

Potential role of group X secretory phospholipase A₂ in cyclooxygenase-2-dependent PGE₂ formation during colon tumorigenesis

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Abstract Although the cyclooxygenase-2 (COX-2) pathway of the arachidonic acid cascade has been suggested to play an important role in colon carcinogenesis, there is little information concerning the identity of phospholipase A₂ (PLA₂) involved in the arachidonic acid release in colon tumors. Here, we compared the potencies of three types of secretory PLA₂s (group IB, IIA and X sPLA₂s) for the arachidonic acid release from cultured human colon adenocarcinoma cells, and found that group X sPLA₂ has the most powerful potency in the release of arachidonic acid leading to COX-2-dependent prostaglandin E₂ (PGE₂) formation. Furthermore, immunohistological analysis revealed the elevated expression of group X sPLA₂ in human colon adenocarcinoma neoplastic cells in concert with augmented expression of COX-2. These findings suggest a critical role of group X sPLA₂ in the PGE₂ biosynthesis during colon tumorigenesis. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Colon tumor; Phospholipase A₂; Secretory phospholipase A₂; Cytosolic phospholipase A₂; Cyclooxygenase; Prostaglandin

1. Introduction

A growing body of evidence has shown that non-steroidal anti-inflammatory drugs (NSAIDs) can suppress colorectal tumorigenesis in both humans and rodents [1]. The molecular basis for their chemopreventive effects has been attributed at least, in part, to the inhibition of cyclooxygenase-2 (COX-2), since COX-2 expression is elevated in human colorectal tumors [2,3]. In addition, inactivation of the COX-2 gene is associated with decreased intestinal tumorigenesis in rodent models of familial adenomatous polyposis (FAP) [4]. COX-2 catalyzes the conversion of arachidonic acid to prostaglandin H₂ (PGH₂) leading to the production of various bioactive

PGs [5]. Among these eicosanoids, PGE₂ is produced in large amounts by colon tumors [6], and supports tumor growth by inducing angiogenesis [7], modulating tumor cell apoptosis [8], and suppressing immune surveillance [9]. A potential role of PGE₂ in colon tumorigenesis has also been demonstrated by a recent study of mice deficient in PGE₂ receptor subtype EP1, which showed reduced development of aberrant crypt foci in treatment with a colon carcinogen [10].

The release of arachidonic acid from cell membrane phospholipids is mainly mediated via the action of phospholipase A₂ (PLA₂) [11]. To date, numerous types of PLA₂s have been identified and classified into different families according to their biochemical features [11]. Among them, secretory PLA₂s (sPLA₂s) are the secreted enzymes that possess several characteristic features, including a low molecular mass (13–18 kDa) and an absolute catalytic requirement for millimolar concentrations of Ca²⁺ [11]. Based on the primary structures, human sPLA₂s are now classified into seven different groups (IB, IIA, IID, IIE, III, V and X) [11–14]. Previous studies have shown that the sPLA₂-IIA gene represents the *Mom1* locus, which is known as a genetic modifier of tumor resistance in the multiple intestinal neoplasia (Min) mouse, a murine model for FAP [15]. However, its functional significance in human colorectal carcinogenesis has not been elucidated, as there is no modifying effect of sPLA₂-IIA polymorphisms on the severity of polyposis in FAP patients [16]. Another type of PLA₂ family, cytosolic PLA₂ (cPLA₂), has been identified as playing pivotal roles in the release of arachidonic acid and the lipid mediator productions in various inflammatory disease models [17,18]. Recent studies have shown that the introduction of a cPLA₂ gene mutation into the *Apc*^{Δ716} knockout mouse, a model for human FAP, results in the reduction of the size of the small intestinal polyps with no alternation in the colonic polyps formation [19]. These findings suggest that cPLA₂ plays a role in the expansion of polyps in the small intestine, but other types of PLA₂s are involved in the polyps formation in the colon.

