. (D) HN SI from a wild-type D110 mouse. Cox-2-positive cells are also present in normal villi (arrows) similar to Apc\textsuperscript{Min/+} mouse SI. 200\times magnification.]

![Fig. 3. Permeability of Apc\textsuperscript{Min/+} and wild-type mouse SI. (A) Urinary lactulose and mannitol excretion, quoted as the lactulose to mannitol (L/M) ratio, of Apc\textsuperscript{Min/+} (Min; bold type) and wild-type (WT; normal type) littermates (D30, \(n = 3\); D60, \(n = 4\); D90, \(n = 4\); D140, \(n = 3\)). Columns and bars represent the mean \(\pm\) SEM values of the L/M ratio. Below each column are the individual mean % lactulose (%L) and % mannitol (%M) recoveries for each group of mice.]

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wild-type SMNCs ($P = 0.06$ for the difference between $Apc^{Min/+}$ and wild-type SMNCs; Mann–Whitney U Test; Figure 6B). A methodological limitation of our studies is that we could not distinguish between isolated $Apc^{Min/+}$ SMNCs originating from within adenomas and those resident in HN mucosa (the lengthy dissection required to separate adenomas from macroscopically normal SI led to a marked decrease in viability of freshly isolated SMNCs). However, comparison of PGE2 production by D70 $Apc^{Min/+}$ and wild-type SMNCs with that by D30 SMNCs suggests that SMNCs from HN mucosa (compare D30 versus D70 wild-type SMNCs), as well as from adenomas (compare $Apc^{Min/+}$ versus wild-type SMNCs at D70), both contributed to increased SMNC PGE2 synthesis in older mice when adenomas are evident macroscopically. By D110, SMNC PGE2 synthesis had decreased in $Apc^{Min/+}$ and wild-type mice compared with respective D70 cells (Figure 6B) although there was no significant difference in the total number of $Apc^{Min/+}$ SMNCs isolated per intestine at D70 ($12.3 \pm 2.4 \times 10^6$ cells (mean ± SEM); $n = 8$) and D110 ($10.6 \pm 2.4 \times 10^6$ cells; $n = 8$).

**PGE2 synthesis by sub-populations of intestinal SMNCs**

Next, we used expression of M-lysozyme and macrophage-specific cell surface markers (F4/80, ER-HR3 and MOMA-1) as tools to identify sub-populations of isolated SMNCs responsible for PGE2 synthesis. We used mice with the enhanced green fluorescent protein (EGFP) gene inserted into the M-lysozyme locus (termed Lys-EGFP-ki mice) in order to specifically label cells of the myelomonocytic lineage, which include macrophages and neutrophil granulocytes (20,34). In addition, we used a mix of three antibodies (Mac-mix) against recognized macrophage-specific antigens (28,29) in order to distinguish between macrophages and neutrophil granulocytes. We limited these experiments to D70 animals as PGE2 production by total SMNCs was highest at D70 and the density of SI Mac-1-positive macrophages in $Apc^{Min/+}$ mice peaked at this time point in an earlier study by Kettunen et al. (35).

Fig. 4. *In situ* hybridization for Cox-2 and Nos2 mRNA in adenomas from $Apc^{Min/+}$ mouse SI. Light (A, C and E) and dark-field (B, D and F) images of adjacent full-thickness sections of a SI adenoma (D140) probed for Cox-2 mRNA (A and B), Nos2 mRNA (C and D) and β-actin mRNA (E and F). Size bar for (A–F) = 125 μm. Note that there is no signal for Nos2 mRNA over the cells that contain Cox-2 transcripts (compare panels B and D). (G) and (H) Dark-field images of adjacent sections of a different SI adenoma probed for Nos2 mRNA (G) and Cox-2 mRNA (H). Size bar for (G–H) = 250 μm. As described previously (25), dysplastic epithelial cells in adenomas do not express Nos2 transcripts (D and G). However, signal for Nos2 mRNA is present over normal epithelial cells overlying the adenoma in panel G (arrow) confirming the sensitivity of the technique for detection of Nos2 transcripts. As in the adenoma pictured in panels (A–F), superficial stromal cells containing Cox-2 mRNA (arrow panel H) do not contain Nos2 transcripts in this adenoma.

