ABSTRACT

Cyclooxygenase-2 (COX-2), the inducible COX isozyme, plays a key role in intestinal tumorigenesis. We have demonstrated recently that COX-2 protein is induced in the polyp stroma near the intestinal luminal surface in the Apc\(^{-/}\) mouse, a model for human familial adenomatous polyposis, and stimulate tumor angiogenesis. However, the precise cell types that express COX-2 are still to be determined. By immunohistochemical analysis, here we show that the majority of COX-2-expressing cells in the intestinal polyps of Apc\(^{-/}\) mice are fibroblasts and endothelial cells. Furthermore, the COX-2-expressing cells in human familial adenomatous polyposis polyps are also fibroblasts and endothelial cells. In contrast, bone marrow-derived cells such as macrophages and leukocytes express little COX-2 protein in the intestinal polyps. These results clearly indicate that fibroblasts and endothelial cells play important roles in polyp expansion by expressing COX-2, resulting in tumor angiogenesis.

INTRODUCTION

Two isozymes of COX\(^{1}\) have been identified, COX-1 and COX-2 (1). They metabolize arachidonic acid to PGG\(_2\), which is converted further to various prostanooids; PGs, prostacyclin and thromboxane A\(_2\). Whereas COX-1 is expressed constitutively and involved in homeostatic functions, COX-2 is induced by various stimuli such as growth factors and cytokines. In human colon cancer, as well as polyps of FAP, COX-2 is significantly induced (2), and levels of PGE\(_2\) are increased (3). Using Apc\(^{-/}\) gene-knockout mice as a model for FAP (4), we have demonstrated recently that COX-2 plays a pivotal role in intestinal tumorigenesis (5, 6). Furthermore, we have shown that PGE\(_2\) receptor EP2 plays a crucial role for both COX-2 induction by a positive feedback mechanism and polyp formation through angiogenesis (6, 7).

Whereas COX-2 is expressed in cancer epithelial cells in human colon and other organs, its expression in benign polyps is found in the stromal cells, in both human FAP and its mouse model Apc\(^{-/}\) mice (5–11). Although myofibroblasts (11) and macrophages (12) have been suggested to express COX-2 in the polyps, quantitative data have been lacking. To determine the cell types that express COX-2 in the polyps, we have quantified immunostained cells with various markers.

MATERIALS AND METHODS

Apc\(^{-/}\) Knockout Mouse Construction. Details of Apc\(^{-/}\) knockout mouse construction were described previously (4).

Histological Analysis, Immunohistochemistry, and Immunofluorescence. To prepare paraffin sections, Apc\(^{-/}\) intestines were fixed in 4% paraformaldehyde, embedded in paraffin wax, and sectioned at 4-µm thickness. Sections were stained with H&E or incubated with a primary antibody specific for COX-2 (Cayman Chemical Co.) and then with a biotinylated secondary antibody followed by treatment with an avidin-biotin-peroxidase complex (Vector Laboratories). For immunofluorescence staining, Apc\(^{-/}\) intestines fixed in 4% paraformaldehyde were embedded in OCT compound, frozen, and sectioned at 7-µm thickness. Sections were incubated with the anti-COX-2 antibody simultaneously with a specific antibody for vimentin, α-SMA (Sigma Chemical Co.) biotinylated with NHS-LC-Biotin (Pierce), CD34 (Santa Cruz Biotechnology), CD31, CD11b (BD Pharmingen), or F4/80 (Serotec Ltd.), followed by Alexa Fluor antibodies (Molecular Probes Inc.) or Fluorescein Avidin DCS (Vector Laboratories).

RESULTS

Fibroblast Appearance of COX-2-expressing Cells in Mouse Intestinal Polyps. We have demonstrated recently that induction of COX-2 plays a critical role in the expansion of Apc\(^{-/}\) intestinal and colonic polyps (5–7). To determine the cell types that express COX-2 in the polyp stroma, we first analyzed small intestinal samples from Apc\(^{-/}\) mice by immunohistochemistry (Fig. 1). Although little COX-2 protein was found in the normal mucosa, abundant COX-2 was expressed in the polyp stroma near the luminal surface (Fig. 1, A and B, arrowheads), consistent with our earlier results (5–7). Histological examinations of these COX-2-expressing cells in more detail revealed that the majority of such cells contained relatively large and ovoid nuclei, prominent nucleoli, and extensive cytoplasm (Fig. 1C, arrowheads). These morphological features for the major COX-2-expressing cell population matched those of active fibroblasts (13).

