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# Potential role of group X secretory phospholipase $A_2$ in cyclooxygenase-2-dependent $PGE_2$ 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 $A_2$ ($PLA_2$) involved in the arachidonic acid release in colon tumors. Here, we compared the potencies of three types of secretory $PLA_2$s (group IB, IIA and X $sPLA_2$s) for the arachidonic acid release from cultured human colon adenocarcinoma cells, and found that group X $sPLA_2$ has the most powerful potency in the release of arachidonic acid leading to COX-2-dependent prostaglandin $E_2$ ($PGE_2$) formation. Furthermore, immunohistological analysis revealed the elevated expression of group X $sPLA_2$ in human colon adenocarcinoma neoplastic cells in concert with augmented expression of COX-2. These findings suggest a critical role of group X $sPLA_2$ in the $PGE_2$ biosynthesis during colon tumorigenesis.** © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

*Key words:* Colon tumor; Phospholipase $A_2$; Secretory phospholipase $A_2$; Cytosolic phospholipase $A_2$; 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 $H_2$ ($PGH_2$) leading to the production of various bioactive PGs [5]. Among these eicosanoids, $PGE_2$ 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 $PGE_2$ in colon tumorigenesis has also been demonstrated by a recent study of mice deficient in $PGE_2$ 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 $A_2$ ($PLA_2$) [11]. To date, numerous types of $PLA_2$s have been identified and classified into different families according to their biochemical features [11]. Among them, secretory $PLA_2$s ($sPLA_2$s) 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 $Ca^{2+}$ [11]. Based on the primary structures, human $sPLA_2$s are now classified into seven different groups (IB, IIA, IID, IIE, III, V and X) [11–14]. Previous studies have shown that the *$sPLA_2$-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 *$sPLA_2$-IIA* polymorphisms on the severity of polyposis in FAP patients [16]. Another type of $PLA_2$ family, cytosolic $PLA_2$ ($cPLA_2$), 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 $cPLA_2$ gene mutation into the $Apc^{\Delta 716}$ 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 $cPLA_2$ plays a role in the expansion of polyps in the small intestine, but other types of $PLA_2$s are involved in the polyps formation in the colon.

Recently, we found that group X $sPLA_2$ ($sPLA_2$-X) has the strongest potency for releasing arachidonic acid from phosphatidylcholine among the $sPLA_2$ 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 $PGE_2$ biosynthesis during colon tumorigenesis. Here, we found a strong potency of $sPLA_2$-X in the arachidonic acid release linked to the COX-2-dependent $PGE_2$ 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; $PLA_2$, phospholipase $A_2$; $cPLA_2$, cytosolic $PLA_2$; $sPLA_2$, secretory $PLA_2$; $sPLA_2$-IB, group IB $sPLA_2$; $sPLA_2$-IIA, group IIA $sPLA_2$; $sPLA_2$-X, group X $sPLA_2$; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PPARδ, peroxisome proliferator-activated receptor δ


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marked expression of $sPLA_2$-X in human colon adenocarcinoma tumors by immunohistochemical analysis.

## 2. Materials and methods

### 2.1. Materials

Purified recombinant human group IB $sPLA_2$ ($sPLA_2$-IB) and $sPLA_2$-X were prepared as described previously [20]. Recombinant human $sPLA_2$-IIA and rabbit anti-$cPLA_2$ antibody were generous gifts from Dr. Ruth Kramer (Eli Lilly, Indianapolis, IN, USA). Rabbit anti-human $sPLA_2$-X antibody was prepared in our laboratories [20], and antibodies against $sPLA_2$-IB, $sPLA_2$-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]. $AACOCF_3$ was obtained from Cayman Chemicals.

