American Journal of Pathology, Vol. 156, No. 2, February 2000

Localization of Cyclooxygenase-2 in Human Sporadic Colorectal Adenomas

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A putative target for the anti-colorectal cancer action of nonsteroidal anti-inflammatory drugs is the inducible isoform of cyclooxygenase (COX), COX-2. COX-2 is expressed within intestinal adenomas in murine polyposis models, but expression has been poorly characterized in human colorectal neoplasms. Therefore, we investigated the localization of the COX-2 protein in human sporadic colorectal adenomas. Immunohistochemistry for COX-2 and CD68 (a tissue macrophage marker) was performed on formalin-fixed, paraffin-embedded (n = 52) and frozen, acetone-fixed (n = 6) sections of human sporadic colorectal adenomas. Forty of 52 (77%) formalin-fixed adenomas expressed immunoreactive COX-2. COX-2 was localized to superficial interstitial macrophages in 39 cases (75%) and to deep interstitial macrophages in 9 cases (17%). COX-2 staining of dysplastic epithelial cells was observed in 15 cases (29%). A logistic regression analysis identified the adenoma site (P = 0.012) and histological type (P = 0.001) as independent predictors of superficial macrophage COX-2 expression. There was no relationship between the number of macrophages within an adenoma and macrophage COX-2 expression. These results indicate that COX-2 is expressed predominantly by interstitial macrophages within human sporadic colorectal adenomas. If COX-2 does indeed play a role in the early stages of colorectal carcinogenesis in man, these data suggest COX-2-mediated paracrine signaling between the macrophages and epithelial cells within adenomas. ***(Am J Pathol 2000, 156:545–553)***

A substantial body of evidence from epidemiological studies and animal models of intestinal tumorigenesis indicates that nonsteroidal anti-inflammatory drugs (NSAID) are effective chemopreventative agents against colorectal cancer.[1,2] The mechanism of the anti-neoplastic activity of NSAIDs remains unclear, but one possible route is via the inhibition of cyclooxygenase (COX).[3] Two isoforms of COX have been described:[4] COX-1, which is constitutively expressed in normal adult human tissues including the colon, [5–9] and an inducible isoform, COX-2, the expression of which is induced in cultured cells by cytokines and growth factors.[4] COX-2 is absent or expressed at low levels in the normal human colon.[5–8,10,11] However, COX-2 expression is upregulated in 85 to 100% of human sporadic colorectal carcinomas,[5,6,10–13] predominantly within neoplastic epithelial cells, in which COX-2 may induce resistance to apoptosis,[14] alter extracellular matrix adhesion,[14] modulate tumor angiogenesis,[15] and increase metastatic potential.[16]

COX-2 also plays an important role at an earlier stage of intestinal tumorigenesis. Within intestinal adenomas of Min and *Apc*$^{\Delta 716}$ mouse models of familial adenomatous polyposis, Cox-2 is localized to interstitial cells, which have been identified as macrophages.[17–19] Disruption of *Ptgs2* (the mouse COX-2 gene) and administration of the selective Cox-2 inhibitor, MF-tricyclic, in the *Apc*$^{\Delta 716}$ mouse have both been shown to dramatically reduce intestinal adenoma development.[17]

Although sporadic colorectal adenoma development and progression are potential targets for colorectal cancer chemoprevention, the expression of COX-2 in human sporadic colorectal adenomas has received little attention. Studies of small numbers of sporadic and familial adenomatous polyposis adenomas have produced variable results with the expression of COX-2 demonstrated in between 0 to 90% of the adenomas.[6,10,13,18,20] We, therefore, performed an immunohistochemical study of COX-2 protein expression and localization in a large series of human sporadic colorectal adenomas.

Materials and Methods

Ethical approval for the study was obtained from the St. James's and Seacroft University Hospitals Research Ethics Committee.

Supported by a Royal College of Surgeons of England Clinical Research Fellowship (K. S. C.) and funding from Yorkshire Cancer Research (E. J. C. and G. H.) and the Medical Research Council of the United Kingdom (M. A. H.).

Accepted for publication October 8, 1999.

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Human Sporadic Colorectal Adenomas

Formalin-fixed, paraffin-embedded specimens of human sporadic colorectal adenomas and adjacent normal colorectal tissue were randomly selected from the histopathology archives at St. James's University Hospital. All specimens had been obtained by endoscopic biopsy, polypectomy, or surgical resection during the preceding nine months. Adenomas from patients with inflammatory bowel disease or familial adenomatous polyposis were excluded from the study.

Adenoma tissue was also obtained immediately after endoscopic polypectomy, embedded in OCT compound (Merck Ltd., Poole, UK), and immediately snap-frozen in isopentane cooled in liquid nitrogen before storage at −70°C.

The age and gender of patients were noted. The size (maximum diameter in millimeters of polypectomy and surgical resection specimens), site (proximal or distal to the splenic flexure), histological type (tubular, tubulovillous, or villous by WHO criteria[21]), and the highest histological grade of dysplasia (mild, moderate or severe) of adenomas were obtained from histopathology data files. Information including a history of NSAID (including low-dose aspirin use), oral corticosteroid use (defined as use at least once a month in 2 or more consecutive months during the previous 2 years), or family history of colorectal cancer (one or more first- or second-degree relatives diagnosed with colorectal cancer) was sought by a case note review (data ascertained in 95% of cases) and by a postal questionnaire (response rate 91%).

