Expression analysis of the group IIA secretory phospholipase A₂ in mice with differential susceptibility to azoxymethane-induced colon tumorigenesis

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The murine non-pancreatic secretory phospholipase A₂ (sPLA₂) has been proposed as a tumor modifier of multiple intestinal neoplasia (Min). A genetic polymorphism in the mouse gene that causes a disruption in exon 3 results in loss of functional protein. Mouse strains with a disrupted sPLA₂ gene are susceptible to the Min phenotype and develop numerous intestinal polyps, whereas mice with normal sPLA₂ develop only a limited number of polyps. The following study was undertaken to test the hypothesis that sPLA₂ plays an equivalent role in murine susceptibility to the colon carcinogen azoxymethane (AOM). sPLA₂ status was confirmed by sequencing in mice that are highly susceptible (A/J), susceptible (SWR/J) and resistant (AKR/J) to AOM-induced tumorigenesis. Constitutive expression of sPLA₂ mRNA was compared in small intestine and colon of untreated mice using semi-quantitative RT–PCR. Whereas mRNA expression was nearly absent in A/J mice, AKR/J mice exhibited extensive expression throughout the intestine. Despite the wild-type sPLA₂ gene, colonic mRNA expression in SWR/J mice was significantly lower relative to AKR/J. Immunohistochemical analysis of sPLA₂ protein confirmed the mRNA data. The effect of AOM on colonic sPLA₂ expression was also examined. Twenty-four weeks after the last of six weekly injections of AOM (10 mg/kg i.p.), RT–PCR analysis of distal colons revealed a significant increase in mRNA in normal-appearing epithelium and tumor tissue from AOM-treated mice relative to controls. However, there was no corresponding increase in protein expression in A/J mice. The absence of sPLA₂ expression within control colons of tumor-susceptible A/J mice together with low expression in SWR/J colons is consistent with its potential role as an intestinal tumor modifier, but the carcinogen-induced increase in expression raises doubts as to the significance of sPLA₂ in inhibiting carcinogenesis.

Introduction

The study of gene–environment interactions and cancer predisposition has led to the identification of tumor modifiers in several organs, including lung, liver and small intestine (1). At least three tumor modifier genes may have a functional role in the intestine, including DNA methyltransferase (Dnmt), cyclooxygenase (COX)-2 (Cox-2) and a genetic factor, Mom1 (modifier of Min1) that decreases tumor multiplicity in the multiple intestinal neoplasia (Min) mouse model (2–4). The latter genetic factor has been mapped to distal mouse chromosome 4 (4). Within this chromosomal region resides a group of non-pancreatic secretory phospholipases of which the group IIA secretory phospholipase A₂ (sPLA₂) is a proposed candidate for Mom1 (5). Mutational analysis of the murine sPLA₂ gene identified a natural mutation in certain strains of mice that causes a disruption in the open reading frame of exon 3, resulting in a premature stop codon and a decrease in mRNA expression (5,6). The mutation is found within a BamHI endonuclease restriction site. Strains with the disrupted sPLA₂ gene are highly susceptible to the Min phenotype and develop numerous intestinal polyps, whereas mouse strains with the normal sPLA₂ gene develop a limited number of polyps. Such evidence suggests that sPLA₂ is the Mom1 genetic factor. Further evidence that Mom1 is, in fact, sPLA₂ was provided by Cormier et al. (7), who introduced the wild-type sPLA₂ allele into Min mice and found a significant reduction in tumor number and size.