### 2.2. Assay for fatty acid release and $PGE_2$ production in $sPLA_2$-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 $12.5\times10^6$ cells/ml. Aliquots of cell suspension (0.4 ml) were preincubated for 10 min at 37°C, and then stimulated with $sPLA_2$s 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 $PGE_2$ production assay, colon carcinoma cells were seeded in 24-well plates at a density of $2.5\times10^5$ 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 $sPLA_2$s 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 $PGE_2$ 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% $H_2O_2$ for 30 min and then treated with 5% normal rabbit serum for 20 min. The slides were incubated with anti-$sPLA_2$-X (6 μg/ml), anti-$sPLA_2$-IB (5 μg/ml), anti-$sPLA_2$-IIA (7 μg/ml), anti-$cPLA_2$ (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% $H_2O_2$. 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 $sPLA_2$-X signals was performed by incubating anti-$sPLA_2$-X antibody with $sPLA_2$-X protein (60 μg/ml) for 2 h before addition to the slides. The availability of the

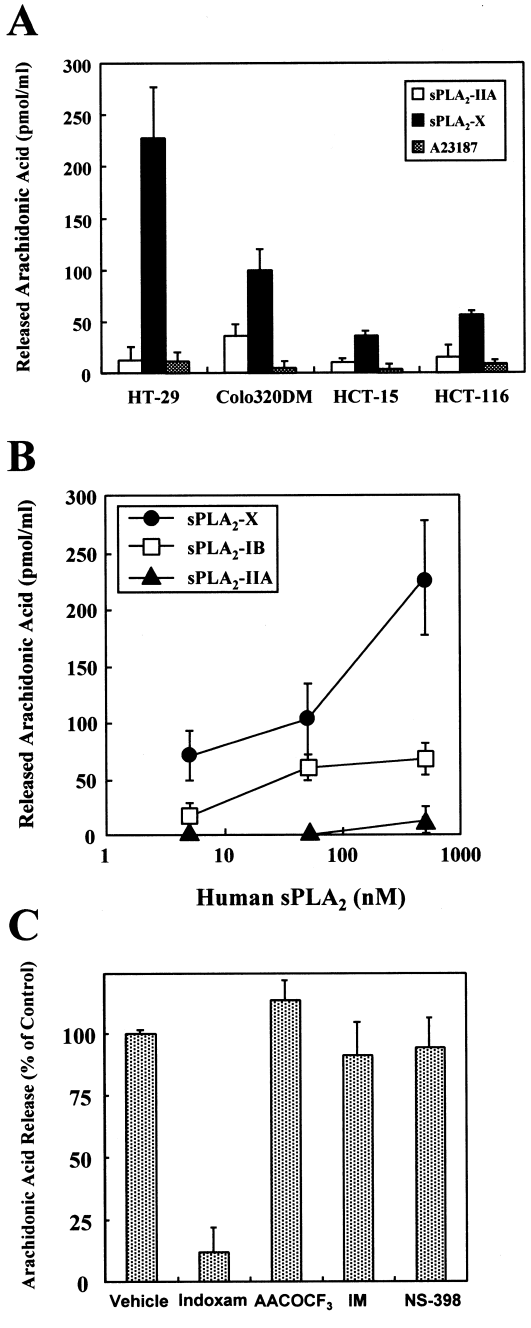


Fig. 1. Release of arachidonic acid from $sPLA_2$-X-stimulated human colon adenocarcinoma cells. A: The amount of arachidonic acid released from HT-29, Colo320DM, HCT-15 and HCT-116 cells by the actions of 500 nM $sPLA_2$-X, 500 nM $sPLA_2$-IIA and 10 μM A23187 for 30 min. B: Dose-dependent release of arachidonic acid from HT-29 cells by the actions of $sPLA_2$-X, $sPLA_2$-IB and $sPLA_2$-IIA for 30 min. C: Inhibitory potencies of $sPLA_2$ inhibitor indoxam, $cPLA_2$ inhibitor $AACOCF_3$, and COX inhibitors, indomethacin (IM) and NS-398 at 10 μM, against the arachidonic acid release from HT-29 cells treated with 100 nM $sPLA_2$-X for 30 min. The results are expressed as the percentage of arachidonic acid released by $sPLA_2$-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 $sPLA_2$-X in arachidonic acid release in cultured human colon tumor cells

