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Increased Expression and Activity of Group IIA and X Secretory Phospholipase A2 in Peritumoral *versus* Central Colon Carcinoma Tissue

LINE TRIBLER[1,2], LOTTE T. JENSEN[1], KENT JØRGENSEN[1], NILS BRÜNNER[2], MICHAEL H. GELB[3], HANS JØRGEN NIELSEN[4] and SIMON S. JENSEN[1]

[1]*LiPlasome Pharma A/S, Technical University of Denmark, DK-2800 Lyngby;*
[2]*Department of Pharmacology and Pathobiology, Faculty of Life Sciences, University of Copenhagen, DK-1855 Frederiksberg;*
[4]*Department of Surgical Gastroenterology, Hvidovre University Hospital, DK-2650 Hvidovre, Denmark;*
[3]*Departments of Chemistry and Biochemistry, University of Washington, Seattle, WA 98195, U.S.A.*

Abstract. *Secretory phospholipase A2 (sPLA2) type IIA and X was analyzed in tumors from 22 patients with colon adenocarcinomas in order to determine the involvement and activity of sPLA2 in colon cancer. Evaluation of immunoreactive sPLA2 IIA by Western blotting showed a significantly higher level in the periphery of the tumors, compared to central tumor regions. Increased levels of sPLA2 IIA protein correlated with a two-fold increase in sPLA2 enzymatic activity in the peripheral regions compared to central regions. Nineteen out of 22 tumors showed high levels of sPLA2 IIA, whereas 7 out of the 22 tumors showed sPLA2 type X. These data demonstrate that both sPLA2 type IIA and X are present in human colon cancer and suggest a role for sPLA2 in colon cancer tumor immunology and tumorigenesis.*

Phospholipase A2 (PLA2) includes a large family of lipases capable of hydrolyzing the *sn*-2 position of phospholipids. The PLA2 family is subdivided into large intracellular 85 kDa lipases (cPLA2 and iPLA2) and small secretory 14 kDa lipases (sPLA2); the latter comprise 10 subtypes in humans to date (1, 2). The sPLA2s are widely expressed at transcript level in various human tissues (3), whereas protein expression is more limited for most sPLA2 subgroups in humans. The most studied sPLA2 subtype in humans is type IIA, involved in inflammatory conditions, such as arthritis (4), acute lung injury (5), psoriasis (6) and cancer (7). The involvement of sPLA2 IIA in cancer and inflammatory diseases has lead to the development of novel prodrug and liposome based drug delivery systems that are specifically activated or degraded by sPLA2 in the diseased tissue, causing a site-specific release of the associated or encapsulated drugs (8-12). The converse strategy of preventing sPLA2 activity with concomitant arachidonic acid and eicosanoid generation has also been suggested. Several natural and synthetic inhibitors against sPLA2 have been identified and developed, while several of these have been evaluated in the treatment of inflammatory conditions (3, 13).

Type IIA and X sPLA2 are of particular interest in relation to human cancer, and increased expression of sPLA2 IIA has been identified by immunohistochemistry, Northern blot, *in situ* hybridization and Western blotting in several human tumors from breast (7, 14), stomach (7, 15, 16) colorectal (7, 17-19), pancreatic (7, 20), prostate (21, 22) and liver cancer (7, 23).

In contrast to sPLA2 IIA, sPLA2 X has been reported only in a colon tumor, where it was identified by immunohistochemistry, co-expressed with COX-2 (24). A study of colorectal carcinomas from 42 patients revealed significantly induced cPLA2-transcript, whereas sPLA2 X transcripts were not significantly altered when compared to normal adjacent mucosa (25).

Although a large number of studies clearly demonstrate that sPLA2 is present in human cancer, it is controversial whether sPLA2 IIA has a pro- or antitumorigenic role in cancer. In prostate and breast cancer, immunohistochemical analysis of sPLA2 IIA showed increased expression with tumor grade and correlated inversely with 5-year patient survival (14, 21, 22). A hypothesis for a pro-tumorigenic role for sPLA2 and cPLA2 in colorectal cancer was also

Correspondence to: Hans Jørgen Nielsen, MD, DMSc, Professor of Surgical Oncology, Department of Surgical Gastroenterology 435, Hvidovre University Hospital, DK-2650 Hvidovre, Denmark. Tel: +1 45 36322436, Fax: +1 45 36323760, e-mail: h.j.nielsen@ofir.dk

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suggested in earlier studies, which showed increased cPLA2 transcript and activity, increased levels of sPLA2 protein, increased level of PLA2 substrates and hydrolysis products, such as platelet activating factor (PAF), lyso-PAF, free fatty acids and arachidonic acid (24, 26-29). In particular the increased arachidonic acid production leads *via* COX-2 to generation of prostaglandin E2 (PGE2), which has been suggested to inhibit host *versus* tumor immune responses (30), and upon secretion acts in an autocrine or paracrine manner to promote cell growth (31).