SMNCs were isolated from D70 Lys-EGFP-ki/$Apc^{Min/+}$ and Lys-EGFP-ki/$Apc^{+/+}$ mice ($n = 4$ each group) and viable cells that excluded PI were analysed for EGFP...
fluorescence and Mac-mix labelling by flow cytometry (see Figure 7 for representative flow cytometric plots). \( \text{Apc}^{+/+} \) and \( \text{Apc}^{\text{Min}+/+} \) mice generated similar numbers of viable SMNCs (Table I). EGFP-expressing cells (G\(^+\)) constituted \( \approx 5\% \) of viable SMNCs in \( \text{Apc}^{+/+} \) and \( \text{Apc}^{\text{Min}+/+} \) mice (Table I). The majority of Mac-mix-positive cells (M\(^+\)) were fluorescent (92.0 ± 5.2 and 83.9 ± 4.4% for \( \text{Apc}^{+/+} \) and \( \text{Apc}^{\text{Min}+/+} \) mice, respectively) consistent with \( M\text{-lysozyme} \) expression by resident macrophages in mouse SI. However, double-positive macrophages (G\(^+\)M\(^+\)) only made up approximately one quarter of the total G\(^+\)SMNC population (Table I), in keeping with significant \( M\text{-lysozyme} \) expression by \( M\text{-lysozyme} \)/null neutrophils in mouse SI. There were no statistically significant differences in the size of each of the SMNC sub-populations between D70 \( \text{Apc}^{\text{Min}+/+} \) and \( \text{Apc}^{+/+} \) mice.

SMNCs were then sorted on the basis of \( M\text{-lysozyme} \) and macrophage marker expression in order to investigate PGE\(_2 \) production by individual SMNC sub-populations tested during a 24 h \textit{ex vivo} culture period. Insufficient numbers of G\(^+\)M\(^+\) cells were obtained to allow measurement of PGE\(_2 \) synthesis (Table II). All three other SMNC populations synthesized PGE\(_2 \) (Table II). G\(^+\)M\(^+\) macrophages were the predominant source of PGE\(_2 \) synthesis in both \( \text{Apc}^{+/+} \) and \( \text{Apc}^{\text{Min}+/+} \) mice (Table II). Differences in PGE\(_2 \) production were not due to variations in cell viability post-sorting (Table II). Although PGE\(_2 \) production by G\(^+\)M\(^+\) and G\(^+\)M\(^{-}\) cells was higher in \( \text{Apc}^{\text{Min}+/+} \) than age-matched \( \text{Apc}^{+/+} \) mice (Table II), these
differences were much smaller than those demonstrated between PGE2 production by respective total SMNCs at D70 (Figure 5B). This may be explained by the prolonged duration of ex vivo handling and culture (and hence lower percentage viability) of sorted SMNCs, compared with total SMNC populations (Table II), leading to selective loss of PGE2-producing cells or decreased activity of the PGE2 biosynthesis pathway. Additionally, sufficient G⁻M⁻ and G⁺M⁺ cells were sorted to allow incubation with the selective Cox-2 inhibitor SC236 (1 µM). Production of PGE2 by G⁺M⁺ macrophages was reduced by 55.2 ± 15.5% (n = 3 for combined Apc⁺/⁺ and ApcMin/+ cells) compared with a 15.9 ± 5.1% reduction in PGE2 synthesis by G⁻M⁻ cells (n = 8) following incubation.

**Table I.** Flow cytometry for EGFP and Mac-mix-positivity on SMNCs from D70 lys-EGFP-ki/Apc⁺/⁺ and lys-EGFP-ki/ApcMin/+ mice

<table>
<thead>
<tr>
<th></th>
<th>Apc⁺/⁺</th>
<th>ApcMin/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>% viability (PI exclusion) of total SMNCs</td>
<td>35.2 ± 6.1</td>
<td>42.3 ± 6.4</td>
</tr>
<tr>
<td>Number of viable SMNCs</td>
<td>9.9 ± 2.6 × 10⁶</td>
<td>10.4 ± 2.8 × 10⁶</td>
</tr>
<tr>
<td>% G⁺ cells</td>
<td>5.2 ± 0.5</td>
<td>3.7 ± 0.45</td>
</tr>
<tr>
<td>% G⁺ positivity of M⁻ cells</td>
<td>92.0 ± 5.2</td>
<td>83.9 ± 4.4</td>
</tr>
<tr>
<td>% M⁺ positivity of G⁺ cells</td>
<td>29.7 ± 8.8</td>
<td>25.3 ± 4.1</td>
</tr>
</tbody>
</table>

*ⁿ = 4 for both groups of D70 mice. Data represent the mean ± SEM.

**Table II.** PGE₂ synthesis by subpopulations of SMNCs from D70 lys-EGFP-ki/Apc⁺/⁺ and lys-EGFP-ki/ApcMin/+ mice

<table>
<thead>
<tr>
<th></th>
<th>Apc⁺/⁺</th>
<th>ApcMin/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>G⁻M⁻</td>
<td>433 ± 56²</td>
<td>372 ± 67²</td>
</tr>
<tr>
<td>G⁺M⁻</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>G⁺M⁺</td>
<td>419 ± 82</td>
<td>508 ± 132</td>
</tr>
<tr>
<td>G⁻M⁻</td>
<td>771 ± 119</td>
<td>844 ± 262</td>
</tr>
</tbody>
</table>

ND, not determined.