We first examined the potencies of $sPLA_2$-IIA, $sPLA_2$-X and $cPLA_2$ 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. $sPLA_2$-X was found to elicit more potent release than $sPLA_2$-IIA and A23187 in four different cell types. Among the carcinoma cells examined, the strongest releases were observed in HT-29 cells, in which $sPLA_2$-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 $sPLA_2$s in the arachidonic acid release in HT-29 cells. Significant release was observed in the $sPLA_2$-X-treated cells at 5 nM, whereas $sPLA_2$-IIA could not induce a significant release even at 500 nM. $sPLA_2$-IB also induced arachidonic acid release at more than 50 nM with lower potency than $sPLA_2$-X. To investigate the mechanisms underlying the $sPLA_2$-X-induced release reactions, the effects of several inhibitors (10 μM) were examined. In contrast to efficient suppression by a specific $sPLA_2$ inhibitor, indoxam [23], a known $cPLA_2$ inhibitor, $AACOCF_3$, and two COX inhibitors, indomethacin and NS-398, did not suppress the responses (Fig. 1C), demonstrating that $cPLA_2$ is not involved in the $sPLA_2$-X-induced fatty acid release reactions.

### 3.2. Potency of $sPLA_2$-X in COX-2-dependent $PGE_2$ formation in cultured colon tumor cells

Next, the potency of $sPLA_2$-X in $PGE_2$ 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 $sPLA_2$-X (500 nM) did not evoke significant $PGE_2$ production for up to 4 h (Fig. 2A). After the induction of COX-2 by pretreatment with TNF-α, $sPLA_2$-X induced a time-dependent increase of $PGE_2$ formation. This reaction was slower than that induced by A23187, and the $PGE_2$ level at 6 h after $sPLA_2$-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 $sPLA_2$-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 $sPLA_2$-X-induced $PGE_2$ formation is completely dependent on COX-2 expression in human colon tumor cells. As shown in Fig. 2B, significant $PGE_2$ production was observed at 10 nM $sPLA_2$-X in TNF-α-pretreated HT-29 cells, whereas $sPLA_2$-IB evoked much weaker responses even at 500 nM and $sPLA_2$-IIA did not provoke any productions. As shown in Fig. 2C, indoxam suppressed the $sPLA_2$-X-stimulated $PGE_2$ production in a dose-dependent manner. In addition, indomethacin and NS-398 blocked the response