A tumor suppressing role for sPLA2 IIA was suggested by constitutively expressing the sPLA2 IIA gene in the Min mouse strain, in which the Apc^{Min} mutation disrupts the mouse homologue of the human polyposis gene, causing multiple intestinal neoplasms. Transgenic Min mice, expressing sPLA2 IIA showed reduction in tumor numbers and size (16). In human pancreatic and gastric cancer, a tumor-suppressing role of sPLA2 IIA was suggested, since sPLA2 IIA expression was associated with increased survival and less frequent metastasis (16, 20).

The objectives of the present study were to analyze sPLA2 type IIA and X in primary tumors from human colon cancer patients at the protein level and determine their expression in peritumoral and central tumor regions. Furthermore, we wanted to assess the relationship between the level of sPLA2 identified by Western blotting and the activity of sPLA2 seen in the tumor specimens. Finally, the serum levels of sPLA2 IIA were tested for association with the findings of sPLA2 in the corresponding tumors and with the clinical stage of tumor development.

Materials and Methods

Patients and samples. Analyses of the presence of sPLA2 IIA and sPLA2 X proteins in solid tumors and serum were conducted on tissue samples obtained from 26 patients (16 males and 10 females) with a mean (range) age of 71.2 (56-90) years who underwent surgical resection for primary colon adenocarcinomas or diverticulosis (3 patients) (confirmed by pathological examination). Serum samples from 20 patients with colon cancer, 3 patients with diverticulosis and one normal individual were analyzed by the sPLA2 IIA specific ELISA-assay (Cayman Chemical, Ann Arbor, MI, USA).

The tumors were localized in the colon and classified as presented in Table I. From each tumor two different tissue samples were excised, the central tissue (T1) taken just outside the necrotic area, and the peripheral tissue (T2). Due to the limited amount of material available, 3 out of the 22 samples consisted only of T2 tissue. Furthermore, adjacent paired colon tissue, localized in the surgically removed specimen was available from 6 out of 22 patients. All samples were stored at –80°C until analysis.

Tissue preparation. From each patient, tumor pieces of 50-100 mg were excised and sonicated in cold RIPA-buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Lysates were subsequently clarified by centrifugation at 12,000 xg at 4°C for 15 minutes.

Table I. *Patients entering the study with colon tumor material were grouped according to Dukes classification system, sex, age and anatomical localization of the tumor in the colon. A total of 26 patients donated tissue material.*

Parameter	Number of patients
Dukes tumor stage	
A	3
B	11
C	6
D	2
Total	22
Diverticulosis	3
Normal	1
Gender (all)	
Male	16
Female	10
Age (CRC patients)	
≤50	0
51-60	5
61-70	3
71-80	6
81	8
Anatomical site	
Right colon	8
Left colon	14

Measurement of the protein concentration was performed using the Pierce BCA Protein Assay (Pierce Biotechnology, Rockford, IL, USA) and adjusted to 1.5 mg/ml in NuPage Sample buffer without reducing agents (Invitrogen, Carlsbad, CA, USA).