The potential significance of sPLA₂ as a tumor modifier in chemical carcinogenesis models, however, is unknown. Repetitive treatment with the methylating carcinogen azoxymethane (AOM) produces colon tumors in mice that exhibit many of the pathological features associated with human forms of the disease (8). As in human populations, the genetic background of mice is a significant component of colon carcinogenesis. We have recently identified a panel of inbred mouse lines that differ markedly in their susceptibility to AOM. A/J and SWR/J mice are susceptible to AOM, although SWR/J mice develop significantly fewer tumors than A/J mice, whereas AKR/J mice are virtually resistant to the carcinogenic properties of this agent (9). The genetic basis of this differential response and the potential mechanisms by which putative tumor modifying genes may exert their influence on multistage carcinogenesis have not been established. Factors that may contribute to differential susceptibility to AOM and its parent compound, 1,2-dimethylhydrazine (DMH), include relative rates of carcinogen activation/detoxification, the extent of DNA alkylation or efficiency of repair, and the balance between proliferation and programmed cell death of initiated colon epithelial cells (10–14). In addition, genetic analysis of colon tumor susceptibility to DMH suggests that AKR/J mice express a protective or resistance factor that impedes the progression of carcinogen-induced foci (15). Thus, the recent identification of Mom1 resistance in AKR/J mice, together with these earlier observations in the DMH model, suggests that AKR/J mice express a powerful cancer modifier(s) that affects tumor multiplicity in two mouse models with potentially non-overlapping pathways.

In the following studies, we have examined the association of sPLA₂ with susceptibility to AOM-induced colon carcino-
Materials and methods

Treatment of animals

SWR/J, A/J and AKR/J mice, purchased from Jackson Laboratories (Bar Harbor, ME), were housed in a ventilated, temperature controlled (23 °C) room with a 12 h light/dark cycle. Mice were allowed access to laboratory rodent chow and water ad libitum up to the time of death. For the sPLA2 expression study, the entire intestinal tract of 6-week-old untreated mice was removed, flushed with ice-cold phosphate-buffered saline (PBS) and divided into discrete anatomical regions (proximal, middle and distal small intestine and colon). A section from each region was frozen in liquid nitrogen for RNA isolation and another section was fixed in formalin and embedded in paraffin. A concomitant study to generate tumor samples was also undertaken. Briefly, 6-week-old mice were injected i.p. once a week for a total of 6 weeks with 10 mg/kg AOM (Sigma Chemical Co., St Louis, MO). Control animals received vehicle saline alone. Twenty-four weeks after the last injection, the mice were killed. The colons were removed, flushed with PBS, opened longitudinally and fixed in 10% neutral buffered formalin. Before fixation, however, a number of macroscopically visible tumors from AOM-treated SWR/J and A/J mice were removed and divided into two equivalent pieces for subsequent RNA isolation and paraffin embedding. After fixation, the colon was stained with 0.2% methylene blue and the number of tumors was determined.

