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Original Paper

High Molecular Weight Phospholipase A_2 and Fatty Acids in Human Colon Tumours and Associated Normal Tissue

A.S. Soydan,[1] I.A. Tavares,[1] P.K. Weech,[2] N.M. Tremblay[2] and A. Bennett[1]

[1]Academic Department of Surgery, The Rayne Institute, King's College School of Medicine and Dentistry, 123 Coldharbour Lane, London SE5 9NU, U.K.; and [2]Merck Frosst Centre for Therapeutic Research, P.O. Box 1005, Pointe Claire, Dorval, Quebec, Canada H9R 4P8

Human colon tumours usually form more prostaglandins (PGs) than associated normal tissues, but the mechanism(s) are not fully understood. We analysed fatty acid compositions, in particular arachidonate, and measured the amount and the activity of high molecular weight cytoplasmic phospholipase A_2($cPLA_2$) of these tissues. Total lipids extracted from homogenised surgical specimens were transesterified and fatty acids were analysed by gas chromatography. $cPLA_2$ was separated by SDS-PAGE, Western-blotted, immunoblotted using a specific antibody to $cPLA_2$ and semiquantified following enhanced chemiluminescence using a scanning densitometer. $cPLA_2$ biological activity was also assayed using 1-stearoyl, 2-[1-^{14}C]-arachidonyl, L-3-phosphatidylcholine. Compared with normal mucosa/submucosa, there was more total arachidonate in tumours ($P < 0.01$), and increased levels of $cPLA_2$ occurred in 6 of 17 tumours. In conclusion, the higher amounts of tumour total arachidonate and the sometimes higher levels of $cPLA_2$, might help to explain why some human colon tumours form increased amounts of PGs. Copyright © 1996 Elsevier Science Ltd

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INTRODUCTION

COLON CANCER is one of the most common neoplastic diseases in developed countries [1]. The influence of dietary fat on human cancers is controversial, but various epidemiological and laboratory studies suggest a relationship between dietary fat and some types of human cancers, such as those of breast and colon [2, 3]. In particular, colorectal cancer is strongly associated with high fat intake [4, 5]. However, the relationships of lipids to malignancy are not fully understood.

Human cancers can produce increased amounts of free arachidonate and its metabolites [6], although Neoptolemos and colleagues [7] and Yazici and coworkers [8] reported different relative amounts of total arachidonate in colorectal tumours and associated normal mucosa/submucosa. Otamiri and Sjodahl [9] found increased PLA_2 activity in colorectal tumours compared with normal mucosal tissue, but they did not specify its type(s). In a preliminary study [10], we demonstrated that human colon tumours and associated normal tissue contain the so-called cytosolic or high molecular weight enzyme ($cPLA_2$) [11, 12].

There has also been some interest in the ratio of stearic acid to oleic acid in cancer patients and tumour-bearing animals. Wood and colleagues [13] found a lower ratio in the erythrocytes of patients with cancer (hepatobiliary, colorectal, pancreatic, breast), and proposed this as a marker for malignancy. The lower stearic:oleic acid ratio in erythrocytes of colorectal cancer patients was not affected by age, gender, Dukes' classification or tumour histopathology, and was therefore not a useful marker in the post-operative follow-up [14]. Similarly, in rats bearing dimethylhydrazine-induced colorectal tumours, the stearic:oleic acid ratio in erythrocytes was also subnormal [15]. The higher mean stearic:oleic acid ratio found in human colon tumours by Yazici and coworkers [8] was not statistically significant.

Reasons why human colon tumours usually form more prostaglandins (PGs) than associated normal tissues [6] might be higher amounts of arachidonate in the tumour phospholipids, more $cPLA_2$ (which hydrolyses arachidonate from phospholipids), more cyclo-oxygenase (which converts arachidonate to PGs) and/or factors that increase enzyme activity.

Correspondence to I.A. Tavares.
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In the present study, we measured fatty acids (FAs) in total lipid extracts, FA ratios, and the amount and the activity of $cPLA_2$ from human colon tumours and associated normal mucosa/submucosa and muscle.