Western blot analysis of sPLA2 IIA and sPLA2 X. Tumor lysates (1.5 mg/ml) were heated for 10 minutes at 80°C, vortexed briefly, spun down at 8,000 xg for five minutes and subsequently loaded on a NuPage™ 10% bis-tris gel under non-reducing conditions. Electrophoresis was carried out according to the manufacturer's recommendations (Invitrogen). A volume of 5 µl of the tumor lysates was loaded onto the gel and human tear fluid (50 µg/ml, diluted x 200) and recombinant bacterially expressed human sPLA2 X protein (0.5 ng/µl) was used as positive control both for sPLA2 IIA and sPLA2 X. Primary antibody was a rabbit antihuman-sPLA2 IIA polyclonal antibody [Cayman Chemical (CPC), or Alexis Biochemicals, San Diego, CA, USA, (APC)], diluted 1:500 (PBS, 0.1% Tween 20, 5% skimmed milk) or a rabbit antihuman sPLA2 X polyclonal antibody (39) diluted 1:1000. Primary antibodies were analyzed for cross-reaction against sPLA2 X and V (sPLA2 IIA antibodies) and sPLA2 IIA and V (sPLA2 X antibodies). A mouse monoclonal antibody (4A1) raised against human sPLA2 IIA confirmed the data seen with CPC and APC, although with much lower affinity (data not shown) (4A1 was kindly donated by K.F. Scott, The University of New South Wales, Australia) (32). Cross-reactions against sPLA2 X and V of antibodies recognizing sPLA2 IIA were evaluated by lack of recognition of 5 ng recombinant sPLA2 V and X, as well as sPLA2 X expressed from genetically modified human HEK293 cells (Kindly donated by M. Murakami, Showa University, Tokyo, Japan). Antiserum against sPLA2 X readily detected 0.5-1 ng of

recombinant sPLA2 X by Western blots but failed to detect 50 ng of several other human sPLA2s. Immuno- detection was accomplished using horseradish peroxidase labeled goat anti-rabbit IgG (H+L) (Vector Laboratories Inc., Burlingame, CA, USA) diluted 1:4000, SuperSignal®West Pico enhanced chemiluminiscence (ECL) reagents (Pierce), and Hyperfilm-ECL (Amersham Pharmacia, Piscataway, NJ, USA). The Simply Blue SafeStain system (Invitrogen) was used for Coomassie staining for control of equal protein content in each lane.

Analysis of sPLA2-IIA in serum. Serum samples (diluted 1:20) from 20 colon cancer patients, 3 control patients with diverticulosis and one healthy person were analyzed for presence of sPLA2 type IIA using the sPLA2 (human synovial) Enzyme Immunoassay Kit (Cayman Chemical) according to the supplier's instructions. The assay was validated against sPLA2 X and V expressed from recombinant modified HEK 293 cells. No cross-reaction was identified against these two sPLA2 proteins.

sPLA2 activity assay. Tissue was excised, and cut into pieces of 10 mg (range 8.6 mg – 11.8 mg) and incubated as tumor chunks at 37°C with stirring for 24 h in 250 μL HEPES buffer containing liposomal substrate as described elsewhere (11). Briefly, the liposomal substrate contained DPPG (1,2-hexadecanoyl-*sn*-glycero-3-phosphoglycerol):DPPC (1-hexadecanoyl-2-(1^{14}C)-hexadecanoyl-*sn*-glycero-3-phosphocholine mixed (99:1), (final 5 μCi/ml), (Larodan Fine chemicals AB, Malmö, Sweden) and (Amersham Biosciences, Bucks, United Kingdom) at a final concentration of 10 mg/ml. Samples were extracted in chloroform:methanol:acetic acid (2:4:1) and analyzed by HPLC according to the manufacturer's instructions using a 5 μM Phenomenex diol spherical column (Torrance, CA, USA). Separation was achieved using gradient elution: Eluent A was: hexane:isopropanol:acetic acid (82:17:1) and eluent B was: isopropanol:H_2O:acetic acid (85:14:1). For detection of radiolabelled lipids, a 150TR Flow Scintillation Analyzer (FSA) was used (Packard Instruments, Meriden, CT, USA).

Control rat breast tissue was taken from 10- to 12-week-old female Sprague-Dawley rats. Rat breast tumor tissue was chemically induced with 20 mg dimethylbenzanthracene (DMBA)/animal, solubilized in 1 ml vegetable oil and administered by intragastric infusion to 50-day-old female Sprague-Dawley rats. After 2-5 weeks, tumors developed and were removed for further analysis. The degree of hydrolysis was calculated by the relative increase in the fatty acid signal. The reported results are the average of two or three experiments, each in duplicate or triplicate.