Recently, we found that group X sPLA₂ (sPLA₂-X) has the strongest potency for releasing arachidonic acid from phosphatidylcholine among the sPLA₂ family enzymes and elicits a marked release of arachidonic acid leading to eicosanoid formation in various inflammatory cells [20–22]. These observations prompted us to examine its potential role in PGE₂ biosynthesis during colon tumorigenesis. Here, we found a strong potency of sPLA₂-X in the arachidonic acid release linked to the COX-2-dependent PGE₂ production in cultured human colon tumor cells, and also presented the evidence for a

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Abbreviations: NSAIDs, non-steroidal anti-inflammatory drugs; COX, cyclooxygenase; FAP, familial adenomatous polyposis; PG, prostaglandin; PLA₂, phospholipase A₂; cPLA₂, cytosolic PLA₂; sPLA₂, secretory PLA₂; sPLA₂-IB, group IB sPLA₂; sPLA₂-IIA, group IIA sPLA₂; sPLA₂-X, group X sPLA₂; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PPAR δ , peroxisome proliferator-activated receptor δ

marked expression of sPLA₂-X in human colon adenocarcinoma tumors by immunohistochemical analysis.

2. Materials and methods

2.1. Materials

Purified recombinant human group IB sPLA₂ (sPLA₂-IB) and sPLA₂-X were prepared as described previously [20]. Recombinant human sPLA₂-IIA and rabbit anti-cPLA₂ antibody were generous gifts from Dr. Ruth Kramer (Eli Lilly, Indianapolis, IN, USA). Rabbit anti-human sPLA₂-X antibody was prepared in our laboratories [20], and antibodies against sPLA₂-IB, sPLA₂-IIA, COX-1 and COX-2 were purchased from Cayman Chemicals. Bovine serum albumin (BSA), indomethacin and A23187 were obtained from Sigma. Indoxam was synthesized at Shionogi Research Laboratories [23]. AACOCF₃ was obtained from Cayman Chemicals.

2.2. Assay for fatty acid release and PGE₂ production in sPLA₂-treated human adenocarcinoma cell lines

Human colon carcinoma cell lines (HT-29, HCT-15, HCT-116 and Colo320DM; obtained from ATCC) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). For the analysis of fatty acid releases, the tumor cells were harvested from the culture dishes and suspended in Hanks' buffered saline (pH 7.6) containing 0.1% BSA at a density of 12.5×10^6 cells/ml. Aliquots of cell suspension (0.4 ml) were preincubated for 10 min at 37°C, and then stimulated with sPLA₂s or 10 μM A23187 in a final volume of 0.5 ml. The reaction was stopped by the addition of 2 ml Dole's reagent, and the released fatty acids were extracted, labeled with 9-anthryldiazomethane (Funakoshi Co.), and analyzed by reverse-phase high performance liquid chromatography on a LiChroCART 125-4 Superspher 100 RP-18 column (Merck), as described previously [20]. For the PGE₂ production assay, colon carcinoma cells were seeded in 24-well plates at a density of 2.5×10^5 cells/well. After the incubation for 24 h, the cells were washed three times with phosphate-buffered saline (PBS) and incubated with or without recombinant human TNF-α (30 ng/ml; R&D Systems Inc.) in 10% FBS/DMEM for 18 h at 37°C. After washing, the cells were preincubated either with or without several inhibitors in Hanks' buffered saline (pH 7.6) containing 0.1% BSA for 10 min at 37°C, and then stimulated with sPLA₂s or 10 μM A23187 in a final volume of 0.5 ml. After the incubation for 37°C, the culture supernatant was collected following centrifugation, and the PGE₂ content was quantified with an enzyme-immunoassay kit (Cayman Chemicals Co.).

2.3. Western blot analysis of COX-1 and COX-2 expression

Human colon carcinoma cells were cultured in 24-well plates and treated with or without TNF-α as described above. After washing with PBS, the cells were solubilized with Laemmli sample buffer containing 5% 2-mercaptoethanol. Equal volumes of the samples were then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using a 4–20% gradient gel (Daiichi Chemicals Co., Ltd.). After proteins were transferred to an Immobilon-P membrane (Millipore Co., Ltd.), Western blot analysis with anti-COX-1 or anti-COX-2 antibody (0.6 μg/ml) was performed as described in the previous paper [20] and the resultant signals were acquired with an image analyzer (Flour-S MultiImager, Bio-Rad).