Reverse transcription (RT)–PCR analysis of gene expression

To determine constitutive expression, RT–PCR analysis was performed on samples from the proximal, middle and distal small intestine and colon from untreated animals of each strain. To determine the effect of AOM on sPLA2 expression, RT–PCR analysis was performed on the distal colon of control and AOM-treated mice and on tumor samples from A/J and SWR/J mice. Using TRIzol reagent (Gibco BRL, Gaithersburg, MD), total cellular RNA was isolated from previously frozen tissues according to the manufacturer’s instructions. RNA was then incubated at 60°C for 10 min and maintained at 4°C before being reverse transcribed. Reverse transcription of 2 μg of total RNA was performed in a volume of 20 μl containing 50 U of MMLV reverse transcriptase, 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 5 mM MgCl2, 1 U/ml RNasin, 1 μM each dATP, dGTP, dCTP and dTTP, and 100 pmol of random hexamers (Gibco BRL), for 60 min at 37°C. The samples were incubated for 10 min at 25°C before reverse transcription and heated to 99°C for 5 min to terminate the reverse transcription reaction. The cDNA was then amplified for a 167 bp region of the sPLA2 gene which contains the BamHI recognition site (5) as well as for hypoxanthine-guanine phosphoribosyltransferase (HPRT), a housekeeping gene which was amplified in parallel. The amplification reaction mixture consisted of 2 μl cDNA, 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl2, 0.2 μM each dATP, dGTP, dCTP and dTTP, 0.25 M each sense and antisense primers, and 1.25 U of Taq DNA polymerase (Gibco BRL) in a final volume of 50 μl. A reaction lacking reverse transcriptase was run as a negative control. The primers used for amplification of sPLA2 were: sense, 5’-CGCAGTTTGGGGAATTGATT-3’; antisense, 5’-TCCAGGCTT-TTGATGACAAACGT-3’. The primers used to amplify HPRT were: sense, 5’-GTAATGATCTGTAACCGGGAC-3’; antisense, 5’-CCAGCAAGCT-TGCAACCTTAAACCA-3’. The primers for sPLA2 were designed so as not to amplify the alternative spliced product from Mom1 sensitive, i.e. A/J, mice (5,6). For amplification of sPLA2 and HPRT, the reaction mixture was first heated at 95°C for 30 s, and amplification was carried out for 34 cycles for HPRT and 28 cycles for sPLA2. For sPLA2, at 95°C for 15 s, 60°C for 30 s and 72°C for 30 s, followed by an incubation for 7 min at 72°C. The number of amplification cycles was previously determined to avoid the plateau effect associated with increased numbers of PCR cycles. PCR products of each specific gene were electrophoresed through a 3% agarose gel containing 0.5 X TBE buffer. For quantitation of PCR products, the intensity of ethidium bromide stained products was measured using a Bio-Rad Image Analysis System (Gel Doc 1000) with Molecular Analyst software (Bio-Rad Laboratories, Hercules, CA). The intensity of each sPLA2 band was normalized to the intensity of the corresponding HPRT control band.

DNA sequencing

PCR products were separated in a 3% agarose gel and visualized by ethidium bromide staining. The resulting 167 bp band was purified from the gel using the Qiaex II Gel Extraction Kit (Qiagen, Santa Clarita, CA). The reverse primer used was the primer for sequencing the antisense strand of the
amplified products. DNA sequencing was carried out using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit and an ABI 377 Prism Automated DNA Sequencer (Applied Biosystems, Foster City, CA).

**Immunohistochemical analysis of sPLA₂**

Four micrometer paraffin-embedded tissue sections were deparaffinized and rehydrated. Endogenous peroxidase activity was ablated with 3% hydrogen peroxide in PBS for 30 min. Non-specific protein binding was suppressed with 10% goat serum. Tissue sections were incubated at 4°C overnight with rabbit polyclonal anti-sPLA₂ antibody at 1:10 000 dilution (16). The sections were then washed and incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) at room temperature for 30 min. After washing, the sections were incubated with avidin–biotin–peroxidase complex at room temperature for 30 min using the Vectastain Elite ABC kit (Vector Laboratories). After color development with 3,3-diaminobenzidine as the substrate, sections were counterstained with Harris’s acid hematoxylin. To demonstrate the specificity of the immunostaining, the primary antibody was replaced with similar protein concentrations of normal rabbit IgG.

**Statistics**

All values represent the means ± SEM of 4–8 individual animals per group. Strain- and treatment-related differences were compared by analysis of variance (ANOVA) followed by the Newman–Keuls post hoc test. A value of \( P < 0.05 \) was considered statistically significant.

**Results**

**Genotype of sPLA₂**

The genotypes of the different strains used in our study were identified in earlier studies. MacPhee et al. (5) showed that A/J mice are Mom1 sensitive and have a corresponding frameshift mutation in the sPLA₂ gene. AKR/J mice, on the other hand, were shown to be Mom1 resistant and have the normal allele. In a related study, Gould et al. (17) showed that SWR/J mice have the same genotype as AKR/J mice. In our first experiment, cDNA was reverse transcribed from RNA isolated from untreated mice. Direct sequencing of PCR products confirmed that the frameshift mutation is present only in the sPLA₂ gene of A/J mice (data not shown).