ⁿ = 4 for both groups of D70 mice. Data represent the mean ± SEM value for PGE₂ production by 10⁵ cells over 24 h.

G⁻ and G⁺, EGFP-negative and positive respectively; M⁻ and M⁺, Mac-mix-negative and positive, respectively.

Mean % pre- and post-culture cell viabilities (determined by trypan blue exclusion) were as follows: wild-type G⁻M⁻, pre = 85.3, post = 57.9; wild-type G⁺M⁻, pre = 81.9, post = 63.0; wild-type G⁺M⁺, pre = 76.3, post = 57.0; ApcMin/+ G⁻M⁻, pre = 87.8, post = 56.7; ApcMin/+ G⁺M⁻, pre = 78.9, post = 58.1; ApcMin/+ G⁺M⁺, pre = 73.9, post = 49.7.

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Fig. 7. Flow cytometric sorting of viable SI SMNCs from wild-type and Lys-EGFP-Ki mice based on EGFP and macrophage-specific cell surface marker expression. (A) Representative plots of flow cytometry of wild-type and Lys-EGFP-Ki SMNCs for EGFP fluorescence. EGFP-positive (G⁺) cells were present only in the Lys-EGFP-Ki mouse. (B) Representative plots of Lys-EGFP-Ki × ApcMin/+ SMNCs labelled with either control rat IgG or the mix of three macrophage-specific cell surface marker antibodies (Mac-mix). Significant labelling of EGFP-negative (G⁻) SMNCs with control rat IgG, despite blocking with pre-immune mouse serum, meant that the Mac-mix (M⁺) gate was set relatively high in order to obtain pure G⁻M⁻ and G⁺M⁺ cell populations.
with SC236. These data suggest that the predominant source of ex vivo PGE$_2$ production by non-macrophage SMNCs is via a Cox-1-dependent pathway, whereas Cox-2 makes a greater contribution to PGE$_2$ synthesis by G$^+$M$^+$ macrophages.

**Discussion**

The fact that systemic selective COX-2 inhibition may not be free of significant side-effects suggests that future chemoprevention strategies, which selectively target COX-2, will need to address inhibition of COX-2 in a tissue-specific manner (36). To this end, one approach is to abrogate COX-2 expression during carcinogenesis. As Cox-2 is predominantly localized to stromal cells at early stages of intestinal tumorigenesis (1–2,8–13), we investigated mechanisms controlling stromal cell Cox-2 expression during Apc$^{Min/+}$ mouse intestinal adenoma development. Stromal macrophages also express Cox-2 in human sporadic colorectal adenomas (14–16) suggesting that our studies in the Apc$^{Min/+}$ mouse are likely to be of relevance to human colorectal carcinogenesis.

Having confirmed that Cox-2 expression by stromal macrophages in Apc$^{Min/+}$ mouse intestinal adenomas was not a property shared by other, distinct macrophage populations (in vitro differentiated BMDM and peritoneal macrophages) in Apc$^{Min/+}$ animals, we searched for local factors that could drive stromal cell Cox-2 expression. As Cox-2 protein is predominantly localized to stromal cells just below surface epithelium, we tested whether increased intestinal permeability to luminal antigen accounted for Cox-2 expression by stromal cells in adenomas. We did not demonstrate any significant difference in permeability to lactulose between Apc$^{Min/+}$ and wild-type mice at different stages of intestinal tumorigenesis, from a stage when micro-adenomata only are present (D30) to a situation in which macroscopic adenomas are noted along the length of the intestine (D140). We can not rule out small, localized changes in epithelial integrity in adenomas on the basis of this test of global intestinal permeability. However, the fact that Cox-2-expressing stromal cells in intestinal adenomas did not co-express Nos2 mRNA is also consistent with the absence of any change in epithelial permeability to luminal antigen, leading to ‘classical’ macrophage activation.

Recently, the concept of phenotypic heterogeneity of macrophages has strengthened with classification of M1 (‘classically’ activated) and M2 (or alternatively activated) macrophages as extremes of a continuum of phenotypic and functional states of these cells (37). Tumour-associated macrophages are often polarized to the M2 phenotype, a key feature of which is absence of Nos2 expression (38). It will be interesting to study other markers of the M2 phenotype (e.g. arginase) in Cox-2-expressing stromal macrophages in Apc$^{Min/+}$ mouse adenomas in order to define the predominant macrophage phenotype in these tumours. This is important as different cytokines (representing potential chemoprevention targets) drive M2, as opposed to M1, polarization e.g. interleukin (IL)-10, IL-4 and IL-13 (37,38).