MATERIALS AND METHODS

Specimen collection

Human colon tumours and associated normal tissues (taken at least 5 cm from the tumour) were collected in glass bottles on ice immediately after their surgical removal. Following macroscopic evaluation by the surgeon or pathologist, tumour was separated from the normal tissues. The layer of mucosa/submucosa in normal colon was cut away from the muscle, and all the tissues were stored in liquid nitrogen until analysis (2–3 weeks).

Quantification of total arachidonate

Aliquots of 100 mg tissue were put into 1 ml ice-cold 154 mM NaCl in glass bottles. Internal standard (17:0, margaric acid) in 100 μl (1 mg/ml in chloroform) was added and the tissues were cut into small pieces and homogenised using a Silverson homogeniser (sealed unit model, micro head, 30 sec). The homogeniser head was washed with 2 ml methanol which was added to the tissue homogenate, and 3.9 ml chloroform were added. Following centrifugation (2000*g*; 10 min; 4°C), the lower (chloroform) phase containing lipids was dried under a stream of O_2-free N_2 at 37°C. The total lipid extract was dissolved in 4 ml methanol/6 M HCl (5:1) in tubes, O_2-free N_2 was passed through the sample for 15 sec, and the lid closed tightly. The sample was incubated at 90°C for 3 h, and cooled in ice-cold water for 2.5 min. Methylated FAs were extracted using 4 ml hexane, evaporated to dryness and redissolved in 100 μl of hexane. Quantification was performed by gas chromatography using a Packard 436 GC fitted with an AT-WAX column (Alltech, 30 m × 0.32 mm, 0.25 μm film thickness), a flame ionisation detector (250°C) and a split injector (250°C). Results were recorded on a Shimadzu C-R3 A integrator. Mean recovery of the internal standard was 58.2 ± 3.3%. For arachidonate, the intra-assay and inter-assay coefficients of variation were 6.8 and 5.9%, respectively, and the detection limit was 2 μg.

Sample preparation for $cPLA_2$ estimation

During experiments, samples were handled in glass bottles either on ice or at 4°C. Tissues were weighed accurately (approximately 150 mg) and placed into 1 ml homogenising buffer (140 mM NaCl, 5 mM KCl, 25 mM Tris pH 7.4, either 1 mM ethylene-diamine-tetra-acetic acid (EDTA), or 100 μM ethylene glycol-bis(β-amino-ethyl ether)-N,N,N′,N′-tetra-acetic acid (EGTA) or 100 μM 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetra-acetic acid (ETA) as calcium-chelating agents), leupeptin, 50 μM pepstatin-A, 1 mM aminoethyl-benzene-sulphonyl fluoride, 1000 kallikrein-inhibitor-units (aprotinin), and 25 mU α_2-macroglobulin). They were cut into small pieces, homogenised for 1 min using a glass manual homogeniser, centrifuged (900*g*, 15 min) to sediment cell debris, and the supernatant was then ultracentrifuged (100000*g*, 1 h) to obtain the cytosolic and mitochondrial/microsomal fractions. The cell debris pellet and the mitochondrial/microsomal pellet were combined and, to aid their solubilisation, they were resuspended in 0.5 ml homogenisation buffer containing 1 M NaCl, homogenised using a teflon-glass manual homogeniser for 1 min, left at 4°C for 1 h, and finally ultracentrifuged (100000*g*, 1 h) to obtain the solubilised pellet supernatant. Proteins in the cytosol and the solubilised pellet supernatant were measured by a Bio-Rad protein assay kit using bovine serum albumin as the standard.

SDS-PAGE and Western blotting

Proteins in the samples were separated by electrophoresis using SDS-polyacrylamide slab gels (7.5 separation and 6% stacking gels) [16]. Purified recombinant human $cPLA_2$ (20 ng) was used as a standard. Samples were diluted 1:4 in buffer (63 mM Tris, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.001% bromophenol blue) and denatured at 60°C for 15 min. After electrophoresis for 45 min at 200 V, the gels were equilibrated in 25 mM Tris/192 mM glycine/20% methanol for 15 min, and the proteins were electroblotted to pure nitrocellulose membrane at 100 V (25 V/cm).