**Constitutive expression of sPLA₂ mRNA**

After confirming the sPLA₂ genotype, mRNA expression patterns were compared in discrete regions of the intestinal tract using semi-quantitative RT–PCR analysis (Figure 1). RT–PCR analysis of mRNA samples isolated from the intestine using primers specific for sPLA₂ and the housekeeping gene HPRT produced DNA fragments of the expected sizes (Figure 1A). The sPLA₂ levels in the proximal, middle and distal regions of the small intestine and colon, quantified by scanning the bands using an image analysis system and normalized to HPRT levels, are shown in Figure 1B. In A/J mice, mRNA expression was barely detectable throughout the entire length of the intestine, consistent with its null genotype (5). In contrast, AKR/J mice produced a strong and consistent signal throughout each region of the gut examined. Although relatively high levels of mRNA expression were found in the small intestine of SWR/J mice, colonic expression was lower relative to the small bowel. Furthermore, when compared with the AKR/J colon, mRNA levels in the SWR/J mice were significantly lower in the proximal (70% lower, \( P < 0.01 \)), middle (58%, \( P < 0.001 \)) and distal (63%, \( P < 0.01 \)) colon. Levels were also significantly lower in the middle (28% lower, \( P < 0.05 \)) and distal (40%, \( P < 0.01 \)) small intestine of SWR/J relative to AKR/J mice.

**Expression of sPLA₂ mRNA in AOM-treated mice**

After examining constitutive expression of sPLA₂ in 6-week-old untreated control animals, we next evaluated the potential effects of AOM treatment on sPLA₂ mRNA expression. Twenty-four weeks after the last of six weekly injections with AOM, distal colons were examined for the presence of tumors. These data are summarized in Table I. AOM exposure produced a significantly greater number of tumors within the colons of the A/J mice relative to the SWR/J mice (28.0 ± 3.6 versus 3.8 ± 0.7, \( P < 0.05 \)). No tumors were found in AKR/J colons. RNA was isolated from the distal colons of control and AOM-treated mice and from tumors of A/J and SWR/J mice. As

**Table I. Induction of tumors in the distal colon by AOM treatment**

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<th>Strain</th>
<th>Treatment</th>
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<td>A/J</td>
<td>Control</td>
<td>0</td>
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<td></td>
<td>AOM</td>
<td>30.1 ± 3.6a</td>
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<tr>
<td>SWR/J</td>
<td>Control</td>
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<td>AOM</td>
<td>4.3 ± 0.67b</td>
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<tr>
<td>AKR/J</td>
<td>Control</td>
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The number of tumors was ascertained as described in Materials and methods.

Data points that are significantly different (\( P < 0.05 \)). Only AOM-treated groups were compared by one-way ANOVA.
shown in Figure 2, AOM produced a significant increase (1.4-fold, $P < 0.05$) in sPLA$_2$ expression within the AKR/J colon. Similarly, mRNA levels in AOM-exposed SWR/J mice were significantly elevated in tumor tissue (2.7-fold, $P < 0.05$) and in normal-appearing epithelium (1.4-fold, $P < 0.05$) relative to controls. While sPLA$_2$ mRNA was barely detectable in A/J control mice, AOM produced a significant increase in mRNA levels in normal-appearing epithelium (8.3-fold, $P < 0.01$) and in tumors (17-fold, $P < 0.001$) compared with colons from control mice. Subsequent DNA sequencing, however, confirmed the presence of the frameshift mutation in the A/J mice (data not shown). Therefore, the AOM-induced elevation in mRNA expression could not be accounted for by a genetic alteration within the sPLA$_2$ gene, at least within the region amplified by our primer set.

