Cytoplasmic Phospholipase A₂ Levels Correlate with Apoptosis in Human Colon Tumorigenesis

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ABSTRACT
Colon cancers often display perturbations in arachidonic acid metabolism, with elevated levels of cyclooxygenase (COX)-2 expression and prostaglandin E₂ (PGE₂) production frequently observed. Whereas COX-2 and PGE₂ are associated with cancer cell survival and tumor angiogenesis, arachidonic acid itself is a strong apoptotic signal that may facilitate cancer cell death. To further explore how cancer cells exploit the progrowth actions of prostaglandins while suppressing the proapoptotic actions of intracellular arachidonic acid, we determined the cytoplasmic phospholipase A₂ (𝑐𝑃𝐿𝐴₂) and COX-2 expression levels in a panel of human colon tumors by immunohistochemistry. Although high levels of 𝑐𝑃𝐿𝐴₂ and COX-2 expression are predicted to facilitate maximal prostaglandin production, tumors frequently displayed a high-COX-2/low-𝑐𝑃𝐿𝐴₂ phenotype. The least represented phenotype was the high expression of 𝑐𝑃𝐿𝐴₂, a characteristic predicted to generate the highest levels of intracellular arachidonic acid. The potential proapoptotic role of 𝑐𝑃𝐿𝐴₂ was supported by a higher frequency of terminal deoxynucleotidyl transferase–mediated nick end labeling staining in 𝑐𝑃𝐿𝐴₂-positive tumors. Moreover, analysis of preneoplastic aberrant crypt foci from high-risk patients suggests that acquisition of the high-COX-2/low-𝑐𝑃𝐿𝐴₂ phenotype may arise at an early stage of colon carcinogenesis. We additionally inhibited 𝑐𝑃𝐿𝐴₂ in HT-29 cells using antisense oligonucleotides. Our results indicate that 𝑐𝑃𝐿𝐴₂ plays an important role in tumor necrosis factor α–induced apoptosis in human colon cancer cells. Our data further support the model in which colon cancer growth is favored when intracellular arachidonic acid levels are suppressed by inhibition of 𝑐𝑃𝐿𝐴₂ or by a high-COX-2/low-𝑐𝑃𝐿𝐴₂ phenotype.

INTRODUCTION
Prostaglandin endoperoxide synthase, commonly referred to as cyclooxygenase (COX), is the key regulatory enzymatic step in the conversion of arachidonic acid to prostaglandins. There are two known isoforms, COX-1 and COX-2. COX-1 is constitutively expressed within the human intestine and plays a fundamental role in maintaining gastrointestinal integrity. COX-2 is normally present at relatively low levels, but can be rapidly induced during infection and inflammation (1). COX-2 is also overexpressed in ~70% to 80% of human colorectal cancers (2, 3). The connection between COX-2 expression and carcinogenesis was first established in studies that showed the efficacy of aspirin and other nonsteroidal anti-inflammatory drugs to reduce the relative risk of colon cancer and to promote tumor regression in a subset of patients with cancer as well as in animal tumor models (4–8).

There is growing evidence that COX-2 and its key metabolite, prostaglandin E₂ (PGE₂), a major eicosanoid produced within the intestine, are associated with enhanced cell proliferation and survival (1). An important source of arachidonic acid for COX-2 is the ubiquitously expressed family of phospholipases (PLA). This complex gene family consists of eight secretory and three cytoplasmic phospholipases (9). The group IV cytoplasmic phospholipases are composed of three forms, α, β, and δ (10). Our focus is on the Ca²⁺-sensitive form, referred to as cytoplasmic phospholipase A₂α (𝑐𝑃𝐿𝐴₂α), which is ubiquitously expressed in most tissues and preferentially hydrolyzes phospholipids in the sn-2 position (10). 𝑐𝑃𝐿𝐴₂ plays a central role in cytokine-induced release of arachidonic acid (11). In cells stimulated by tumor necrosis factor α (TNF-α), 𝑐𝑃𝐿𝐴₂ undergoes immediate activation via mitogen-activated protein kinase–mediated phosphorylation and Ca²⁺-directed translocation from the cytoplasm to the endoplasmic reticulum and nuclear membrane (12, 13). At the endoplasmic reticulum, it hydrolyzes phospholipids to arachidonic acid, which in turn are metabolized by the actions of colocalized COXs to produce prostaglandin (PG) metabolites (9).