2.4. Immunohistochemistry

Preparations of human normal colon tissues (four males; age 40–53 years) and colon adenocarcinoma tissues (five males and one female; ages 45–73 years) were purchased from Biochain Inc. (San Leandro, CA, USA). The tissue slides were dewaxed, incubated in methanol containing 0.3% H₂O₂ for 30 min and then treated with 5% normal rabbit serum for 20 min. The slides were incubated with anti-sPLA₂-X (6 μg/ml), anti-sPLA₂-IB (5 μg/ml), anti-sPLA₂-IIA (7 μg/ml), anti-cPLA₂ (1/1000 dilution), or anti-COX-2 antibodies (0.25 μg/ml) in PBS containing 0.1% BSA for 1 h at room temperature. After washing with PBS, they were incubated with biotin-conjugated goat anti-rabbit antibody for 30 min followed by treatment with horseradish peroxidase avidin–biotin complex reagent (Vector Laboratories). After washing, the peroxidase activity was visualized by 10 min incubation in 50 mM Tris–HCl (pH 7.6) containing 200 mg/ml of diaminobenzidine and 0.006% H₂O₂. The nuclei were counterstained with 0.4%

hematoxylin and the preparations were mounted in Entellan new medium. Positive signals were detected as diaminobenzidine deposits of dark-brownish color. Neutralization of sPLA₂-X signals was performed by incubating anti-sPLA₂-X antibody with sPLA₂-X protein (60 μg/ml) for 2 h before addition to the slides. The availability of the

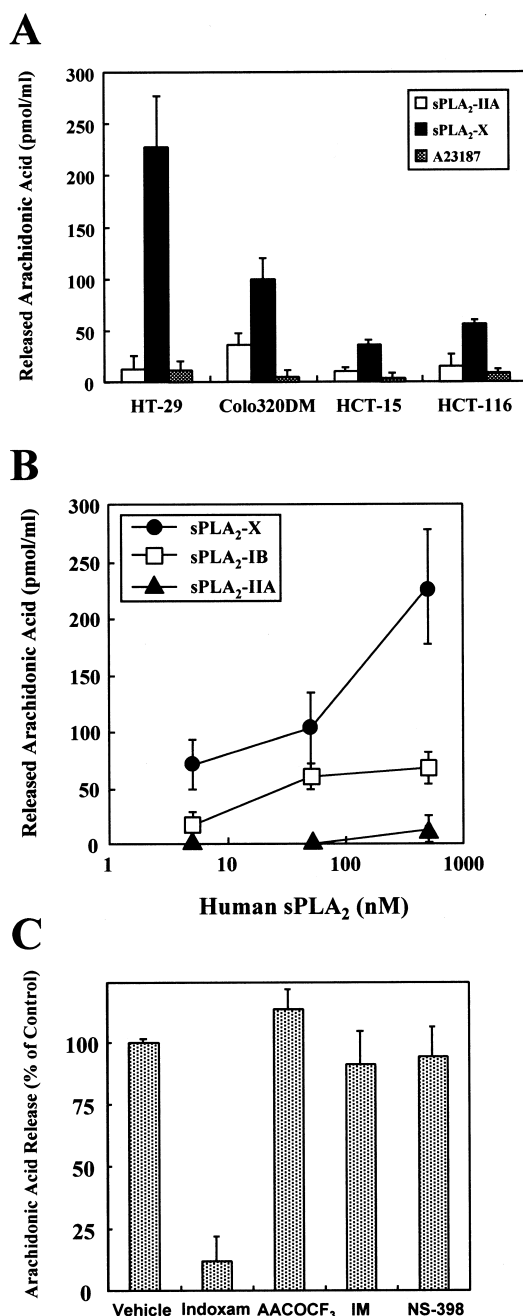


Fig. 1. Release of arachidonic acid from sPLA₂-X-stimulated human colon adenocarcinoma cells. A: The amount of arachidonic acid released from HT-29, Colo320DM, HCT-15 and HCT-116 cells by the actions of 500 nM sPLA₂-X, 500 nM sPLA₂-IIA and 10 μM A23187 for 30 min. B: Dose-dependent release of arachidonic acid from HT-29 cells by the actions of sPLA₂-X, sPLA₂-IB and sPLA₂-IIA for 30 min. C: Inhibitory potencies of sPLA₂ inhibitor indoxam, cPLA₂ inhibitor AACOCF₃, and COX inhibitors, indomethacin (IM) and NS-398 at 10 μM, against the arachidonic acid release from HT-29 cells treated with 100 nM sPLA₂-X for 30 min. The results are expressed as the percentage of arachidonic acid released by sPLA₂-X in the absence of these agents. Each point represents the mean S.D. of triplicate measurements. The data are representative of three experiments.

antibodies for immunohistochemical analysis was confirmed with each positive tissue preparation.