Statistical analysis. Due to the non-normal distribution of the serum sPLA2 IIA data, non-parametric tests were chosen for significance tests. The Mann-Whitney *U*-test was used to examine the differences of sPLA2 IIA contents in serum from colon cancer patients and control patients. Examination of the differences between the different cancer stages and serum sPLA2 IIA values were performed using the Kruskal-Wallis non-parametric test. This test was also used to investigate differences between colon cancer stage according to Duke's classification system and the localization of sPLA2 IIA in the tumor. To examine the significance of the localization of sPLA2 IIA in the tumors the χ^2-test was used. Activity assays were analyzed by either Wilcoxon matched pairs signed rank test or Wilcoxon rank sum test. $P<0.05$ was considered as significant.

Table II. *Evaluation of sPLA2 IIA and X protein amounts in lysates of colon tumors derived from the central tumor just outside the necrotic region (T1) and in the periphery of the tumor (T2) evaluated by Western blot. The relative signals were judged from Western blot in comparison to the human sPLA2 IIA control protein, derived from human tear fluid. Nineteen samples from T1 and 22 samples from T2 tumors were analyzed, 19 were paired from the same tumor, and three T2 samples were analyzed without a corresponding T1 sample. There was a significantly stronger expression of sPLA2 IIA alone (and with type X sPLA2) in T2 regions than in T1 regions, as analyzed by χ^2-test ($p<0.005$). Overview of findings in sPLA2 type IIA and X by Western blotting.*

	Absent	Weak	Moderate	Strong
sPLA2 IIA				
T1	14/19	5/19	0/19	0/19
T2	5/22	7/22	5/22	5/22
sPLA2 X				
T1	17/19	1/19	1/19	0/19
T2	16/22	4/22	1/22	1/22
sPLA2 IIA and X				
T1	13/19	5/19	1/19	0/19
T2	3/22	8/22	5/22	6/22

Results

Western blot analysis of sPLA2 IIA with two different antibodies raised against human sPLA2 IIA was carried out in order to evaluate the expression in colon carcinoma lysates. The intensity of the signal was judged in comparison to human sPLA2 IIA controls loaded on each gel, and evaluated as: not detectable (absent), weak, moderate and strong intensity (Table II). Fourteen of the 19 samples from the central region of the tumor (T1) showed no sPLA2 IIA, while 5 showed weak sPLA2 IIA content. In contrast, the region from the tumor periphery (T2) showed sPLA2 IIA in 17 out of 22 samples, of which 10 showed strong to moderate expression. Using χ^2-test for statistical analysis, T2 regions showed significantly more sPLA2 IIA than T1-regions ($p<0.005$). A representative Western blot is shown in Figure 1A, where non-tumor controls from patients with diverticulosis showed no sPLA2 IIA (panel II and III, lane 4 and 5), and a patient with colon cancer showed no sPLA2 IIA in neither T1 nor T2 (lane 6 and 7). Lanes 8 to 13 show tumors from 3 patients with different levels of sPLA2 IIA in the periphery of the tumor and no sPLA2 IIA in central regions. An estimation of the amount of sPLA2 IIA protein from patient 181T2 (Pt2), representing one of the highest amounts of sPLA2 IIA seen in this study, approximated 100 ng sPLA2 IIA/mg total protein lysate, based on the concentration of sPLA2 IIA in human tear fluid at 50 μg/ml (33).

A similar analysis was carried out using a polyclonal antibody recognizing specifically human sPLA2 type X

(Table II and Figure 1B). Six out of the 22 tumors examined contained sPLA2 X in the peripheral region, while 2 tumors showed sPLA2 X in the central region. The total levels of sPLA2 IIA and/or X were found to be significantly increased in the peripheral compared to the central regions ($p<0.005$) (Table II). An example of a Western blot against sPLA2 X on tumor lysates is shown in Figure 1B. Patient 180 (Pt1) showed presence of sPLA2 X in the periphery and virtually undetectable levels in the central region (lane 4 and 5). There was no significant difference between the sPLA2 X localization in T1 and T2 tumor sites ($p>0.05$), nor could any association of redundant or mutually exclusive expression be seen, since two tumors contained neither sPLA2 IIA nor sPLA2 X, whereas 6 tumors contained both. Analysis of adjacent tissue to 6 paired tumor samples revealed expression of sPLA2 at these sites also, suggesting a tumor dependent inflammation in adjacent tissue (not shown) (30, 34).