Immunoblotting

Tween-20 (0.1%) in phosphate buffered saline (T-PBS, pH 7.4) for 2 h was used for blocking the nitrocellulose membrane. Specific antibody (MF 142) to high molecular $cPLA_2$ was raised to a peptide fragment of $cPLA_2$ (S731–A749) in rabbits (P. Weech, Merck Frosst). A 1:1000 dilution of MF 142 in T-PBS was incubated for 1.5 h at room temperature with the electroblotted nitrocellulose membrane. Following rinsing, the second antibody (horseradish-peroxidase-conjugated goat antirabbit, 1:3000 dilution in T-PBS), was incubated at room temperature for 1 h with the membrane. Amersham's enhanced chemiluminescence was used for development, and the image was recorded on Amersham Hyperfilm-ECL with an exposure of 30 sec to give a reading that was submaximal and on the calibration curve. Quantification was by laser scanning densitometry in comparison with a calibration curve (2.5, 5 and 10 ng) of pure recombinant human cytosolic $cPLA_2$ loaded in the same gel as the samples.

$cPLA_2$ activity assay

The method used for assaying $cPLA_2$ activity was a composite of three techniques: (1) Nakamura and coworkers [17] (incubation of sample with radiolabelled substrate), (2) Dole and coworkers [18] (extraction of released arachidonate and intact phospholipid), and (3) Tremblay and coworkers [19] (separation of released arachidonate from intact phospholipids). $cPLA_2$ activity was assayed using 200 μg protein from the sample, 5 mM Ca^{2+} and 1-stearoyl, 2-[1-^{14}C]-arachidonyl, L-3-phosphatidylcholine (500 pmoles, 0.025 μCi). Samples were pretreated with 3 mM dithiothreitol (DTT) for 15 min at 37°C, the substrate was added, and incubation continued for 30 min at 37°C. Following incubation, 1.25 ml of Dole's extraction solution (1 N H_2SO_4/heptane/isopropyl alcohol, 0.05:1:4) [18] was added to stop the reaction, and the sample was vortex-mixed for 30 sec and left at room temperature for 5 min. To extract released arachidonate, 0.5 ml water and 0.75 ml heptane were added, vortex-mixed for 30 sec, and left at room temperature for 5 min. The upper organic phase was passed through a 100 mg silica column which had been equilibrated previously with 0.5 ml heptane. Released arachidonate was eluted from the column with 1 ml diethyl ether and the radioactivity was measured (intra-assay coefficient of variation, 5.6%).

Statistical analysis

Results were analysed using the Wilcoxon signed rank test for paired data and analysis of variance where appropriate (two-tailed).

RESULTS

The patients were usually elderly (median age 69 years, range 37–90; 12 female, 16 male). Histopathology of the tumours showed moderately differentiated adenocarcinomas except for one well-differentiated adenocarcinoma. The tumour sites were: caecum 1, ascending colon 4, descending colon 2, sigmoid colon 3, rectosigmoid 12, rectum 6. Abdominal lymph nodes were infiltrated in 8 patients (Dukes' stage C); the other tumours were Dukes' stage A (6 patients) or stage B (14 patients).

FA analysis was carried out in 20 of 28 tumours and associated normal tissues (Table 1). There was a trend for more FAs in tumours compared with mucosa and muscle. In tumours, the mean amounts of 18:0, 20:3, 20:4, 22:4, 22:6 and the ratios of 18:0/18:1, 20:3/20:4, and saturated/unsaturated FAs were greater ($P<0.01$) than in the normal mucosa/submucosa. The 18:2/20:4 ratio was lower ($P<0.05$) in tumour compared with normal mucosa. Compared with muscle, there were greater amounts of tumour 18:0, 20:3, 20:4, 22:6, total n-3 FAs, total n-6 FAs ($P<0.01$), 20:1 and 20:5 ($P<0.05$) and a higher 20:3/20:4 ratio ($P<0.01$), but less 18:3 ($P<0.05$). Multiple regression analysis found no correlation between the site of tumour, stage of disease, age, gender or tumour FA composition ($P>0.1$).

Increasing amounts of purified $cPLA_2$ (1–20 ng), when immunoblotted with the antibody MF 142, gave bands of increasing density (linear standard curve). Immunoblots (Figure 1) of high molecular weight $cPLA_2$ from tumour and associated normal mucosa/submucosa of both the cytosolic fraction and the further solubilised pellet fractions were run since $cPLA_2$ was found in the pellet despite using different amounts of EDTA (2, 20, 100 mM), EGTA (2, 20, 100 mM) or ETA (1, 10 mM) as calcium-chelating agents in an attempt to prevent $cPLA_2$ translocating from the cytosol to the membrane fractions.