3. Results

3.1. Potency of sPLA₂-X in arachidonic acid release in cultured human colon tumor cells

We first examined the potencies of sPLA₂-IIA, sPLA₂-X and cPLA₂ activator A23187 in releasing arachidonic acid from four different types of human colon carcinoma cell lines. Each reaction reached a steady state level within 30 min, and Fig. 1A summarizes the amount of arachidonic acid released within 30 min. sPLA₂-X was found to elicit more potent release than sPLA₂-IIA and A23187 in four different cell types. Among the carcinoma cells examined, the strongest releases were observed in HT-29 cells, in which sPLA₂-X induced the release of other unsaturated fatty acids (oleic acid: 1060 pmol/ml and palmitoleic acid: 700 pmol/ml) more efficiently than arachidonic acid (230 pmol/ml). Fig. 1B shows the dose-dependence of three types of sPLA₂s in the arachidonic acid release in HT-29 cells. Significant release was observed in the sPLA₂-X-treated cells at 5 nM, whereas sPLA₂-IIA could not induce a significant release even at 500 nM. sPLA₂-IB also induced arachidonic acid release at more than 50 nM with lower potency than sPLA₂-X. To investigate the mechanisms underlying the sPLA₂-X-induced release reactions, the effects of several inhibitors (10 μM) were examined. In contrast to efficient suppression by a specific sPLA₂ inhibitor, indoxam [23], a known cPLA₂ inhibitor, AACOCF₃, and two COX inhibitors, indomethacin and NS-398, did not suppress the responses (Fig. 1C), demonstrating that cPLA₂ is not involved in the sPLA₂-X-induced fatty acid release reactions.

3.2. Potency of sPLA₂-X in COX-2-dependent PGE₂ formation in cultured colon tumor cells

Next, the potency of sPLA₂-X in PGE₂ production was examined in HT-29 cells, in which COX-1 was constitutively expressed and COX-2 was strongly induced by treatment with several pro-inflammatory cytokines such as TNF-α [24]. In the resting cells, COX-2 protein could not be detected and sPLA₂-X (500 nM) did not evoke significant PGE₂ production for up to 4 h (Fig. 2A). After the induction of COX-2 by pretreatment with TNF-α, sPLA₂-X induced a time-dependent increase of PGE₂ formation. This reaction was slower than that induced by A23187, and the PGE₂ level at 6 h after sPLA₂-X stimulation was calculated to be 1.6 nM, which was about 2.5-fold higher than the maximum level induced by 10 μM A23187 (at 30 min incubation). In contrast, there was no detectable production in sPLA₂-X-treated HCT-15 and HCT-116 cells (data not shown), where COX-1 was constitutively expressed and COX-2 could not be induced even after treatment with pro-inflammatory cytokines [25]. These findings suggest that the sPLA₂-X-induced PGE₂ formation is completely dependent on COX-2 expression in human colon tumor cells. As shown in Fig. 2B, significant PGE₂ production was observed at 10 nM sPLA₂-X in TNF-α-pretreated HT-29 cells, whereas sPLA₂-IB evoked much weaker responses even at 500 nM and sPLA₂-IIA did not provoke any productions. As shown in Fig. 2C, indoxam suppressed the sPLA₂-X-stimulated PGE₂ production in a dose-dependent manner. In addition, indomethacin and NS-398 blocked the response