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections (3 μm thick) were mounted on 3-aminopropyltriethoxysilane-coated glass slides. Sections were dewaxed in xylene (three times for 5 minutes), and rehydrated through a graded alcohol series (three times for 5 minutes). Endogenous peroxidase activity was blocked with 0.6% hydrogen peroxide in 100% methanol for 15 minutes at room temperature. After washing in water, nonspecific binding sites were blocked with 5% swine serum (DAKO Ltd., High Wycombe, UK) in phosphate-buffered saline (PBS) for 30 minutes at room temperature. We used affinity-purified rabbit polyclonal anti-human COX-2 IgG, (IBL, Gunma, Japan; 100 μg/ml),[22,23] which was generated by immunization with a synthetic 17-mer peptide (TVKDTQAEMIYPPQVPE) corresponding to residues 251 to 267 of human COX-2. The primary antibody was diluted 1:25 in PBS and incubated with sections overnight at 4°C. After washing in PBS (two times for 10 minutes), sections were incubated with a 1:200 dilution of biotinylated swine anti-rabbit IgG (DAKO) for 25 minutes at room temperature. After further PBS washes, sections were incubated with streptavidin/biotin-horseradish peroxidase complex (DAKO) in 80 mmol/L Tris-HCl, pH 7.6, for 30 minutes at room temperature. Sections were visualized using 3,3′-diaminobenzidine tetrahydrochloride (0.7 mg/ml) and 0.05% hydrogen peroxide in 80 mmol/L Tris-HCl, pH 7.6, for 10 minutes. The sections were counterstained in Mayer's hematoxylin for 3 minutes before dehydration, using a sequential alcohol and xylene series. The sections were mounted in diphenylxylene (BDH, Poole, UK).

Frozen sections (5 μm thick) were mounted on Superfrost Plus slides (BDH), fixed in 100% acetone for 2 minutes at room temperature, and air-dried for 10 minutes immediately before performing COX-2 immunohistochemistry as above.

The negative controls included omission of the primary antibody and antibody preadsorption with its cognate peptide (4 μg/ml, IBL) for 2 hours at 4°C. Human sporadic colorectal carcinomas ($n = 3$) were used as a positive tissue control.[5] In addition, we confirmed the specificity of the primary antibody for COX-2 by Western blot analysis of purified ovine Cox-1 and Cox-2 (Cayman Chemical Co., Ann Arbor, MI) and whole-cell lysates of human umbilical vein endothelial cells in the absence (COX-2-negative) or presence of 20 ng/ml phorbol 12-myristate 13-acetate for 6 hours (COX-2-positive).

We also performed COX-2 immunohistochemistry on formalin-fixed, paraffin-embedded sections from the same adenoma series, using rabbit anti-mouse COX-2 antiserum (Cayman Chemical Co.), which we have described previously.[19] The antiserum was generated by immunization with a synthetic 17-mer polypeptide (CY-SHSRLDDINPTVLIK), which corresponds to a C-terminal sequence in murine COX-2 (residues 584–598) with 80% homology to human COX-2. This antibody has previously been shown to recognize human COX-2 but not COX-1.[24] Immunohistochemistry was performed as above except that the sections were placed in 10 mmol/L citrate buffer, pH 6.0, and heated to 80°C for 10 minutes in a microwave oven after the blocking of the endogenous peroxidase activity. The negative controls for this antibody, including antibody preadsorption, were performed as described.[19]

Adjacent sections were stained with mouse monoclonal anti-human CD68 IgG (clone KP1; DAKO), which recognizes mature tissue macrophages.[25] The sections underwent antigen retrieval by pressure cooking (100°C, pressure 15 psi) in a 10 mmol/L citrate buffer, pH 6.0, for 60 seconds before cooling in tap water. The primary antibody, diluted 1:100 in PBS, was incubated with sections for 60 minutes at room temperature, and biotinylated rabbit anti-mouse IgG (DAKO) was used at a 1:200 dilution. The primary antibody was omitted as a negative control and human tonsil was used as a positive tissue control.[26]

Immunofluorescence

Colocalization of COX-2 and CD68 was performed using dual-labeling indirect immunofluorescence on frozen sections and using polyclonal anti-human COX-2 (IBL) and anti-CD68 antibodies. Nonspecific binding sites were blocked using 5% goat serum (Sigma Chemical Co., St Louis, MO) in PBS for 30 minutes at room temperature. COX-2 and CD68 antibodies (both diluted 1:25 in PBS) were simultaneously applied for 60 minutes at room

temperature. After washes with PBS (four times for 5 minutes), sections were incubated with tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (IgG; COX-2; Sigma) and fluorescein isothiocyanate-conjugated goat anti-mouse IgG (CD68; Sigma) for 60 minutes at room temperature at dilutions of 1:200 and 1:25, respectively. After washing in PBS four times for 5 minutes, sections were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and visualized with a Zeiss Axioplan fluorescence microscope equipped with a dual filter for fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate (Zeiss filter set 487924). The controls included the omission of one or both of the primary antibodies and the omission of one or both of the labeled secondary antibodies.