Each immunoblot of human samples gave several bands, one of which was located at the same level as the standard purified $cPLA_2$ from U937 cells; most of the other bands were below the standard (Figure 1). To determine whether the band corresponding with the standard represented high molecular weight $cPLA_2$, MF 142 was pretreated with an excess of peptide S731-A749 (the antigen used to produce the $cPLA_2$ antibody). The band corresponding with the standard, and one lower band, virtually disappeared with MF142 pretreatment, indicating homology or identity to cystolic $cPLA_2$ (Figure 2); this lower band may be a degradation product of $cPLA_2$ since it was still immunospecific. In contrast, all the other bands remained, and appeared to be non-specific interactions.

$cPLA_2$ amounts were analysed by immunoblotting and laser

Table 1. Amounts of fatty acids from human colon tumours and associated normal mucosa/submucosa and muscle

Fatty acid	Tumour (n = 20)	Mucosa (n = 20)	Muscle (n = 10)
14:0	289 (119)	242 (41)	302 (68)*
16:0	3050 (475)	2780 (26)	2320 (381)
16:1n-7	436 (163)	337 (52)	457 (118)*
18:0	1430 (82)	1190 (98)‡	814 (77)‡
18:1n-9	4450 (974)	4340 (401)	3750 (708)
18:2n-6	1810 (2571)	1840 (169)	1270 (263)
18:3n-3	49 (21)	46 (9)	52 (11)†
18:4n-3	54 (20)	36 (4)	42 (8)
20:0	62 (13)	48 (7)	34 (5)
20:1n-9	65 (11)	55 (14)	57 (18)†
20:2n-6	88 (15)	64 (10)	56 (18)
20:3n-6	209 (19)	113 (8)‡	70 (8)‡
20:4n-6	1290 (82)	1050 (60)‡	703 (40)‡
20:5n-3	70 (6)	62 (6)	33 (7)†
22:2n-6	289 (32)	276 (24)	230 (41)
22:4n-6	113 (13)	63 (5)‡	71 (6)*
22:6n-3	290 (24)	207 (14)‡	130 (14)‡
n-3	438 (42)	404 (67)	214 (30.0)‡
n-6	5130 (1570)	3400 (230)	2380 (323)‡
S	4840 (650)	4260 (381)	3470 (515)
U	9150 (1410)	8460 (670)	6860 (1160)
T	14000 (2060)	12700 (104)	10300 (1650)
16:0/16:1	10.2 (0.7)	10.4 (1.0)	12.1 (5.3)
18:0/18:1	0.40 (0.03)	0.29 (0.08)‡	0.34 (0.09)
18:2/20:4	1.7 (0.5)	1.8 (0.2)†	1.8 (0.4)
20:3/20:4	0.17 (0.02)	0.11 (0.01)‡	0.1 (0.01)‡
n-6/n-3	9.4 (0.44)	10.2 (0.4)*	11.8 (1.0)*
S/U	0.54 (0.01)	0.50 (0.01)‡	0.54 (0.03)

Results are means and (S.E.M.), μg/g tissue given to three significant figures; S, saturated; U, unsaturated; T, total FAs. *$P<0.1$; †$P<0.05$; ‡$P<0.01$, tumour versus mucosa or muscle (Wilcoxon signed-rank test for paired data).