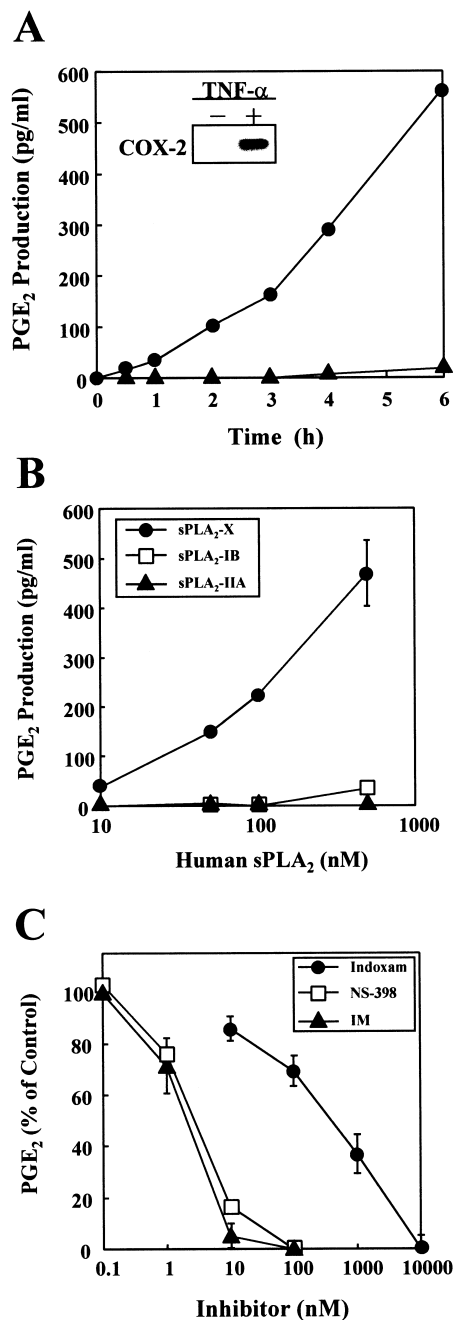


Fig. 2. COX-2-dependent PGE₂ production in sPLA₂-X-stimulated HT-29 cells. A: Time-dependent PGE₂ formation by sPLA₂-X in TNF-α-pretreated HT-29 cells. HT-29 cells were pretreated with (circles) or without (triangles) 30 ng/ml TNF-α for 18 h. After washing, the cells were stimulated with 500 nM sPLA₂-X for the indicated times. The expression of COX-2, as assessed by Western blotting, is shown in the inset. B: Dose-dependent production of PGE₂ by sPLA₂s in TNF-α-pretreated HT-29 cells. After pretreatment with TNF-α, HT-29 cells were incubated with various concentrations of sPLA₂-X, sPLA₂-IB and sPLA₂-IIA for 3 h. C: Inhibition of sPLA₂-X-induced PGE₂ production. After pretreatment with TNF-α, HT-29 cells were preincubated with indoxam, NS-398 and indomethacin (IM), and then stimulated with 100 nM sPLA₂-X for 3 h. The results are expressed as the percentage of PGE₂ produced by sPLA₂-X in the absence of these agents. Each point represents the mean S.D. of triplicate measurements. The data are representative of three experiments.