Serum samples collected prior to surgery from 20 of the 22 colon cancer patients were analyzed by ELISA. Serum levels of normal individuals have been reported previously with the upper 95 percentile at 3.6 ng/ml (7). Our findings range between 1-22 ng/ml for colon cancer patients, while one normal and three patients with diverticulosis showed levels below 3.6 ng/ml, which was considered as normal (Figure 2). The serum levels were plotted as a function of Dukes' classification, where 4 patients from group A and B had high levels above 15 ng/ml, whereas the rest of the patients had levels below 10 ng/ml. No significant differences between group A, B, C, D and patients with diverticulosis were seen ($p>0.05$). No clear associations were found between serum levels and the amount of sPLA2 IIA seen in the tumor by Western blotting of the corresponding patient.

Finally, tumors showing higher sPLA2 content in the T2 region compared to T1 were analyzed for hydrolytic activity of sPLA2 using substrates poor for the cellular phospholipases c/iPLA2 (35). Tumor pieces from the periphery showed significantly higher hydrolytic activity compared to T1 regions (Figure 3). As control for normal hydrolytic activity in non-inflamed non-cancerous tissue, Sprague-Dawley rat breast tissue was analyzed and showed activity similar to the T1 region from human colon tumors. DMBA-induced tumors from Sprague-Dawley rats showed significantly increased hydrolytic activity compared to rat control tissue, indicating that increased sPLA2 activity in tumors is conserved between humans and rats (36). Surprisingly, patients with diverticulosis also exhibited increased sPLA2 activity, which suggests potential involvement of other sPLA2 subtypes in diverticulosis, since neither sPLA2 IIA nor sPLA2 X were identified in these samples.

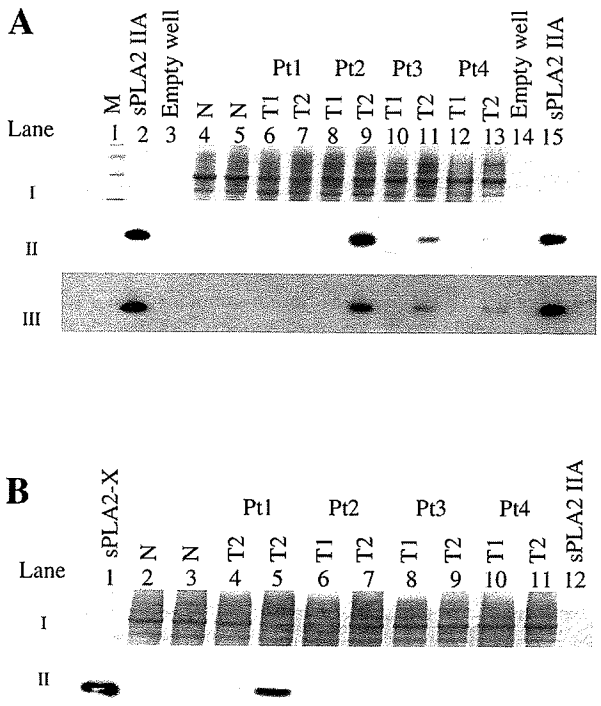

Figure 1. *Western blot analyses showing secretory PLA2-type IIA and X protein in tumor lysate from colon cancer patients. A) Representative analyses of lysates from patients with diverticulosis denoted N (Lanes 4 and 5) and colon cancer lysate from four patients (Pt1-4) (lanes 6-13). Lysates from patient 1-4 are shown pairwise with central (T1) and peripheral (T2) samples. Lane 1 is a molecular weight marker, lane 2 and 15 control sPLA2 IIA protein. Panel I shows a Coomassie-stained upper part of the gel as control for equal loading of protein lysates. Panel II and III show Western blot of identical lysates probed with polyclonal antibodies against sPLA2 IIA APC and CPC respectively. B) Lysates from patients in lane 2 to 11 are identical to A). Upper panel shows Coomassie-stained gel and panel II the membrane probed with a polyclonal antibody against sPLA2 X. sPLA2 IIA was derived from human tear fluid, diluted x200 to approximately 250 ng/ml sPLA2 IIA. Recombinant human bacterially expressed sPLA2 X (2.5 ng) was loaded as control for human sPLA2 X.*