Arachidonic acid has been shown to be an important mediator of TNF-α induced apoptosis, possibly via activation of the ceramide pathway, or by a generalized disruption of membrane integrity (1, 14–18). In addition, Scorrano et al. (19) recently showed that Ca²⁺ release from the endoplasmic reticulum via arachidonic acid is a key mechanism for controlling apoptotic death. In contrast, PGs, especially PGE₂, have been shown to possess potent proliferative and tumorigenic capacity. Given the central role of 𝑐𝑃𝐿𝐴₂ and COX-2 in maintaining the balance of arachidonic acid and PG levels, the coordinated regulation of these two proteins may be a critical mechanism for maintaining the balance between proliferation and apoptosis.
In our previous study, we reported that cPLA₂ levels were barely detectable in murine colon tumors induced by the organotropin colon carcinogen, azoxymethane (15). Despite the minimal expression of cPLA₂, COX-2 levels were markedly increased with concomitant overproduction of PGE₂ (15). We hypothesized that the absence of coordinated regulation between cPLA₂ and COX-2 expression may attenuate the apoptotic signals that are mediated by arachidonic acid while further potentiating the proliferative signals elicited by PGE₂. To further explore the potential for coordinated regulation of cPLA₂ and COX-2 expression in human colon tumors, a total of 27 human colon cancer specimens were analyzed for cPLA₂ and COX-2 expression and correlated with apoptotic index. In addition, the role of cPLA₂ in TNF-α–induced apoptosis was clearly shown in a human colon tumor cell line.

MATERIALS AND METHODS
Colorctal Tumor and Aberrant Crypt Foci Samples.
A total of 27 matched human colorectal cancer specimens collected from surgically resected primary colorectal cancers were provided by the Department of Pathology at UCHC after approval by the Institutional Ethics Committee. A summary of the human tumor data is provided under Table 1. For the analysis of human preneoplastic aberrant crypt foci, close-focus, magnifying colonoscopy (Olympus Corporation, Lake Success, NY) was done to identify aberrant crypt foci in the colons of high-risk patients. Methylene blue staining was done in vivo to reveal colonic crypts. Biopsy specimens were immediately frozen in ornithine carbamyl transferase and sectioned for subsequent histopathologic and immunohistochemical analyses.

Immunohistochemistry. Five-micrometer formalin-fixed, paraffin-embedded serial tissue sections were incubated with anti–COX-2 antibody (Cayman Chemical, Ann Arbor, MI) at 1:200 dilution, or anti-cPLA₂ antibody (Upstate, Lake Placid, NY) at a 1:100 dilution overnight at 4°C. Sections were washed with PBS and incubated with biotinylated goat IgG (1:1,000, Vector Laboratories, Burlingame, CA) at room temperature for 30 minutes. After washing, the sections were incubated with avidin-biotin peroxidase complex provided by Vectastain Elite ABC kit (Vector Laboratories) at room temperature for 30 minutes. Color was developed with 3,3’-diaminobenzidine as the substrate. Sections were counterstained with hematoxylin. As a negative control, the duplicate sections were immunostained with goat serum in place of the primary antibody. For staining of aberrant crypt foci, frozen tissue sections were air-dried briefly and then fixed in a mixture of methanol-acetone (1:1) at −20°C for 20 minutes. The remaining staining procedures were carried out as described above.

Terminal Deoxynucleotidyl Transferase–Mediated Nick-End Labeling Assay. Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay was carried out according to the instructions provided by the ApopTag peroxidase in situ Apoptosis Detection Kit (Intergen Company, Purchase, NY). Briefly, deparaffinized tissue sections were treated with proteinase K followed by incubation with terminal deoxynucleotidyltransferase enzyme in the presence of digoxigenin-labeled deoxynucleotides (dUTPs) at 37°C for 60 minutes. The integrated dUTPs were then conjugated to antidigoxigenin peroxidase and color was developed with 3,3’-diaminobenzidine. Sections were counterstained with hematoxylin. For each section, five tumor crypts were randomly selected and the number of apoptotic cells were counted at 200× magnification and recorded as a percentage of total cells within one crypt. In addition to

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brown staining, cells classified as apoptotic also displayed condensed nuclei with integral cell membrane.

**Cell Culture and Treatments.** Equal numbers of HT-29 cells were seeded on 24-well plates and grown to 85% confluence in DMEM supplemented with 10% fetal bovine serum and 100 IU/mL penicillin/streptomycin. High-performance liquid chromatography–purified cPLA2 antisense oligonucleotides or control oligonucleotides (Biomol, Plymouth Meeting, PA) were transfected according to the LipofectAMINE 2000 protocol (Life Technologies, Inc., Gaithersburg, MD) into HT-29 cells. Twenty-four hours after transfection, cells were sensitized to TNF-α–induced cell death by incubating with 25 μg/mL cyclohexamide for 1 hour. TNF-α (25 ng/mL) was added to each well and the cells were incubated for an additional 6 hours. Data represent the result of experiments done in triplicate.