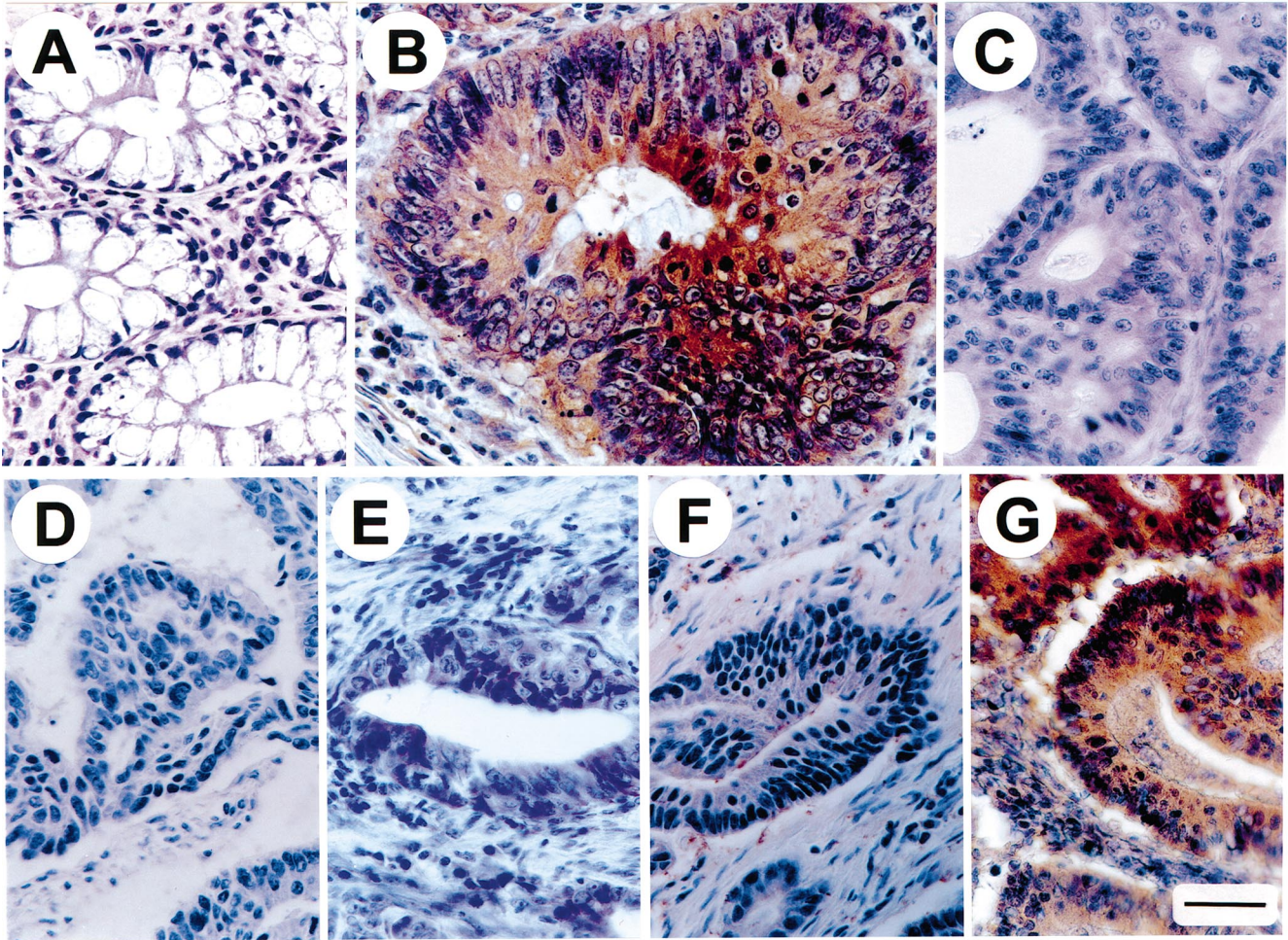


Fig. 3. Immunostaining for sPLA₂s, cPLA₂ and COX-2 in normal colon and colorectal adenocarcinoma tissues. Human normal colon (A) and well-differentiated colorectal adenocarcinoma tissues (B) were immunostained with anti-sPLA₂-X antibody (6 µg/ml). Control staining with anti-sPLA₂-X antibody absorbed with human sPLA₂-X protein (60 µg/ml) (C) was completely negative in colorectal adenocarcinoma tissues. Human colorectal adenocarcinoma tissues were also immunostained with anti-sPLA₂-IB (D), anti-sPLA₂-IIA (E), anti-cPLA₂ (F) and anti-COX-2 antibodies (G). The positive signals were detected as diaminobenzidine deposits of dark-brownish color, and the nuclei were counterstained with hematoxylin. Bar indicates 100 microns.

with IC₅₀ values of about 2 nM, demonstrating complete dependency of PGE₂ biosynthesis on COX-2 activity.