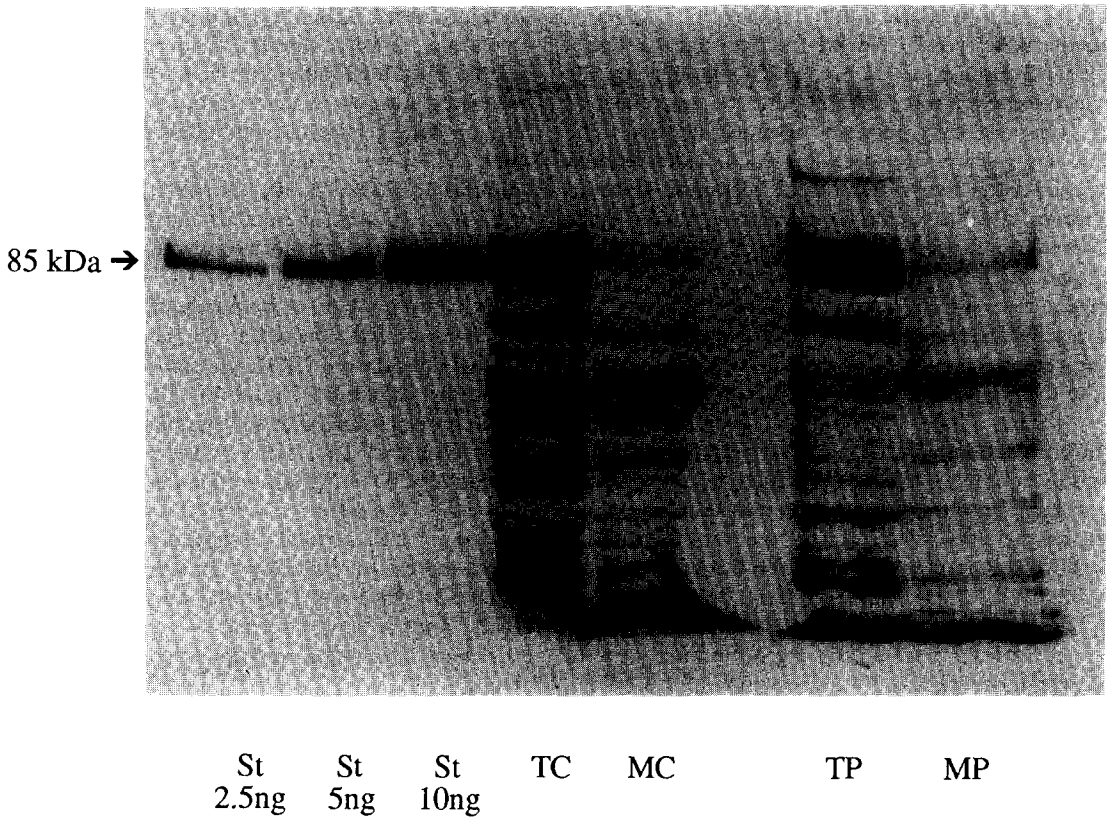

Figure 1. Immunoblot of cytosolic and pellet fractions from human colon tumour and associated normal mucosa/submucosa. St, standard; TC, tumour cytosol; MC, mucosa/submucosa cytosol; TP, tumour pellet; MP, mucosa/submucosa pellet. Proteins loaded were 120 μg for cytosol and 48 μg for pellet.

scanning densitometry in 17 of the 28 specimens studied, and were found to be similar in the tumours and associated mucosal tissues (median values and interquartile ranges of 79 (46–97) and 82 (57–122) ng/mg protein, respectively, $P = 0.57$; Figure 3). However, there was more $cPLA_2$ in six of 17 tumours and less in the other 11 compared with normal tissue. In two tumours, $cPLA_2$ was not found by immunoblotting but was detected by the more-sensitive radioactivity assay. All the associated normal tissues showed $cPLA_2$ with both immunoblotting and the radioactivity assay. The median values for $cPLA_2$ activity were similar in the tumours and their associated normal mucosa/submucosa as determined by [^{14}C]-AA release from [^{14}C]-phosphatidylcholine (median values and interquartile ranges of 163 (68–253) and 171 (147–200) pmol released arachidonate per mg protein in 30 min respectively, $P = 0.906$, Figure 4). Compared with the mucosa, the tumour $cPLA_2$ biological activity was greater in the same six of 17 tumours which showed increased $cPLA_2$ amounts and less in the others. Although amounts of $cPLA_2$ found by immunoblotting correlate well with the $cPLA_2$ activity in tumour samples ($P < 0.001$), the correlation was poor in the associated normal tissue ($P = 0.2$).

In eight tumours and associated normal tissues, both FA and $cPLA_2$ analyses were carried out. Again in these tumours the $cPLA_2$ content correlated well with the $cPLA_2$ activity ($P < 0.001$), but poorly in the associated normal tissues ($P = 0.2$). There was no correlation between $cPLA_2$ and the total arachidonate in tumours or mucosal tissues.