**Immunohistochemistry for sPLA$_2$**

To gain further insight into the cellular distribution of sPLA$_2$ within the colonic epithelium and to compare expression between the strains, immunohistochemical analyses were performed on tissue sections from control mice. As shown in Figure 3, sPLA$_2$ protein was strongly localized to Paneth cells found within the lower crypt compartment throughout the small intestine, confirming earlier studies in Balb/c mice (16,18). This staining pattern was comparable in both AKR/J and SWR/J mice (Figure 3A and B, respectively). As predicted on the basis of mRNA expression patterns, A/J mice did not demonstrate immunoreactivity in any region of the small intestine examined (Figure 3C). Although Paneth cells are not present within the colonic epithelium, sPLA$_2$ staining was evident in the AKR/J colon. Immunostaining was localized primarily within the lower crypt compartment, although intermittent staining was apparent in cells at higher positions within individual crypts. Immunoreactivity is evident throughout the entire colon but not in every crypt (200×). (E) Normal colonic epithelium from an AKR/J mouse. The colons of A/J mice are uniformly negative for sPLA$_2$ staining (200×).

Figure 3. Immunohistochemical analysis of sPLA$_2$ in 6-week-old untreated mice. (A) Normal small intestine epithelium from an AKR/J mouse. Staining is localized to Paneth cells in crypts at the base of villi and is found throughout the entire small intestine (200×). (B) Normal small intestine epithelium from an SWR/J mouse. The pattern is the same as in the AKR/J mouse in (A) (200×). (C) Normal small intestine epithelium from an A/J mouse. There is no immunoreactivity in any region of the small intestine examined (200×). (D) Normal colonic epithelium from an AKR/J mouse. Immunostaining is localized primarily within the lower crypt compartment, although intermittent staining is apparent in cells at higher positions within individual crypts. Immunoreactivity is evident throughout the entire colon but not in every crypt (200×). (F) Normal colonic epithelium from an A/J mouse. The colons of A/J mice are uniformly negative for sPLA$_2$ staining (200×).

Figure 4. Immunohistochemical analysis of sPLA$_2$ in colons and tumors from AOM-treated mice. (A) Colonic epithelium from a saline-treated AKR/J mouse (200×). (B) Normal-appearing colonic epithelium from an AOM-treated AKR/J mouse (200×). (C) Colonic epithelium from a saline-treated SWR/J mouse (200×). (D) Adenoma from a SWR/J mouse. Staining is evident within the adenoma but not in the surrounding normal-appearing mucosa (200×). (E) Colonic epithelium from a saline-treated A/J mouse. There is no staining in the mucosa of the colon (200×). (F) Adenoma from an A/J mouse. No staining is evident either in the adenoma or the surrounding normal-appearing mucosa (100×).
underlying stroma. There was no staining in either normal-appearing colonic mucosa or in tumors from AOM-treated A/J mice, despite the increase in mRNA expression described earlier.

Discussion

These studies were undertaken to assess the potential role of the *Mom1* candidate, sPLA2, in a mouse carcinogen model. AOM has been shown to produce colonic epithelial lesions in AKR/J, SWR/J and A/J mice, however, tumor progression occurs only in the latter two strains (9). Sequencing analysis of these mouse lines with varying susceptibilities to AOM confirmed the presence of the wild-type form of the sPLA2 gene in both the AKR/J (resistant) and SWR/J (susceptible) mice. In contrast, A/J mice, which have been shown to develop a high number of tumors, were found to have the identical frameshift mutation as that present in C57BL/6J mice, a background strain on which the *Min* intestinal tumor phenotype is fully expressed (4,5). It was further demonstrated that colonic sPLA2 mRNA and protein levels in untreated or control mice are inversely correlated with tumor susceptibility to AOM. In fact, colons from A/J mice lacked sPLA2 mRNA and protein expression altogether. These data suggest that colonic sPLA2 may have a role in chemically induced tumorigenesis that is equivalent to its tumor modifier role in the *Min* model.

On the other hand, if the hypothesis is correct that susceptibility to AOM is inversely related to levels of sPLA2, then all the strains having the frameshift mutation in the gene should be susceptible to the carcinogen. While no one has studied the induction of tumors by AOM in C57BL/6J mice, it has been shown that the strain is susceptible to tumor formation by DMH (19), the parent compound of AOM, and by methylazoxymethanol, a metabolite of AOM (20). However, this strain was only relatively susceptible, having a lower incidence and frequency of tumors than SWR/J mice. It is possible, of course, that C57BL/6J mice have other resistance genes which prevent complete susceptibility to colon carcinogenesis.