**Western Blot Analysis.** Twenty-four hours after transfection, cells were harvested, washed in ice-cold PBS, and lysed by sonication in radioimmunoprecipitation assay buffer. The mixture was centrifuged at 10,000 × g for 10 minutes at 4°C. Protein concentrations in the supernatant were measured using the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Fifty micrograms of protein were separated on a 10% SDS-PAGE gel and electrotransferred onto a nitrocellulose membrane. The membrane was probed with 2 μg/mL anti-cPLA2 antibody overnight at 4°C. The membrane was washed, incubated with anti-mouse horseradish peroxidase–conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and visualized using the ECL Western blot analysis system (Santa Cruz Biotechnology).

**Cytoplasmic Phospholipase A2 Activity.** cPLA2 activity was measured upon incubation of cell lysates with the substrate, arachidonyl thio phosphatidylcholine, according to instructions provided with the cPLA2 assay kit (Cayman, Ann Arbor, MI). Briefly, equal numbers of cells transfected with either control or antisense oligonucleotides were homogenized in 500 μL ice-cold PBS buffer containing 1 mmol/L EDTA and centrifuged at 10,000 × g for 15 minutes at 4°C. Supernatants were treated with 5 μmol/L bromoelcol lactone for 15 minutes at 25°C to inhibit the activity of calcium-independent PLA2 and filtered through a cellulose membrane filter with a molecular weight cutoff of 30,000 (Millipore, Bedford, MA) to remove any residual secretory PLA2. The purified sample was then incubated with the substrate, arachidonyl thio phosphatidylcholine. Hydrolysis of the substrate at the sn-2 position by the PLA2 enzyme releases free thiol, which is then detected colorimetrically using Ellman’s reagent on a microplate reader (405 nm).

**Caspase-3 Activity.** After a 6-hour incubation with TNF-α, cells were harvested, washed in ice-cold PBS, and lysed by sonication in lysis buffer. The mixture was centrifuged at 10,000 × g for 10 minutes at 4°C. After determining the protein concentrations in the supernatant, cell lysates containing 80 μg of protein were incubated with the colorimetric caspase-3 substrate I (Calbiochem, San Diego, CA) for 30 minutes at 37°C. Absorbance at 405 nm was recorded using a microplate reader.

**Statistical Analysis.** A two-tailed unpaired Student’s t test was done to determine statistical significance between levels of apoptosis.

**RESULTS**

**Expression Analysis of cPLA2 and COX-2 Human Colorectal Cancers.** To examine the relative expression levels of cPLA2 and COX-2 in human colorectal cancers, 27 archival human tumor specimens with matching normal control tissue were examined by immunohistochemical analysis. As summarized under Table 1, the tumors were grouped into four distinct phenotypes as follows: COX-2+/cPLA2+, COX-2+/ cPLA2–, COX-2–/cPLA2+, and COX-2–/cPLA2–. Shown in Fig. 1 are representative serial tumor sections and normal colonic mucosa stained for COX-2 and cPLA2. When both proteins are present in normal tissues, our data show perinuclear colocalization (Fig. 1A and B), an observation that is consistent with their presumed functional coupling (19, 20). Figure 1C highlights a crypt-specific staining pattern that suggests monoclonality, a feature of cPLA2 staining that we consistently observe in adjacent normal tissue.
colonic epithelium. This crypt-specific cPLA₂ staining, however, was accompanied by uniform COX-2 staining, as shown in a serial section (Fig. 1D). Those crypts that stained positive for cPLA₂ in adjacent normal tissue showed intense COX-2 staining as well (Fig. 1E and F). Finally, Fig. 1G and H shows representative serial sections of colon tumor that are cPLA₂ negative and COX-2 positive. Further analysis of the frequency of cPLA₂ staining show its absence in 84.6% (11/13) of the COX-2–overexpressing tumors. These observations suggest that COX-2 and cPLA₂ expression are not coordinately regulated within a significant percentage of human colorectal cancers.