Semiquantitative Assessment of COX-2 and CD68 Immunostaining

COX-2 staining was assessed by two independent observers who were blinded to the origin of the sections. COX-2 protein expression in epithelial cells, interstitial cells immediately below the luminal surface of the adenoma (superficial interstitial cells), and interstitial cells deep within the body of the adenoma (deep interstitial cells) were scored separately on a scale of 0–3 based on both degree and intensity of staining (0, no staining; 1, small numbers, ie <10%, of COX-2-positive cells; 2, 10–50% of the cells were positive for COX-2; 3, >50% of cells were COX-2-positive with intense cellular staining). Data are expressed as the median COX-2 expression score and interquartile range (IQR). An interobserver concordance of COX-2 scores was 85% for superficial interstitial cells, 78% for deep interstitial cells, and 78% for epithelial cells.

The density of the CD68-positive macrophage infiltrate within adenomas was also measured. The mean number of CD68-positive cells counted in three random high-power fields (hpf) was scored on a scale of 1 to 3 (1, less than 25 CD68-positive cells/hpf; 2, 25–49 CD68-positive cells/hpf; 3, 50 or more CD68-positive cells/hpf).

Statistical Analysis

A logistic regression with forward conditional selection was performed to identify the factors that predicted COX-2 protein expression by adenomas. The following factors were included in the model: patient age, gender, NSAID/corticosteroid use, family history of colorectal carcinoma, adenoma site, adenoma size (for polypectomy and surgical specimens), histological type, grade of dysplasia and mode of excision (biopsy or polypectomy/surgical resection). The significance of the differences in COX-2 protein expression related to the adenoma site, histological type, grade of dysplasia, and patient age was tested using either a Mann-Whitney *U* test or Kruskal-Wallis one-way analysis of variance. The relationship between the interstitial cell COX-2 expression score and macrophage density was tested with the Spearman rank correlation coefficient. Statistical significance was assumed if the *P* value was less than or equal to 0.05.

Results

Sporadic Human Colorectal Adenomas

Fifty-two formalin-fixed, paraffin-embedded adenoma specimens (14 endoscopic biopsies, 33 endoscopic polypectomies, 5 surgical polypectomies) were studied from 44 patients (24 male, 20 female, age range 44–84 years). Mean adenoma diameter was 11.9 ± 1.0 (SEM) mm. An examination of the sections stained with hematoxylin and eosin (H&E) revealed 27 (52%) tubular, 22 (42%) tubulo-villous, and 3 (6%) villous adenomas. Of the 52 adenomas, 20 (38%) exhibited mild dysplasia, 25 (48%) moderate dysplasia, and 7 (14%) severe dysplasia; 42 (81%) of the adenomas were located distal to the splenic flexure, and 10 (19%) were proximal to the splenic flexure. Of the 44 patients, 23 (46%) described a history of NSAID or corticosteroid use, with daily or alternate-day basis in 13 (30%). Five patients (11%) had a family history of colorectal cancer.

Six frozen, acetone-fixed specimens were studied from six patients (3 male, 3 female; age range 57–78 years). The mean adenoma size was 13.0 ± 2.9 (SEM) mm. An examination of the H&E-stained sections revealed four tubular adenomas and two tubulo-villous adenomas. Two adenomas exhibited mild dysplasia, two moderate dysplasia, and two severe dysplasia. Four adenomas were located distal to the splenic flexure, and two were proximal to the splenic flexure.

COX-2 Localization

Overall, COX-2 protein was detected in 40/52 (77%) human sporadic colorectal adenomas. Immunoreactive COX-2 was localized to superficial interstitial cells at the luminal surface of the adenoma in 39 (75%) cases (Figure 1, A-D). Aggregates of COX-2-positive interstitial cells, which were located just below the surface epithelium, were separated by interstitial tissue containing no COX-2-positive cells (Figure 1A). The degree of superficial interstitial cell COX-2 immunostaining varied between adenomas (Table 1). In 17 adenomas, widely scattered small groups of COX-2-positive cells made up a small minority of the superficial interstitial cell infiltrate of the adenoma. In other adenomas (n = 10), COX-2-positive interstitial cells accounted for the majority of the superficial interstitial cells (scored 3; Figure 1, A and C). COX-2-positive interstitial cells were frequently observed at the tips of dysplastic villi (Figure 1, B-D), often beneath the flattened epithelium (Figure 1D). The close proximity of COX-2-positive cells to capillaries was a prominent feature (Figure 1, B-D).