Discussion

Our finding, that both sPLA2 type IIA and X are present in colon carcinomas, raises several questions in relation to the involvement of these sPLA2 subtypes in tumorigenesis. The fact that 6 of the 7 tumors proven positive for sPLA2 X also contained sPLA2 IIA could indicate that the two sPLA2 subtypes have divergent roles in colon cancer. The finding of differences in substrate specificity between the two lipases supports this idea. sPLA2 X displays high affinity for phosphatidylcholine (PC) vesicles and is able to bind well to the PC-rich outer layer of mammalian cells and induce fatty acid release (37-41). On the other hand sPLA2 IIA binds extremely weakly to PC-rich membranes, is not able to efficiently release fatty acids when added exogenously to a

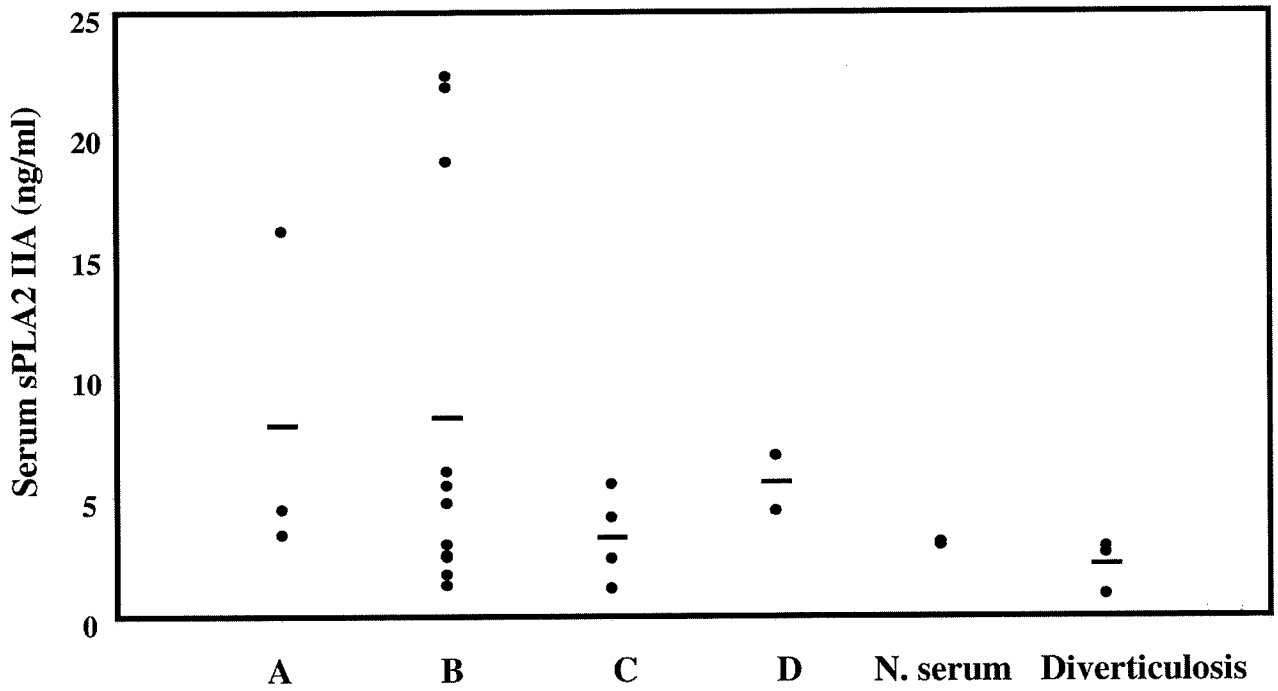

Figure 2. *Diagram showing the distribution of the sPLA2-IIA serum levels from a total of 24 persons, 20 colon cancer patients classified according to Duke's classification system, in one healthy individual and 3 diverticulosis patients. (•) Serum sPLA2-IIA value, (-) mean value in each group. Each data point is the average of two determinations performed each in quadruplicate using the human sPLA2 IIA-specific ELISA-assay.*