Coexpression of COX-2 and Vimentin in Polyp Stromal Cells. To investigate whether most COX-2-expressing cells were fibroblasts, we next investigated the expression of intermediate filament protein vimentin, a fibroblast marker (14). As expected, vimentin was also expressed in the polyp stroma near the luminal surface (Fig. 1E) where COX-2 was expressed (Fig. 1D). In fact, 85% of COX-2-expressing cells were also expressing vimentin as shown in the merged photograph (Fig. 1F; Table 1). It should be noted that the fluorescence signals for COX-2 and vimentin were not precisely superimposed inside a single expressing cell, because subcellular localizations of these two markers were different. Whereas COX-2 was expressed in the endoplasmic reticulum and nuclear membrane, intermediate filament vimentin was in the cytoplasm.

Little Coexpression of COX-2 and α-Smooth Muscle Actin. It has been reported that submucosal myofibroblasts, another fibroblast subclass, also express vimentin (14–16), and that myofibroblasts express COX-2 in the polyp stroma of Apc\(^{-/}\) mice (11). Accordingly, we double stained the polyp sections for COX-2 and α-SMA, a marker for myofibroblasts (14–16). As we reported previously (6), α-SMA immunostaining was rarely found in the polyp stroma near the intestinal lumen where COX-2-expressing cells were found (Fig. 1, G–I). In fact, only 0.6% of the COX-2-expressing cells were concomitantly stained for α-SMA (Table 1).

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3 The abbreviations used are: COX, cyclooxygenase; Apc, adenomatous polyposis coli; α-SMA, α-smooth muscle actin; FAP, familial adenomatous polyposis; PG, prostaglandin; VWF, von Willebrand factor; PGE\(_2\), prostaglandin E\(_2\).
Coexpression of COX-2 and CD34 in Some Polyp Endothelial Cells. Because endothelial cells can also express COX-2 (17), we then investigated the spatial relationship between the COX-2-expressing cells and vascular endothelial cells by a double staining for COX-2 and CD34, a marker for endothelial cells (Fig. 2, A and B). As shown in the merged photograph (Fig. 2 C), endothelial cells accounted for 4.7% of all of the COX-2-positive cells (Table 1). However, it is worth noting that the COX-2 staining in the endothelial cells showed a punctate pattern (Fig. 2 C) different from that in the fibroblasts where endoplasmic reticulum and nuclear membranes were stained (Fig. 1 F). Essentially the same results were obtained using antibody for CD31, another representative endothelial cell marker (data not shown).

Little Coexpression of COX-2 and F4/80 in Polyp Macrophages. Because macrophages have been reported to express COX-2 protein in the polyp stroma of the ApcMin mouse intestines (12), we next stained the ApcMin polyp sections for F4/80, one of the representative macrophage markers (18). As reported (12), F4/80 immunostainings were found in the stroma near the polyp base (data not shown). Although some macrophages were found also in the polyp stroma where COX-2-positive cells were found (Fig. 2, D and E), they expressed little COX-2 (Fig. 2 F). In fact, they constituted only 1% of the total COX-2-expressing cells (Table 1). Likewise, immunostaining for bone marrow-derived cell marker CD11b was also found in the polyp stromal cells different from those expressing COX-2.

Table 1 Colocalization of COX-2 and cell-type markers in the stroma of ApcMin polyps

<table>
<thead>
<tr>
<th>Marker (Cell type)</th>
<th>No. of marker-expressing cells</th>
<th>No. of COX-2-expressing cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vimentin (fibroblast)</td>
<td>1338/1574 (85.0%)</td>
<td></td>
</tr>
<tr>
<td>CD34 (endothelial cell)</td>
<td>111/2370 (4.7%)</td>
<td></td>
</tr>
<tr>
<td>(\alpha)-SMA (myofibroblast)</td>
<td>16/2755 (0.6%)</td>
<td></td>
</tr>
<tr>
<td>F4/80 (macrophage)</td>
<td>15/1480 (1.0%)</td>
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a Results from 10–15 representative polyps.

b These populations can partly overlap because of coexpression of some markers.