COX-2 staining was also detected in the deep interstitial cells within the body of the adenoma in nine (17%) cases (Figure 1E). These COX-2-positive cells were smaller and more widely separated than superficial inter-

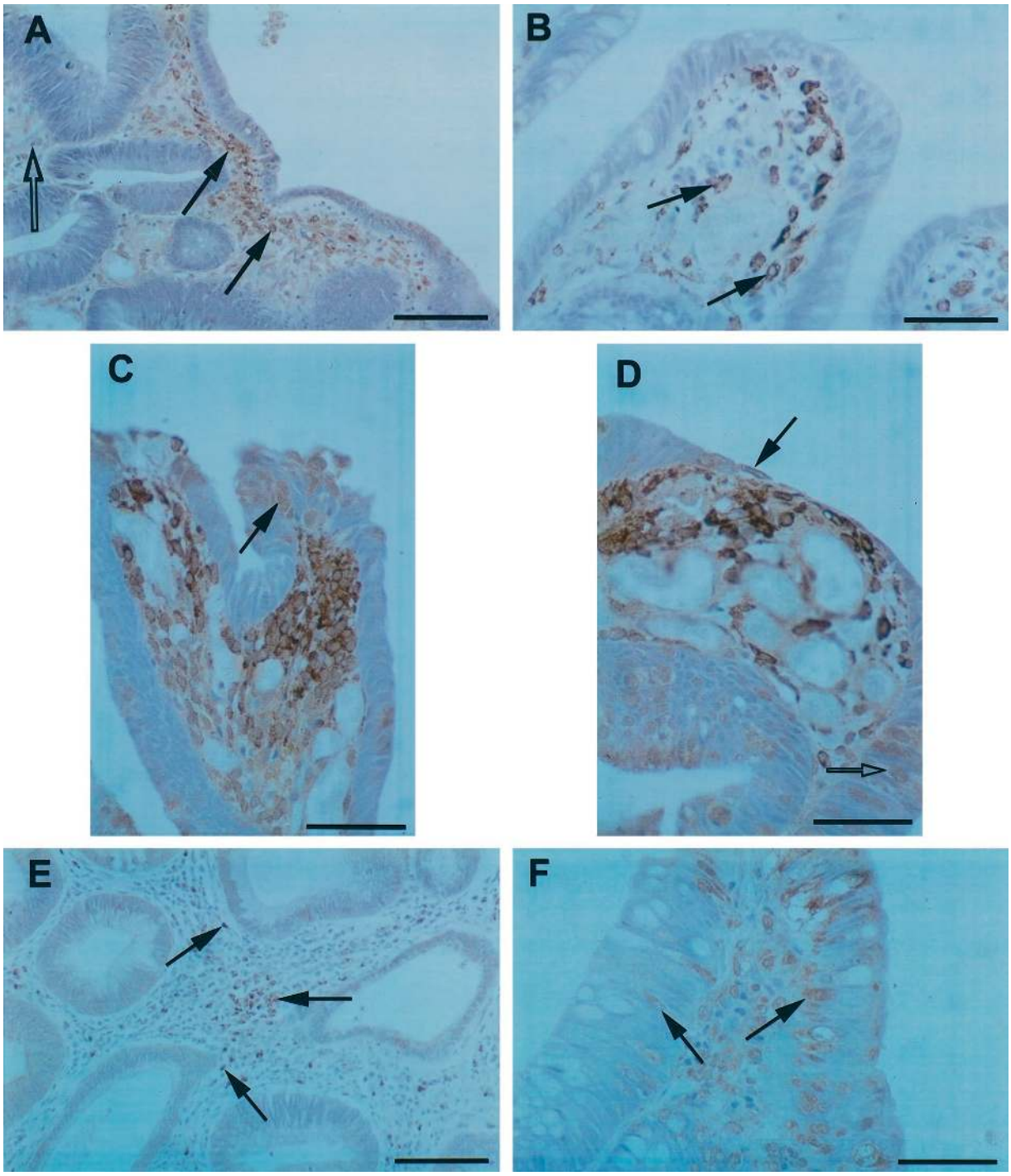
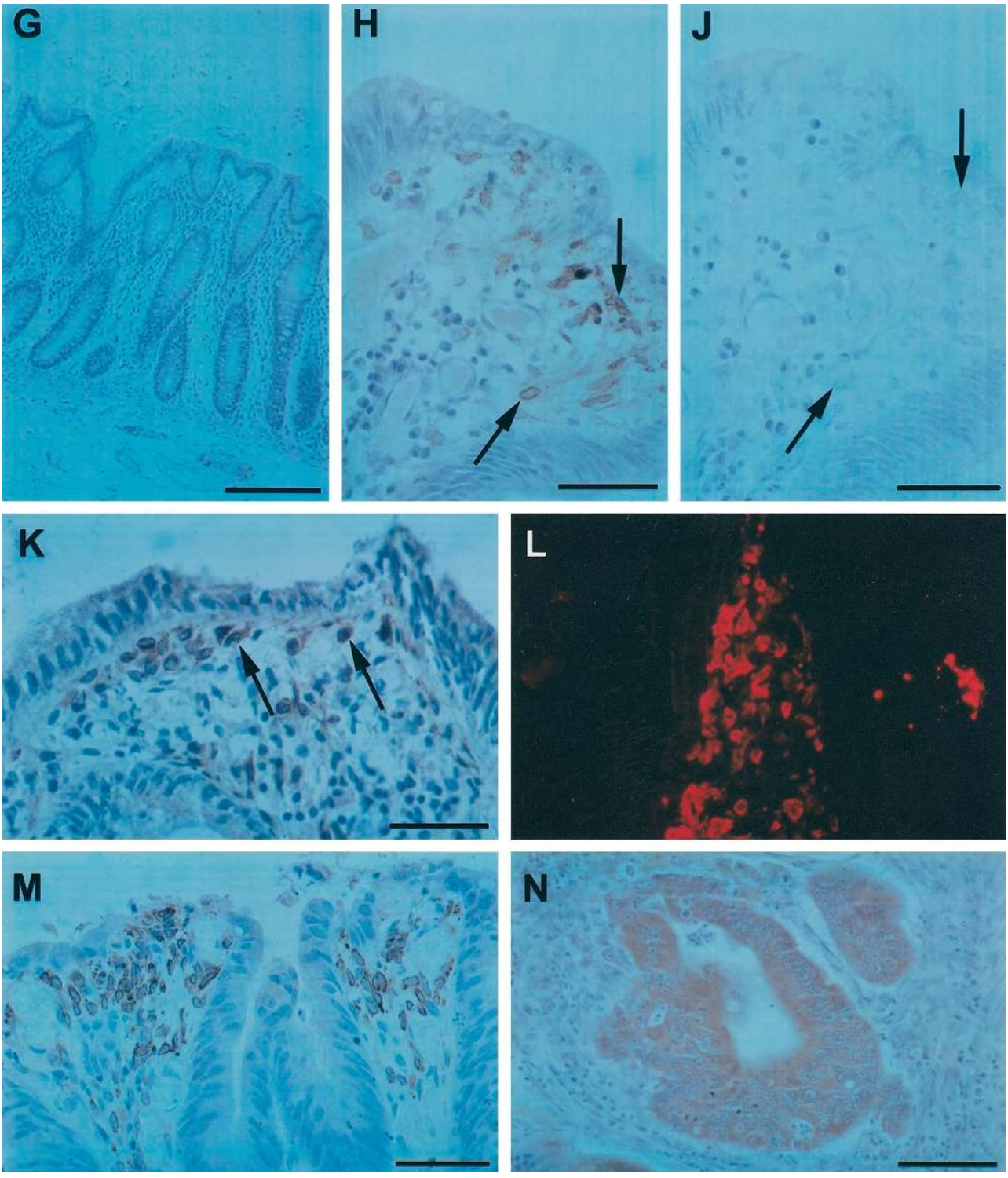