By learning more about the function of sPLA2, it may be possible to understand if this protein can modulate tumorigenesis in two cancer models with potentially non-overlapping molecular pathways. The sPLA2 protein catalyzes the release of fatty acid from the sn-2 position of phospholipids, yielding free fatty acids, such as arachidonic acid, and lysophospholipid. A number of products released by the catalytic action of sPLA2 have been implicated in local and systemic inflammation (21,22). sPLA2 also has biological properties that associate it with tumor progression. Recently, it was reported that membranes of cells undergoing apoptotic death are highly susceptible to type II sPLA2, providing a potential pathway for elimination of transformed cells (23). These observations suggest biochemical properties of sPLA2 that could directly or indirectly involve this protein in colon tumorigenesis.

As noted, there are two potentially contrasting activities for sPLA2 in colon cancer. Genetic and functional studies clearly place sPLA2 within the context of an intestinal tumor modifier (4–7). However, rationalizing the mechanism by which sPLA2 might suppress colon carcinogenesis is complicated by observations that COX-inhibiting non-steroidal anti-inflammatory drugs (NSAID)s suppress colon cancer development, a mechanism that implicates prostaglandins as tumor promoters. Since arachidonic acid is the main substrate for COX, sPLA2 is predicted to increase prostaglandin production in the colon (reviewed in ref. 24). It has been proposed, however, that cancer suppression by NSAIDs is unrelated to its influence on prostaglandin synthesis; non-COX-inhibiting NSAID derivatives (e.g. sulindac sulfone) are also effective at suppressing cancer development without reducing prostaglandin levels in the colon (25–27). This raises an alternative possibility that some arachidonic acid metabolites may have tumor suppressing activities while others are tumor promoters. These contrasting properties may depend, for example, on the specific effects of particular eicosanoids on the immune response.

Data regarding human sPLA2 are also complicated. A recent report of sPLA2 gene loss in a sporadic colon tumor suggests that loss of heterozygosity of the sPLA2 locus is common in sporadic cancer, suggesting that sPLA2 plays a suppressive role in cancer development and progression (29). In addition, ~30–40% of human colon cancers have abnormalities involving chromosome 1p35–36, the region which corresponds to mouse chromosome 4 (30–32). However, in contrast to findings in mice, the absence of gene mutations in the human homolog of sPLA2 in sporadic and FAP colorectal tumors suggests that sPLA2 is not a tumor modifier in humans (33,34). In fact, overexpression of sPLA2 is found in a number of different human cancers, including colon, breast and liver cancer (35–37). Our findings of increased sPLA2 mRNA expression in AOM-treated colons and tumors corroborate these recent observations regarding sPLA2 expression in human colorectal tumors. It should be noted, however, that the increase in sPLA2 message in A/J mice was not accompanied by an increase in protein. Our findings of increased colonic expression upon AOM exposure are consistent with a recent study in which 1-hydroxy-9-anthraquinone, a colon carcinogen, produced an increase in sPLA2 mRNA levels in tumors from F344 rats (38). In addition, ursodeoxycholic acid, a chemopreventive agent that is active within the colon, was shown to decrease levels of sPLA2 mRNA and protein in AOM-treated rats. This effect was directly correlated with a decrease in the number of aberrant crypt foci, putative preneoplastic lesions (39).

Data from 6-week-old untreated mice, in which constitutive sPLA2 expression is correlated with differential susceptibility to AOM, suggest a tumor modifying role for this protein within the colon, at least during the early stage of carcinogenesis. However, the carcinogen-induced increase in expression noted in this study and in the aforementioned studies argues that sPLA2 may have actions that involve it in tumor progression. Additional studies aimed at further defining the biological role of sPLA2 will be required to more fully understand its potential significance in intestinal tumorigenesis.

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