Fig. 1. COX-2 expression in the ApcMin small intestinal polyp stroma. A, COX-2 immunostaining in a section containing a polyp and the normal mucosa (hematoxylin counterstaining). B, higher magnification of the boxed area in (A), showing COX-2-expressing stromal cells with relatively large and ovoid nuclei (arrowheads). C, a serial section of B, showing polyp stromal cells with prominent nucleoli and extensive cytoplasm (arrowheads; H&E). D-F, a cryosection stained for COX-2 (D), vimentin (E), and the merged image (F) showing fibroblasts as the major population of the COX-2-expressing cells. G-I, a cryosection stained for COX-2 (G), \(\alpha\)-SMA (H), and the merged image (I), showing little COX-2 expression in myofibroblasts. Bars, 100 \(\mu\)m in (A) and 10 \(\mu\)m in (B) to (F).
To determine whether fibroblasts and endothelial cells express COX-2 in human FAP polyps as in the mouse model, we additionally performed immunostainings on FAP polyp sections. Like in the Apc<sup>Δ716</sup> polyps, COX-2 immunostaining was found in the stromal cells near the luminal surface (Fig. 3A). Interestingly, the majority of these cells again showed morphological characteristics of active fibroblasts. Consistent with the mouse polyp results, the human COX-2-expressing cells were also stained for vimentin (Fig. 3B) but not for α-SMA (Fig. 3C), indicating that the majority of these cells were fibroblasts. Furthermore, immunostaining of vWF, another representative endothelial cell marker, was also found in some of the COX-2-expressing cells (Fig. 3D). These results indicate that fibroblasts and endothelial cells express COX-2 in FAP polyps as in the mouse model.

**DISCUSSION**

Accumulating evidence indicates that COX-2 and one of its downstream products, PGE<sub>2</sub>, play key roles in intestinal tumorigenesis. When the COX-2 gene (Ptgs2) is knocked out in Apc<sup>Δ716</sup> mice, formation of the intestinal polyph is markedly suppressed (5). Furthermore, disruption of the gene for EP2, one of four PGE<sub>2</sub> receptors, in Apc<sup>Δ716</sup> mice causes similar suppression to the COX-2 gene disruption, whereas mutation in either EP1 or EP3 gene shows little effect (7). This effect is largely because of the blockade of the positive feedback mechanism for COX-2 expression by PGE<sub>2</sub> in the polyp stromal cells.

To determine what type of cells express COX-2, we performed histological and marker-immunostaining studies. In a histological analysis, the COX-2-expressing cells had a characteristic morphology of active fibroblasts; i.e., relatively large and ovoid nuclei, prominent nucleoli, and extensive cytoplasm (13). Confirmed by their vimentin expression, these cells have been identified as fibroblasts. This is consistent with our earlier results of immuno-electron-microscopic study showing the COX-2 gene promoter activity in fibroblasts of the Apc<sup>Δ716</sup> polyp stroma (19). Interestingly, vimentin-positive stromal cells were found recently to express COX-2 also in human gastric cancer (20), suggesting that fibroblasts play a key role in not only intestinal but also gastric tumorigenesis by expressing COX-2.

As a subclass of fibroblasts in the intestines, submucosal myofibroblasts should be discriminated from the active or mature fibroblasts for their distinct features; namely, expression of α-SMA simultaneously with vimentin and the contractile characteristic (15, 16). It has been reported that myofibroblasts express COX-2 in the Apc<sup>Δ716</sup> polyps, or interleukin 10 knockout mice, a model for inflammatory bowel disease and colitis-associated carcinoma (11). However, in the present study, we detected little simultaneous immunostainings of COX-2 and α-SMA in the polyp stroma of Apc<sup>Δ716</sup> mice or human FAP. Because the anti-α-SMA antibody 1A4 that we and Shattuck-Brandt et al. (11) used was generated by a mouse hybridoma, endogenous mouse immunoglobulin may be stained as background signals by the secondary antimouse antibody. To avoid this problem, we first biotinylated the 1A4 antibody and detected it with fluorescein-conjugated avidin. With this improved sensitivity for α-SMA, myofibroblasts were unambiguously identified for their elongated morphology (Fig. 1H) and epithelium-associated location (data not shown). Although myofibroblasts expressed little COX-2 in this study, they might contribute to tumorigenesis in other ways, such as cytokine production and immune system modulation (15, 16). In addition to myofibroblasts, macrophages are suggested to express COX-2 in Apc<sup>Δ716</sup> mice (12). Although we found some macrophages in the stroma near the
polyp base, we detected little COX-2 expression in this subregion of the polyp stroma. The results are consistent with our earlier immunostaining study of LacZ reporter protein of which expression is driven by endogenous COX-2 promoter (5). Although we found little COX-2 expression in a polyp of an FAP patient (hematoxylin counterstaining), respectively, in a polyp of an FAP patient (hematoxylin counterstaining).

In conclusion, these results clearly show significant roles of fibroblasts and endothelial cells in the intestinal polyp development through COX-2 expression.

ACKNOWLEDGMENTS

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REFERENCES


Fig. 3. COX-2 expression in fibroblasts and endothelial cells, but not in myofibroblasts in human FAP polyps. A–D, serial sections immunostained for COX-2 (A), vimentin (B), α-SMA (C), and vWF (D), respectively, in a polyp of an FAP patient (hematoxylin counterstaining). Bars, 50 μm.
Cyclooxygenase-2 Expression in Fibroblasts and Endothelial Cells of Intestinal Polyps

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