Figure 1. Immunohistochemistry for COX-2 on human sporadic colorectal adenomas. Affinity-purified rabbit polyclonal anti-human COX-2 IgG was used unless stated otherwise. **A:** COX-2 immunostaining of superficial interstitial cells (**closed arrows**) adjacent to COX-2-negative epithelial cells at the flat luminal surface of an adenoma. Within adenomas, aggregates of COX-2-positive superficial interstitial cells were separated by interstitium containing no COX-2-positive cells (**open arrow**). Scale bar = 100 μm. **B:** COX-2-positive superficial interstitial cells (**arrows**) at the tip of a dysplastic villus. Cells were mononuclear with large ovoid nuclei. COX-2 immunoreactivity was demonstrated in a perinuclear distribution (**arrows**; see also panels **C**, **D**). Scale bar = 50 μm. **C:** Numerous COX-2-positive superficial interstitial cells in close proximity to capillaries (see also **D**). The epithelium contained occasional COX-2-positive cells (**arrow**; see also **D**, **F**). Scale bar = 50 μm. **D:** COX-2-positive cells adjacent to flattened epithelium (**closed arrow**) at the tip of a villus. Note the densely vascular area immediately adjacent to the COX-2-positive cell infiltrate. COX-2-positive epithelial cells were also observed (**open arrow**). Scale bar = 50 μm. **E:** COX-2-positive interstitial cells deep within an adenoma (**arrows**). In contrast to superficial interstitial cells (see **A–D**), deep interstitial cells were smaller and less closely aggregated. Scale bar = 100 μm. **F:** COX-2 localization to epithelial cells (**arrows**). Staining was abolished by antibody preadsorption with its cognate peptide. Scale bar = 50 μm. **G:** Normal colonic mucosa. No COX-2 immunoreactivity was detected. Scale bar = 200 μm. **H:** COX-2 localization to superficial interstitial cells (**arrows**). Scale bar = 50 μm. **J:** Preadsorption of COX-2 antibody with its cognate peptide. There was abolition of specific COX-2 staining compared with panel H (**arrows**). Scale bar = 50 μm. **K:** COX-2 immunohistochemistry on frozen, acetone-fixed tissue, using peroxidase detection. Localization of COX-2 to superficial interstitial cells (**arrows**) was similar to that demonstrated in fixed tissue (compare with **A**) and was abolished by antibody preincubation with its cognate peptide. Faint staining of the epithelium was non-specific and was not abolished by antibody pre-adsorption. Scale bar = 50 μm. **L:** COX-2 immunohistochemistry on frozen, acetone-fixed tissue, using fluorescent detection (TRITC). Numerous COX-2-positive interstitial cells (red) were observed. No epithelial cell staining was detected. **M:** COX-2 immunohistochemistry on formalin-fixed, paraffin-embedded sections, using murine COX-2 antiserum. There was a similar distribution of immunoreactivity to that obtained using rabbit polyclonal anti-human COX-2 IgG (see **A–D**). Scale bar = 50 μm. **N:** Human sporadic colorectal carcinoma. Diffuse cytoplasmic COX-2 staining was apparent in cancer cells. Scale bar = 50 μm.



stitial COX-2-positive cells. In the majority of adenomas (n = 37), COX-2 was not detected in epithelial cells. Dysplastic epithelial cells expressed COX-2 protein in only 15 (29%) cases (Figure 1, C, D, and F). COX-2 staining was noted in the superficial epithelial cells that were adjacent to COX-2-positive interstitial cells (Figure 1, C, D, and F), but was also observed in epithelial cells deep within adenomas. No immunoreactive COX-2 protein was detected in paired samples (n = 16) of histologically normal colorectal mucosa (Figure 1G).