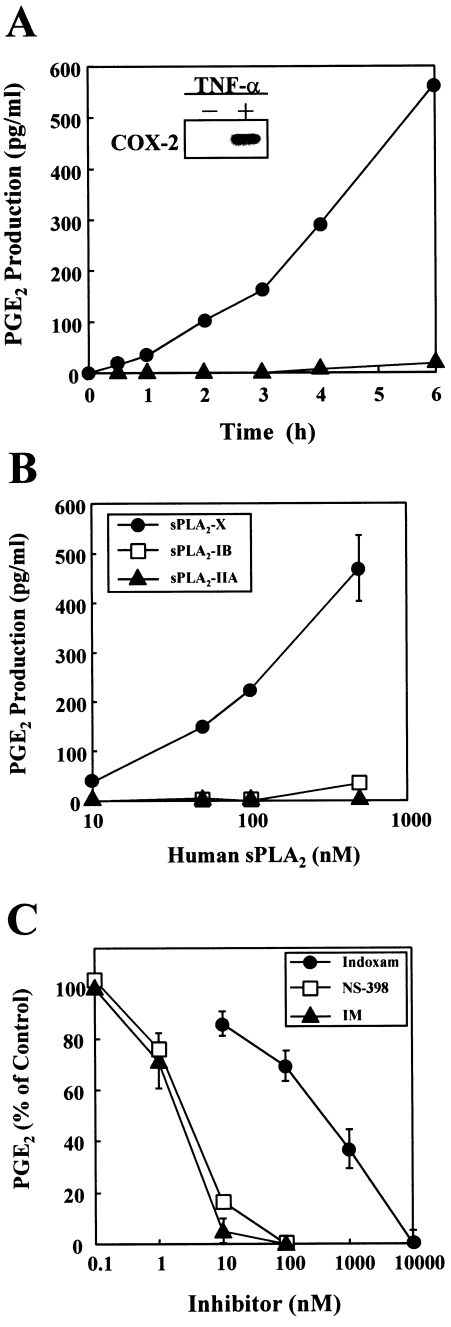


Fig. 2. COX-2-dependent $PGE_2$ production in $sPLA_2$-X-stimulated HT-29 cells. A: Time-dependent $PGE_2$ formation by $sPLA_2$-X in TNF-α-pretreated HT-29 cells. HT-29 cells were pretreated with (circles) or without (triangles) 30 ng/ml TNF-α for 18 h. After washing, the cells were stimulated with 500 nM $sPLA_2$-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 $PGE_2$ by $sPLA_2$s in TNF-α-pretreated HT-29 cells. After pretreatment with TNF-α, HT-29 cells were incubated with various concentrations of $sPLA_2$-X, $sPLA_2$-IB and $sPLA_2$-IIA for 3 h. C: Inhibition of $sPLA_2$-X-induced $PGE_2$ production. After pretreatment with TNF-α, HT-29 cells were preincubated with indoxam, NS-398 and indomethacin (IM), and then stimulated with 100 nM $sPLA_2$-X for 3 h. The results are expressed as the percentage of $PGE_2$ produced by $sPLA_2$-X in the absence of these agents. Each point represents the mean S.D. of triplicate measurements. The data are representative of three experiments.