wide variety of mammalian cells (40, 41) and displays high activity on anionic phospholipid-rich membranes, such as bacterial membranes (42). The relative role of sPLA2 X and sPLA2 IIA in liberating arachidonic acid from mammalian cellular membranes for the production of eicosanoids is an area of active investigation. An interesting question raised by our study, is the origin of the different sPLA2 subtypes identified in the tumors. sPLA2 IIA has previously been reported associated with macrophages from human pleural effusions (7), whereas sPLA2 X has been identified in granules and vesicles of human neutrophils (39). The finding that sPLA2 X transcript was not induced in 38 colorectal cancer tumors (25) correlates with release of sPLA2 X protein from intracellularly stored organelles from neutrophils invading the tumor. Hence, neutrophils which are an important effector cell related to the inflammatory response, could release sPLA2 X in the tumor upon activation, which would not involve sPLA2 X regulation at the transcriptional but only the secretory level (39). A similar phenomena could be involved in the presence of sPLA2 IIA protein in tumors from colon cancer, since the sPLA2 IIA transcript was not shown to be increased in colorectal tumors from 44 patients (28), whereas the sPLA2 IIA protein has been identified in several studies (7, 18, 19). The presence of sPLA2 IIA protein in colon cancer tumors in the absence of increased sPLA2 IIA transcript could be

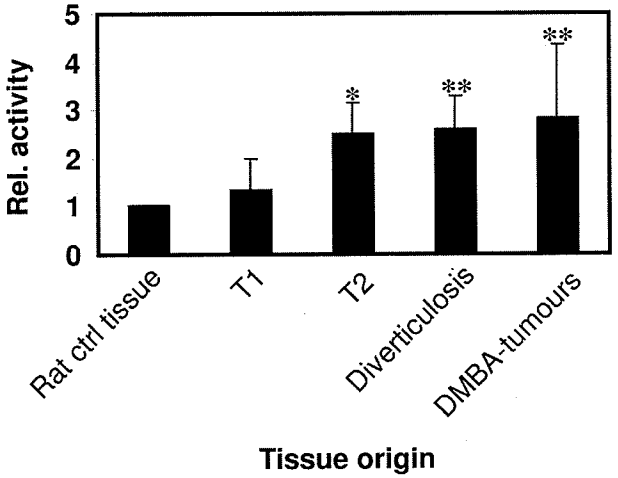

Figure 3. *Hydrolytic activity of sPLA2 from approximately 10 mg selected tissue was analyzed after 24 h incubation at 37°C. A total of 86 samples (each measured 3-7 times) at an average size of 10.19±0.82 mg (stdv) (range 8.6-11.8) were analyzed. The hydrolytic activity of normal breast tissue from Sprague Dawley rats was used as control. Comparison was made between central tumor pieces (T1) and excised peripheral samples (T2) for which sPLA2 IIA or X was higher in T2 than in T1. Significance was evaluated using Wilcoxon matched pairs signed rank test. Peripheral regions showed significantly higher activity than the central region (N=7 pairs, *p<0.05, two-sided). Colon tissue pieces excised from patients with diverticulosis (n=3) and rat DMBA induced breast tumor pieces (n=10) showed significantly higher sPLA2 hydrolytic activity compared to rat control breast tissue (n=7) using Wilcoxon rank sum test (**p<0.01, two-sided).*

mediated *via* secretion from invading macrophages, neutrophils or mast cells, known to contain secretory granules of sPLA2 IIA (43-45). The idea that sPLA2 in tumors originate from invading immune related cells was further supported by Praml *et al.*, showing that the sPLA2 IIA transcript is rarely expressed in colon cancer cell lines (16). In this context, our study supports the earlier studies on identification of sPLA2 protein in the periphery of colon and colorectal tumors (18, 24).

The finding of increased sPLA2 IIA transcript in colorectal tumors from familial adenomatous polyposis (FAP) patients is in contrast with the above hypothesis, which could indicate that FAP patients display a differential sPLA2 expression pattern (19).

Two tumors in our study did not show sPLA2 IIA or X expression. An attempt to clarify the potential involvement of other sPLA2 subtypes such as IID, IIE, IIF, III or XII in such tumors could be interesting, but at present the major limitation in such studies are the lack of antibodies recognizing these subtypes. Analysis against sPLA2 V in our present tumor lysates did not show expression of this subtype.

In summary, our study has shown an association between increased expression of sPLA2 IIA and X protein in human colon carcinoma and increased activity towards lipid substrates optimal for these lipases. The study has confirmed the peritumoral localization of sPLA2 IIA in colon cancer, identified using Western blot with immunospecific antibodies not cross-reacting with other sPLA2 proteins known at present to be involved in human malignancies. Our study suggests that the hydrolytic activity of sPLA2 plays a role in human colon cancer and warrants further investigation of the different sPLA2 subtypes role in human cancer.

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