The control sections confirmed the specificity of COX-2 staining observed with polyclonal anti-human COX-2 IgG. The omission of the primary antibody was associated with no positive staining. Preadsorption of the antibody with its cognate peptide abolished staining in interstitial cells (compare Figure 1, panels H and J) and in epithelial cells

Table 1. Distribution of COX-2 Expression Scores in 52 Formalin-Fixed, Paraffin-Embedded Sporadic Colorectal Adenomas

Score	Superficial interstitial cells	Deep interstitial cells	Epithelial cells
0	13	43	37
1	17	7	9
2	12	0	5
3	10	2	1

(data not shown). COX-2 immunohistochemistry performed on acetone-fixed, frozen sections using both peroxidase- and tetramethylrhodamine isothiocyanate-conjugated secondary antibodies demonstrated identical COX-2 immunostaining to that obtained with formalin-fixed sections (Figure 1, K and L). Immunohistochemistry with murine COX-2 antiserum (n = 11) produced an identical pattern of COX-2 staining to that seen with polyclonal anti-human COX-2 IgG (Figure 1M). Staining with this antibody was also abolished by antibody preadsorption with its cognate peptide. Concordance between COX-2 expression scores for the two antibodies was 91% for superficial interstitial cells, 91% for deep interstitial cells, and 82% for epithelial cells. A diffuse cytoplasmic staining of cancer cells was consistently demonstrated in human colorectal carcinoma specimens (Figure 1N) as has been demonstrated by others.[5]

Immunohistochemistry for CD68 (n = 26) revealed large numbers of interstitial macrophages within adenomas. There was a wide variation in the macrophage number in the adenomas examined (score 1, n = 11; 2, n = 8; 3, n = 7). A proportion of the CD68-positive macrophage population was observed at the tips of villi in a similar distribution to COX-2-positive superficial interstitial cells. Furthermore, COX-2-positive superficial interstitial cells had characteristic features of macrophages (mononuclear cells with large ovoid nuclei; Figure 1B). Therefore, we proceeded to test whether COX-2 localized to CD68-positive macrophages, using dual-labeling immunofluorescence. The control sections showed no cross-reactivity between the primary and secondary antibodies. Numerous interstitial CD68-positive cells (Figure 2A) and a smaller number of interstitial COX-2-positive cells (Figure 2B) were observed by single immunofluorescence. Observation with the dual filter demonstrated that the COX-2 protein expression was restricted to a subgroup of CD68-positive macrophages (Figure 2C). COX-2 localized to both superficial and deep CD68-positive macrophages. Immunoreactive COX-2 was not detected in CD68-negative cells (Figure 2C).