We have demonstrated that viable SMNCs can be isolated from Apc$^{Min/+}$ animals and that phenotypically distinct sub-populations of SMNCs can be sorted for further in vitro studies. An important component of future studies will be improvement in overall SMNC viability, which will allow more refined ex vivo experimentation. We used Lys-EGFP-ki mice as a tool for investigation of myelomonocytic cells in the SI lamina propria, for the first time. Simultaneous labelling with a cocktail of antibodies to macrophage-specific epitopes (designed to maximize detection of phenotypically heterogeneous macrophage sub-populations) demonstrated that M-lysozyme is expressed by the majority of SI lamina propria macrophages and that neutrophils make a significant contribution to M-lysozyme expression in mouse SI. Previously, lysozyme mRNA was not detected in resident macrophages in mouse SI by in situ hybridization using a [35S]-labelled anti-sense lysozyme riboprobe (39). However, it is likely that this in situ hybridization technique was not sufficiently sensitive to detect relatively low level expression of M-lysozyme transcripts. Experiments with sorted SMNCs suggested that several cell types contribute to PGE2 production including G$^+$M$^+$ macrophages and G$^+$M$^-$ neutrophils. The relative contribution of Cox-1 and Cox-2 to PGE2 production appears to vary depending on the cellular source of PGE2 in the SI with Cox-2 being the predominant isoform responsible for PGE2 synthesis in G$^+$M$^+$ macrophages.

There was a significant increase in Cox-2-dependent PGE2 synthesis by isolated SMNCs from both Apc$^{Min/+}$ and wild-type mice between D30 and D70. This may be related to changes in diet and/or luminal flora in the post-weaning period. At D70, Cox-2-dependent PGE2 synthesis by isolated SMNCs was higher in Apc$^{Min/+}$ mice compared with wild-type littermates, despite little difference in Cox-2 mRNA or protein levels. This is consistent with previous data demonstrating a higher PGE2 content of histologically normal SI of Apc$^{Min/+}$ mice compared with wild-type SI (17). Our data imply that another rate-limiting step for PGE2 synthesis also controls PGE2 synthesis in Apc$^{Min/+}$ mice, perhaps involving Cox substrate (arachidonic acid) availability by phospholipases and/or generation of PGE2 by PGE synthases (40,41). To date, the role of different phospholipase A2 and PGE synthase isoforms in PGE2 synthesis by SMNCs and their role in intestinal tumorigenesis has received little attention compared with COX-2 (40–43), despite the fact that they represent potential targets for novel immunomodulatory and cancer chemoprevention therapy.

Consistent with our data showing decreased PGE2 synthesis by isolated SMNCs from Apc$^{Min/+}$ and wild-type mice between D70 and D110, Kettunen et al. (35) have demonstrated that PGE2 levels in the luminal content of the distal SI of Apc$^{Min/+}$ and wild-type mice decrease by 30–50% between 56 and 105 days of age. We consistently measured PGE2 synthesis by the same number of SMNCs from Apc$^{Min/+}$ and wild-type mice at each age. Therefore, selective depletion of Cox-2-expressing cells from the SI lamina propria of Apc$^{Min/+}$ mice by D110 could explain the overall decrease in PGE2 production by SMNCs despite ongoing growth of intestinal tumours containing Cox-2-positive stromal cells. Apc$^{Min/+}$ mice exhibit haematopoietic abnormalities, including peripheral lymphodepletion, from approximately day 80 onwards (44), which might impact on a specific population of PGE2-synthesizing cells in the intestinal mucosa. The peak in PGE2 synthesis by SMNCs at D70, at a time of active tumour growth in Apc$^{Min/+}$ mice, suggests that there may be an optimal period for targeting PGE2 synthesis and/or downstream PGE2 signaling during intestinal tumorigenesis.

In conclusion, we have addressed the novel concept of cell-specific inhibition of Cox-2 as a chemoprevention strategy by investigating the regulation of stromal Cox-2 expression during intestinal tumorigenesis in the Apc$^{Min/+}$ mouse. Simple
antigenic stimulation of stromal macrophages is unlikely to explain stromal cell Cox-2 expression in adenomas or the changes in PGE2 synthesis by isolated SMNCs at different stages of tumorigenesis. Further work is now required to define the precise mechanistic basis of stromal cell Cox-2 induction and changes in PGE2 synthesis during tumorigenesis.

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Conflict of Interest Statement

We declare that we have no financial or personal relationships that could inappropriately influence our work.

References


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