Involvement of cPLA₂ in TNF-α-Induced Apoptosis in Human Colon Tumor Cells. cPLA₂ activation by TNF-α has been shown to be a critical component of TNF-α–induced apoptosis. This has been shown both in tumor cells and in immune cells (1, 14–18). To determine whether cPLA₂ has a comparable functional role in human colonocytes, we interfered with cPLA₂ expression in human HT-29 cells using antisense oligonucleotides. These cells have been shown to express high levels of COX-2 (21). As shown in Fig. 2A and B, treatment of HT-29 cells with antisense oligonucleotides against cPLA₂ produced a dose-related decrease in both the levels and functional activity of cPLA₂ compared with cells treated with control oligonucleotides. Treatment of HT-29 cells with TNF-α, however, was less effective in activating caspase 3 when cPLA₂ expression was reduced. These in vitro data clearly extend the role of cPLA₂ to colonocytes and further support its involvement in TNF-α–mediated cell death.

Enhanced Cell Death in Human Colorectal Cancers with Deregulated cPLA₂ and COX-2 Expression. The above data raise the possibility that during tumorigenesis, transformed colonocytes may, in the absence of cPLA₂, acquire differential sensitivity to immune-mediated (TNF-α) apoptotic cell death. To further evaluate this possibility, we carried out TUNEL staining in human tumors that were positive for COX-2 and either positive or negative for cPLA₂. Representative TUNEL staining is shown in Fig. 3. Only minimal staining was found in cPLA₂-negative tumors (Fig. 3A and B). In striking contrast, TUNEL staining was markedly increased in tumors that stained positive for cPLA₂ (Fig. 3C and D). As shown in Fig. 3E, the fraction of apoptotic cells was significantly (P < 0.05) higher in cPLA₂-expressing tumors relative to nonexpressing tumors (9.82 ± 6.23 versus 4.67 ± 3.04, respectively).

Analysis of cPLA₂ and COX-2 in Preneoplastic Human Aberrant Crypt Foci. Aberrant crypt foci represent a morphologically distinct alteration that occurs within the colonic mucosa, occurring in some cases many decades before neoplasia (22). To develop a more comprehensive understanding of potential metabolic changes that are present within these early preneoplastic lesions, we used close-focus, magnifying colonoscopy to identify and harvest a total of 24 abnormal-appearing colonic crypt foci from 17 high-risk patients undergoing colorectal cancer screening. Representative macroscopic images of human colon are shown in Fig. 4A and B (magnification ×40). Aberrant crypt foci were frozen-sectioned, stained with H&E and examined by board-certified pathologists (M.M.S. and F.F.; Fig. 4C, magnification ×100; Fig. 4D, magnification ×200). COX-2 staining was evident in 12 of the 24 aberrant crypt foci examined. Representative colon sections with COX-2 staining are shown in Fig. 4E and F (magnification ×400 and ×200, respectively). Figure 4F (inset) shows a dysplastic aberrant crypt foci at higher magnification (×400), demonstrating intense perinuclear COX-2 staining, consistent with the staining patterns observed in tumors and adjacent normal tissues. However, cPLA₂ staining was not present in the aberrant crypt foci examined (data not shown), indicating that dysregulation of arachidonic acid metabolism can occur during very early stage of colon tumorigenesis.
DISCUSSION

The functional activity of cPLA2 is regulated by a variety of stimuli including hormones, neurotransmitters, antigens, and mitogens (10). cPLA2 is functionally linked to COX-2 within the perinuclear membrane and is the primary source of arachidonic acid production for COX-2 (19, 20). Despite its critical role in the generation of PGs, deletion of cPLA2 protected against intestinal tumorigenesis in Apc
\textsuperscript{Min} and Apc
\textsuperscript{-776} mice (23, 24). On the contrary, the absence of cPLA2 cPLA2 increased tumor multiplicity and size within the small intestine of Apc mutant mice. However, an opposite effect was observed in the colon, whereby the absence of cPLA2 resulted in a modest increase in the number or size of colon tumors (24, 25). These disparate observations from the Apc heterozygous intestinal tumor models raise the possibility that the cPLA2 effect is tissue specific.

Previously, the levels of cPLA2 were examined in human colon tumors, with somewhat ambiguous results. Using reverse transcription–PCR, Dimberg et al. (25) reported that cPLA2 expression was sometimes increased, although there was no correlation with COX-2 expression. Soydan et al. (26) also reported elevated cPLA2 protein levels in 6 of 17 human colorectal tumors, although the data were highly variable. Using a dot-blot method to estimate cPLA2 expression, Osterstrom et al. (27) found a significant increase in cPLA2 levels in human tumors. Wendum et al. (28) recently compared COX-2, secretory PLA2, and cPLA2 protein expression in small bowel adenocarcinomas and colorectal adenocarcinomas. Consistent with our findings, they reported that only 35% of tumors with moderate to strong staining of COX-2 had strong cPLA2 positivity. Using an immunohistochemical approach, our findings clearly show that cPLA2 protein is markedly reduced in 11 (84.6%) of 13 human colorectal tumors expressing high levels of COX-2 (Table 1). The relative infrequency of the low-COX-2/high-cPLA2 phenotype suggests that cells harboring these alterations are less likely to progress to tumors.