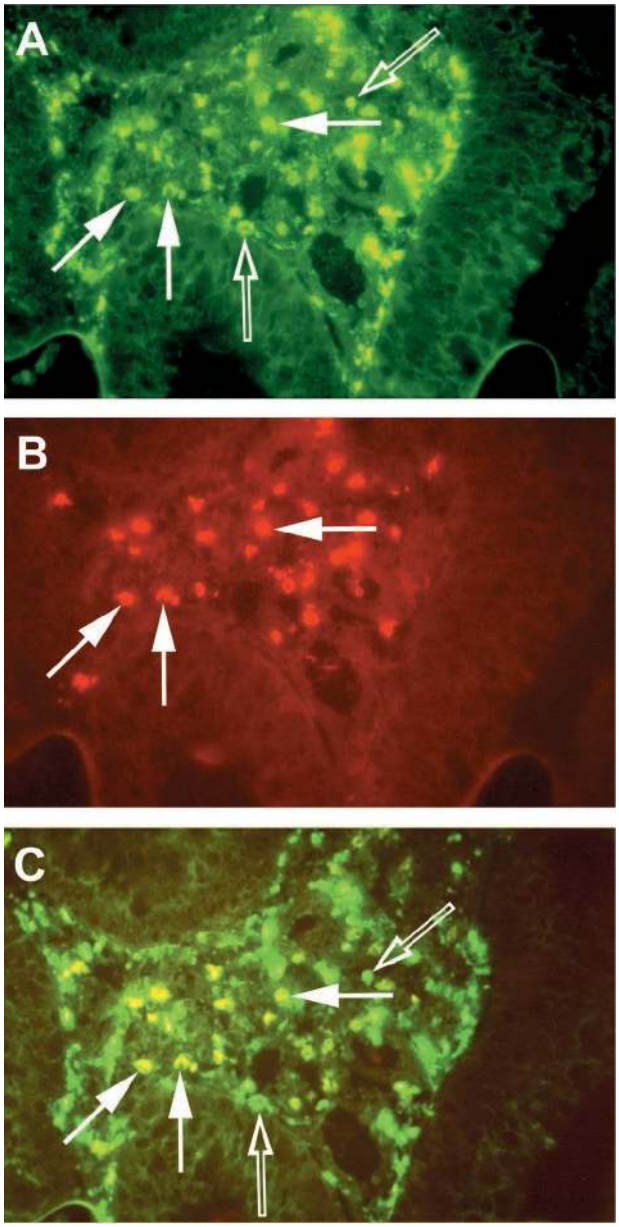


Figure 2. Colocalization of COX-2 and CD68 in human sporadic colorectal adenoma by dual labeling immunofluorescence. **A:** Numerous CD68-positive macrophages (green; **open** and **closed arrows**). **B:** COX-2-positive interstitial cells (red; **arrows**). **C:** Colocalization of COX-2 and CD68 using a dual wavelength filter. **Closed arrows** indicate COX-2-positive, CD68-positive macrophages (yellow), **open arrows** indicate COX-2-negative, CD68-positive macrophages (green).

Factors Influencing COX-2 Expression

A logistic regression analysis identified the adenoma site (P = 0.012) and histological type (P = 0.001) as significant independent predictors of superficial interstitial cell (macrophage) COX-2 protein expression. Age (P = 0.025) was the only significant independent predictor of COX-2 protein expression by deep interstitial cells. The degree of epithelial cell dysplasia did not predict epithelial cell COX-2 protein expression (P = 0.19, Kruskal-Wallis one-way analysis of variance).

The superficial interstitial cell COX-2 expression scores in adenomas distal to the splenic flexure were increased significantly (median COX-2 expression score 1; IQR 1–2.25; n = 42) compared with adenomas proximal to the splenic flexure (median score 0.5; IQR 0–1.25; n = 10; P = 0.03, Mann-Whitney U test). The superficial interstitial cell COX-2 expression scores for villous adenomas were significantly less (median score 0, n = 3) than corresponding values for both tubular (median score 1; IQR 1–2; n = 27) and tubulo-villous adenomas

(median score 1; IQR 0–2; $n = 22$; $P = 0.002$, Mann-Whitney U test; combined tubular/tubulo-villous *versus* villous adenomas). COX-2 staining in deep interstitial cells in patients aged less than 65 years was increased significantly compared with those patients aged 65 years or more ($P = 0.001$, Mann-Whitney U test). The degree of macrophage infiltration in adenomas did not correlate with the COX-2 expression score for superficial and deep interstitial macrophages ($r = 0.05$; $P = 0.79$, Spearman rank correlation coefficient).

Discussion

This immunohistochemical study has revealed that 77% of human sporadic colorectal adenomas contain COX-2-expressing cells. Using two COX-2 antibodies that recognize different epitopes, we have demonstrated the consistent localization of COX-2 to superficial interstitial macrophages in 75% of adenomas. By contrast, we detected immunoreactive COX-2 in dysplastic epithelium in only a minority (29%) of adenomas.

Existing immunohistochemical data on COX-2 expression in human and murine adenomas are conflicting.[17–20,27] Therefore, we studied two different antibodies on both frozen and formalin-fixed sections and ensured that COX-2 staining with both antibodies was specific by preadsorption with the appropriate immunizing peptide. In addition, we demonstrated identical COX-2 immunoreactivity in colorectal cancer cells to that previously reported,[5] and confirmed previous reports that histologically normal colonic mucosa does not express COX-2 protein.[5–8,10,11] Moreover, the series of sporadic colorectal adenomas studied has wide clinical relevance as the adenomas had similar characteristics to those reported in the National Polyp Study.[28] Because it remains unclear whether NSAIDs can attenuate expression, as well as inhibit the activity of COX-2,[29–31] we also obtained data on NSAID use to ensure this did not confound our COX-2 expression data.

The finding that COX-2 is expressed predominantly by macrophages in human sporadic colorectal adenomas is in agreement with data from murine ($Apc^{+/-}$) polyposis models. We have previously reported macrophage-specific Cox-2 expression in adenomas of Min mice.[19] Similarly, Oshima et al used a *lacZ* reporter under control of the *Ptgs2* promoter and localized Cox-2 expression to interstitial cells within adenomas of $Apc^{\Delta 716}$ mice.[17] By contrast, Williams et al have described COX-2 immunostaining of epithelial cells in Min mouse adenomas.[27] However, this group did not use controls confirming the specificity of staining for COX-2 in fixed tissue sections that had been predigested with trypsin. The only published immunohistochemical study on COX-2 localization in human sporadic adenomas reported that COX-2 was localized to dysplastic epithelial cells with only weakly positive staining of interstitial cells.[20] However, this study did not confirm antibody specificity on formalin-fixed sections by antibody preadsorption and demonstrated epithelial cell staining in normal colonic mucosa, which conflicts with existing data.[5–8,10,11] We did not demonstrate COX-2 protein expression by other interstitial cell types, such as the fibroblast, in our series, although others have reported COX-2 expression by fibroblasts (identified by morphological criteria) in adenomas of $Apc^{\Delta 716}$ mice[32] and in *Helicobacter pylori* gastritis in humans.[33]