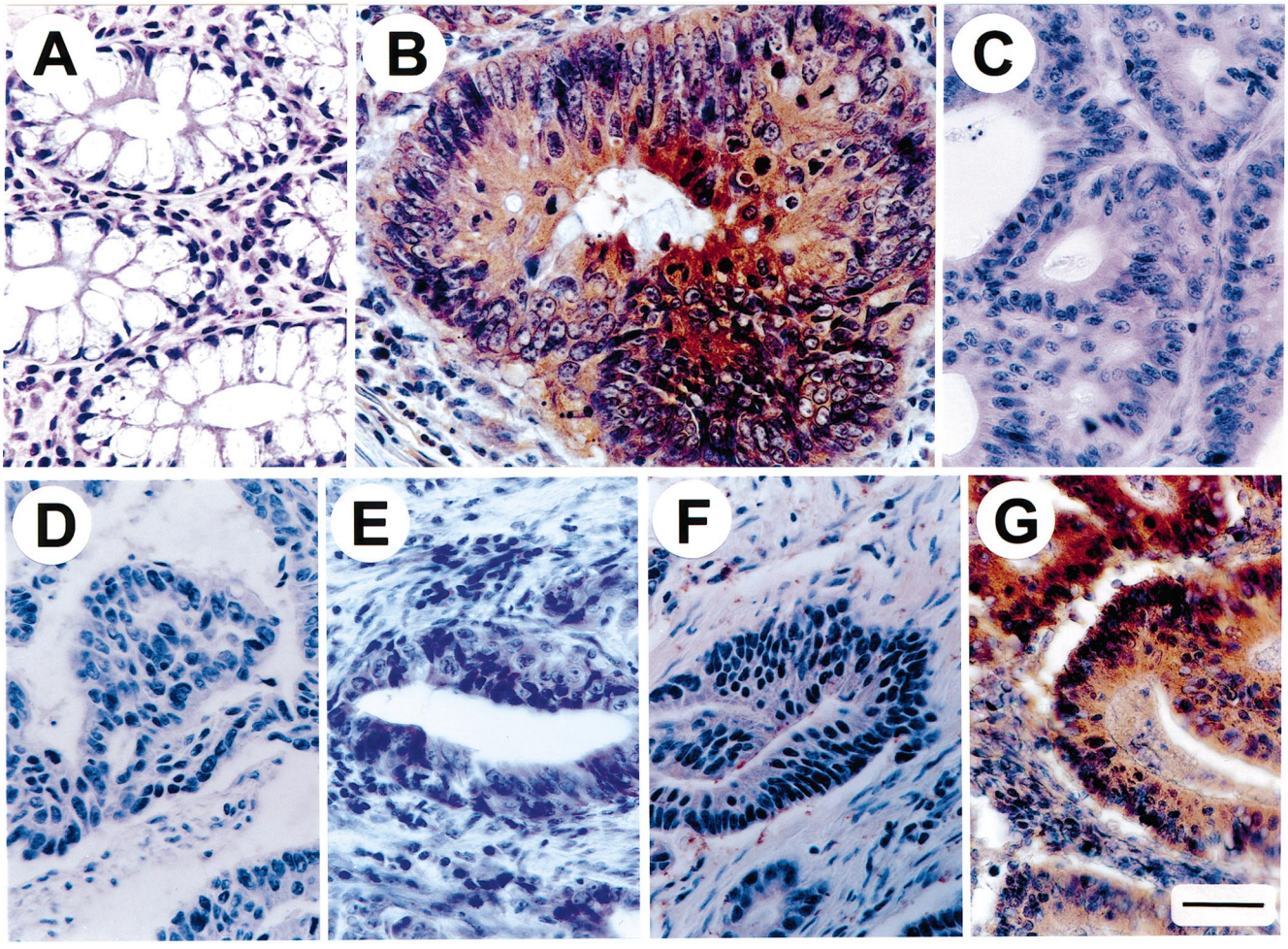


Fig. 3. Immunostaining for $sPLA_2$s, $cPLA_2$ and COX-2 in normal colon and colorectal adenocarcinoma tissues. Human normal colon (A) and well-differentiated colorectal adenocarcinoma tissues (B) were immunostained with anti-$sPLA_2$-X antibody (6 μg/ml). Control staining with anti-$sPLA_2$-X antibody absorbed with human $sPLA_2$-X protein (60 μg/ml) (C) was completely negative in colorectal adenocarcinoma tissues. Human colorectal adenocarcinoma tissues were also immunostained with anti-$sPLA_2$-IB (D), anti-$sPLA_2$-IIA (E), anti-$cPLA_2$ (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 $IC_{50}$ values of about 2 nM, demonstrating complete dependency of $PGE_2$ biosynthesis on COX-2 activity.

### 3.3. *Immunohistochemical analysis of $sPLA_2$-X expression in human colon adenocarcinoma tumor tissues*

We have recently developed a polyclonal antibody that specifically recognizes $sPLA_2$-X among the human $sPLA_2$ species [20]. Immunohistological analysis of normal human colon tissues revealed little, if any, expression of $sPLA_2$-X (Fig. 3A). In contrast, marked expression of $sPLA_2$-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 $sPLA_2$-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 $sPLA_2$-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 $PLA_2$ 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 $sPLA_2$-IB and $sPLA_2$-IIA (Fig. 3D,E), whereas slight but significant signals for $cPLA_2$ 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 $sPLA_2$-X and COX-2 are greatly enhanced in colorectal tumor cells.

## 4. Discussion

The present study demonstrates that $sPLA_2$-X induces a marked release of arachidonic acid leading to COX-2-dependent $PGE_2$ formation in cultured human colon adenocarcinoma cells. In human colorectal cancer tissues, the expression of $sPLA_2$-X was greatly up-regulated and correlated well with an elevated expression of COX-2, suggesting a pivotal role of $sPLA_2$-X in the COX-2-dependent $PGE_2$ biosynthesis during colon tumorigenesis. The expression of $cPLA_2$ could also be

detected in colon adenocarcinoma tissues (Fig. 3). Compared with cPLA$_2$ activator A23187, sPLA$_2$-X could elicit more potent release of arachidonic acid in various colon tumor cells (Fig. 1A). In addition, the sPLA$_2$-X-induced PGE$_2$ 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 sPLA$_2$-X plays a role in the sustained PGE$_2$ production in colon tumor cells. The enzymatic activity of sPLA$_2$-X is known to be regulated by the conversion of its pro-form to the mature enzyme via proteolytic removal of the propeptide [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 sPLA$_2$-X could be accelerated during the development of colon tumors. Because recent gene deletion studies have shown that cPLA$_2$ 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 sPLA$_2$-X in colon tumorigenesis deserves attention in the future. Further studies such as the analysis of the promoter regions of sPLA$_2$-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 (PPARδ) gene expression [27]. In the present study, sPLA$_2$-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, sPLA$_2$-X might modify the spectrum of PPARδ ligands leading to the alteration of PPARδ 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 sPLA$_2$ inhibitor can suppress the formation of various types of sPLA$_2$-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 sPLA$_2$-X inhibitors for colon cancer.

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