There is accumulating evidence that cellular arachidonic acid balance plays a key role in regulating apoptosis (1). For example, diminished production of arachidonic acid as a result of reduced cPLA2-dependent generation or enhanced utilization through elevated COX-2 may deplete intracellular arachidonic acid pools, thereby attenuating apoptotic signals and facilitating tumorigenesis (1, 14). In an earlier study, we reported that in young adult mouse colon cells, pharmacologic inhibition of cPLA2 with arachidonyl trifluoromethylketone attenuated apoptosis in response to TNF-\(\alpha\) (15). In the present study, we show for the first time that cPLA2 is an important mediator of TNF-\(\alpha\) induced apoptosis in human colon tumor cells as well.

We have also evaluated the possibility that the balance of cPLA2 and COX-2 may be disrupted during early stages of colon tumorigenesis, perhaps providing a stimulus for tumor cell proliferation. The development of human colorectal cancer has been proposed to follow a stepwise progression, with the formation of dysplastic aberrant crypt foci constituting an important transitional event (29, 30). Genetic alterations occurring during this stage may allow the transformed tumor cells to escape apoptosis and undergo uncontrolled proliferation. Several key genetic alterations that are associated with colorectal cancer, including loss of Apc or K-ras activating mutations, have been identified in aberrant crypt foci and may represent important initiating events in the pathogenesis of colon cancer (31). Until recently, however, there have been only limited efforts to more fully characterize aberrant crypt foci at the molecular level, largely due to limitations in imaging technology that has the requisite sensitivity to distinguish aberrant crypt foci within the colonic epithelium. In the present study, we have used close-focus magnifying colonoscopy to identify aberrant crypt foci. We found that COX-2 expression was markedly up-regulated in a subset of aberrant crypt foci procured from the colons of high-risk individuals (12/24). This is the first time that COX-2 overexpression has been shown within these early precursor lesions and raises important questions about the actions of COX-2 as an early driving force in colorectal cancer. Considering the importance of COX-2 in colon cancer, these initial observations may provide some insight as to why aspirin is able to prevent colon cancer within the general population, including those individuals that are not necessarily

\[\text{Fig. 3 Comparison of apoptotic index in COX-2–overexpressing tumors in the presence/absence of cPLA2. TUNEL staining was done on serial sections of tumor tissue. A and B, cPLA2-negative tumors (magnification \times 200). Note the minimum TUNEL staining within most of the tumor crypts. C, representative of a cPLA2-positive tumor (magnification \times 100). Note the abundance of apoptotic nuclei within the tumor crypts (arrows). D, higher magnification (\times 400) of C. E, graphical representation of five randomly selected tumor crypts counted and presented as a percentage of total cells. Note that cPLA2-positive tumors scored significantly more (P < 0.05) apoptotic cells than the tumors without cPLA2.}\]
at high risk. More importantly, we also found that within aberrant crypt foci expressing COX-2, there was no concomitant elevation in cPLA2, a coordinated response that we established often occurs within the normal colonic epithelium.

PLA2s and COX-2 are commonly activated in response to immune stimulation and their coordinate activation plays a key role in regulating cell survival or death. Evasion from immune surveillance may play an important role in the early stages of tumorigenesis. Obviously, transformed cells that have acquired genetic alterations that enable survival against immune-mediated attack will be able to undergo further clonal expansion. Our findings of COX-2 elevation without concomitant cPLA2 induction potentially provides a pathway that may confer a survival advantage to early precancerous lesions in the colon, thereby facilitating aberrant crypt foci expansion and the ultimate development into adenocarcinomas. Thus, this model predicts enhanced cell survival in the presence of immune activation by attenuated apoptotic response (15). In summary, our results show that within the normal colonic epithelium, COX-2 and cPLA2 expression are coordinately regulated; that is, COX-2 up-regulation is generally accompanied by cPLA2 overexpression. However, in most colon tumors, this coordinate induction response is obscured. It is argued that up-regulation of COX-2 without a corresponding increase in cPLA2 activity may lead to diminished arachidonic acid production and enhanced arachidonic acid utilization. This imbalance may lead to an environment in which arachidonic acid levels are depleted within tumor cells, potentially removing several key apoptotic pathways that ultimately favor tumor promotion.

REFERENCES
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