A variable number of macrophages within adenomas expressed COX-2 protein, which could not be accounted for by differences in the density of the macrophage infiltrate within individual adenomas. It is unclear why only a variable proportion of the total macrophage population within an adenoma expressed COX-2. However, a similar phenomenon has been noted in a study of human colorectal cancers, in which tumor necrosis factor α expression was localized to less than 10% of tumor-infiltrating macrophages.[34]

It is likely that the COX-2-expressing macrophages within adenomas represent a population of activated macrophages. Human macrophage/monocytes express COX-2 following activation by several agents including bacterial lipopolysaccharide,[35] interleukin-1β,[36] interferon-γ, and tumor necrosis factor-α.[37] The nature of an activating stimulus for macrophages within adenomas remains open to speculation. A direct activation of murine peritoneal macrophages by multiple tumor cell lines has been reported,[38] suggesting that activation may be due to an antigenic stimulation from the adjacent adenomatous epithelial cells. Alternatively, the passage of a luminal antigen such as bacterial endotoxin across the dysplastic epithelial cell layer could be responsible for the activation of superficial macrophages.

Coculture studies with human colorectal cancer cell lines and *in vivo* studies in animals have demonstrated that macrophages can stimulate tumor cell proliferation and migration.[39–43] If COX-2 does indeed play a role in the early stages of colorectal carcinogenesis in man, COX-2 localization to interstitial macrophages implies a paracrine-signaling pathway between macrophages and the neighboring epithelial cells. Macrophages synthesize large quantities of prostaglandin D_2,[44] derivatives of which are capable of activating the transcription factor, peroxisome proliferator-activated receptor γ,[45] thus providing an auto- and/or paracrine mechanism whereby gene expression could be altered. Other eicosanoids, such as PGE_2, have also been shown to directly increase the proliferation of human colorectal cancer cell lines.[46] In the only published study of eicosanoid synthesis by macrophages isolated from adenomas, it was reported that such macrophages do not synthesize excess PGE_2, compared with tissue macrophages from a normal colon.[47] However, this study only examined villous adenomas, which, in our study, did not contain COX-2-positive macrophages. Alternatively, COX-2-positive macrophages may play a role in the adenoma progression via promotion of angiogenesis. COX-2 expression has been shown to stimulate angiogenesis in an *in vitro* coculture model using Caco-2 cells.[15] In this regard, the close proximity demonstrated in our study of COX-2-positive macrophages to capillaries is noteworthy.

COX-2-positive deep interstitial macrophages, noted in 17% of adenomas, were smaller than superficial COX-2-positive macrophages and probably represent a dis-

tinct macrophage population that can be isolated from the total tissue macrophage population of different tumors based on differential sedimentation characteristics.[48] It is unclear why adenomas from older patients were significantly less likely to contain COX-2-positive deep interstitial macrophages. However, aged (greater than 65 years) human peripheral blood monocytes secrete decreased amounts of interleukin-1 after stimulation with lipopolysaccharide.[49] This suggests that adenomas from older patients may contain fewer activated macrophages and, hence, a decreased macrophage COX-2 protein content.

COX-2 protein expression by macrophages was greater in the distal adenomas compared with the adenomas proximal to the splenic flexure. It is recognized that colorectal cancers exhibiting microsatellite instability have reduced COX-2 protein expression.[50] It is possible that the predilection of mismatch repair-defective colon cancers, characterized by microsatellite instability, for the proximal colon could account for the differential COX-2 expression in adenomas demonstrated in our study.

We did not demonstrate macrophage COX-2 protein expression in villous adenomas, in contrast to tubular and tubulo-villous adenomas. Whether a causal relationship exists between the absence of macrophage COX-2 expression and the well-recognized increased malignant potential of villous adenomas remains to be determined.

Adenomatous epithelial cells expressed COX-2 protein in 29% of adenomas in our series. We found no relationship between the degree of dysplasia within adenomas and epithelial cell COX-2 protein expression, which is in agreement with data from Hao et al.[20] In contrast, COX-2 is consistently expressed by the neoplastic epithelium in human colorectal carcinomas.[5,11] This suggests that, in the majority of cases, up-regulation of COX-2 expression by epithelial cells is a relatively late event in colorectal carcinogenesis. Interestingly, COX-2 expression during development of esophageal adenocarcinoma from Barrett's mucosa has similarities with our findings, in that COX-2 expression in the premalignant state (Barrett's mucosa) is restricted to interstitial cells compared with established esophageal adenocarcinomas, which have been shown to contain COX-2-expressing malignant epithelial cells.[51]

A significant proportion of the adenomas (23%) that were studied did not express COX-2 protein detectable by our immunohistochemical technique. This has obvious implications for the development of selective COX-2 inhibitors as chemopreventative agents for colorectal cancer. The variable COX-2 expression by human sporadic colorectal adenomas should be taken into account when chemoprevention trials with selective COX-2 inhibitors are performed.

In summary, we have demonstrated that COX-2 protein is localized predominantly to superficial interstitial macrophages within human sporadic colorectal adenomas. If COX-2 plays a role at this stage of colorectal carcinogenesis, these data imply a paracrine signaling mechanism between macrophages and epithelial cells that warrants further investigation.

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