3.3. Immunohistochemical analysis of sPLA₂-X expression in human colon adenocarcinoma tumor tissues

We have recently developed a polyclonal antibody that specifically recognizes sPLA₂-X among the human sPLA₂ species [20]. Immunohistological analysis of normal human colon tissues revealed little, if any, expression of sPLA₂-X (Fig. 3A). In contrast, marked expression of sPLA₂-X was detected in human well-differentiated adenocarcinoma tissues, especially in the neoplastic epithelium and in some inflammatory cells present within the stroma (Fig. 3B). The signal was specific, since preincubation of the antibody with an excess amount of sPLA₂-X protein resulted in its complete abolishment (Fig. 3C). In addition, there was no signal when non-immunized control IgG was used (data not shown). Enhanced expression of sPLA₂-X was detected in the well-differentiated carcinomas of six different patients, and their expression levels were similar to those detected in the moderately and poorly differentiated cases (data not shown). We then examined the expression profiles of other PLA₂ species with their specific

antibodies, and found no signals in normal colon tissues (data not shown). In colon adenocarcinoma tissues, there was no detectable signal for sPLA₂-IB and sPLA₂-IIA (Fig. 3D,E), whereas slight but significant signals for cPLA₂ were detected in colon cancer cells and in some inflammatory cells of colonic stroma (Fig. 3F). The expression of COX-2 was strongly detected within colon neoplastic cells and stroma cells (Fig. 3G) in contrast to no significant signal in normal tissues. These findings demonstrate that the expressions of sPLA₂-X and COX-2 are greatly enhanced in colorectal tumor cells.

4. Discussion

The present study demonstrates that sPLA₂-X induces a marked release of arachidonic acid leading to COX-2-dependent PGE₂ formation in cultured human colon adenocarcinoma cells. In human colorectal cancer tissues, the expression of sPLA₂-X was greatly up-regulated and correlated well with an elevated expression of COX-2, suggesting a pivotal role of sPLA₂-X in the COX-2-dependent PGE₂ biosynthesis during colon tumorigenesis. The expression of cPLA₂ could also be

detected in colon adenocarcinoma tissues (Fig. 3). Compared with cPLA₂ activator A23187, sPLA₂-X could elicit more potent release of arachidonic acid in various colon tumor cells (Fig. 1A). In addition, the sPLA₂-X-induced PGE₂ formation was much slower in contrast to a prompt response by A23187 in COX-2-expressing HT-29 cells (Fig. 2A). These findings suggest that sPLA₂-X plays a role in the sustained PGE₂ production in colon tumor cells. The enzymatic activity of sPLA₂-X is known to be regulated by the conversion of its pro-peptide to the mature enzyme via proteolytic removal of the pro-peptide [20,21]. Since several proteases such as trypsin are expressed at high levels in colon cancer tissues and colon tumor cells actively secrete trypsinogens and several proteases [26], the conversion rate from pro-form to active sPLA₂-X could be accelerated during the development of colon tumors. Because recent gene deletion studies have shown that cPLA₂ plays a role in the expansion of polyps in the small intestine, but not in the initiation process neither the polyps formation in the colon [19], the functional role of sPLA₂-X in colon tumorigenesis deserves attention in the future. Further studies such as the analysis of the promoter regions of sPLA₂-X genes may also offer more information regarding the regulatory mechanisms of its expression during colon carcinogenesis.

Recent studies have shown the presence of COX-2-independent mechanisms underlying the NSAIDs-induced apoptosis of colon tumor cells, including the suppression of peroxisome proliferator-activated receptor δ (PPAR δ) gene expression [27]. In the present study, sPLA₂-X elicited potent releases of various types of unsaturated fatty acids from human carcinoma cells, and these fatty acids and eicosanoids can act as endogenous ligands and modifiers of PPAR activity [27,28]. In this context, sPLA₂-X might modify the spectrum of PPAR δ ligands leading to the alteration of PPAR δ activity in colon tumors. Furthermore, potent fatty acid releases from membrane phospholipids could cause enhanced production of a variety of lysophospholipids, which are known to induce diverse biological responses in non-specific or Edg-receptor-mediated fashions, including tumor cell proliferation [29]. In contrast to selective inhibition of COX-2 inhibitor for PG production, the sPLA₂ inhibitor can suppress the formation of various types of sPLA₂-X-induced lipid mediators, including unsaturated fatty acids, eicosanoids, and lysophospholipids. Further elucidation of the biological functions of these lipid mediators in colon tumorigenesis should give us more information concerning the therapeutic potential of sPLA₂-X inhibitors for colon cancer.

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