DISCUSSION

Our studies, including the preliminary report [10] demonstrate for the first time that human colon tumours and associated normal mucosa/submucosa contain high molecular weight cytoplasmic PLA_2. The amount and the activity of this $cPLA_2$ have been semiquantified in human colon tumours and associated normal mucosa/submucosa. Amounts of $cPLA_2$ correlated well with the enzyme activity in tumour, but not in mucosa. Factors which might influence the amounts and activity of PLA_2 could be differences in cellularity between specimens, and the delay of several minutes from excision of the tissues until their freezing.

Inactive $cPLA_2$ occurs in the cytosol, and following a rise in the cytosolic Ca^{2+} concentration, activation of the enzyme occurs by phosphorylation with consequent translocation to the membrane. In spite of using Ca^{2+} chelating agents, as much as 60% of the $cPLA_2$ still translocated to the membrane and was detected in the further solubilised pellet fraction (cell debris, microsomal–mitochondrial fractions). Hence, the total $cPLA_2$ is the sum of the amounts in the cytosolic fraction and the further solubilised pellet fraction.

The immunoblot method we used for detecting the $cPLA_2$ required at least 1 ng of protein, which presumably explains why in two tumours $cPLA_2$ activity was detected by [^{14}C]-AA release but not immunologically. The phospholipase activity was resistant to DTT, so excluding the involvement of low molecular weight type II PLA_2 [20]. Furthermore, [^{14}C]-phosphatidylcholine, which was used as the assay substrate in this study, is not a good substrate for type II PLA_2.

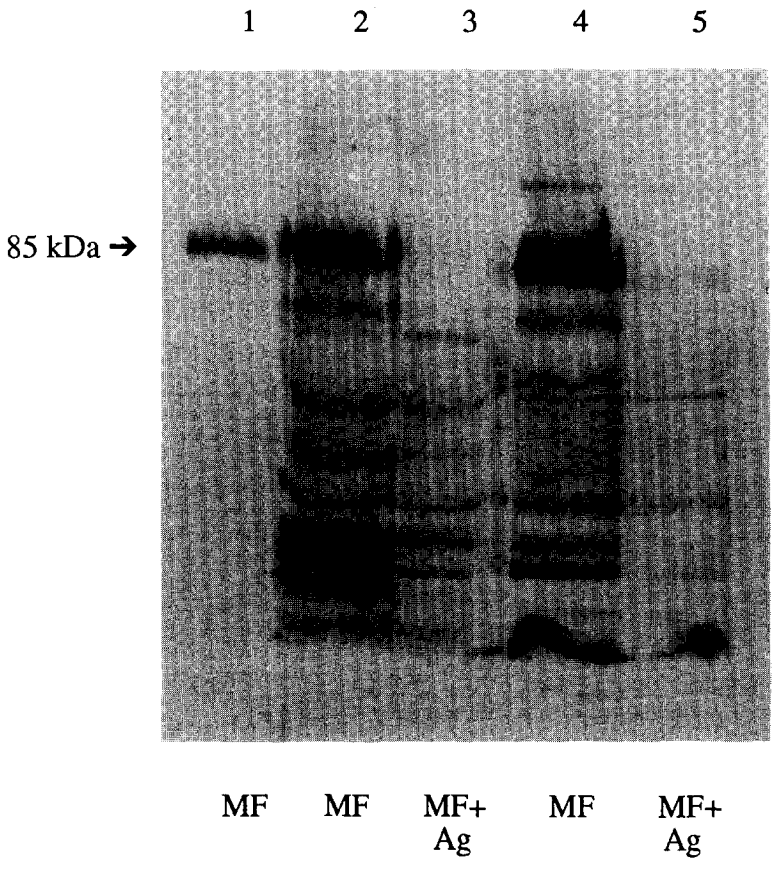

Figure 2. Specificity of MF142 for high molecular weight PLA$_2$. Lane 1, purified PLA$_2$; lanes 2 and 3, tumour cytosol; lanes 4 and 5, tumour pellet; loaded in the same gel. Following blocking, individual strips were incubated with either MF142 alone (1, 2 and 4) or MF142 pretreated with excess antigen peptide, Ag (3 and 5).

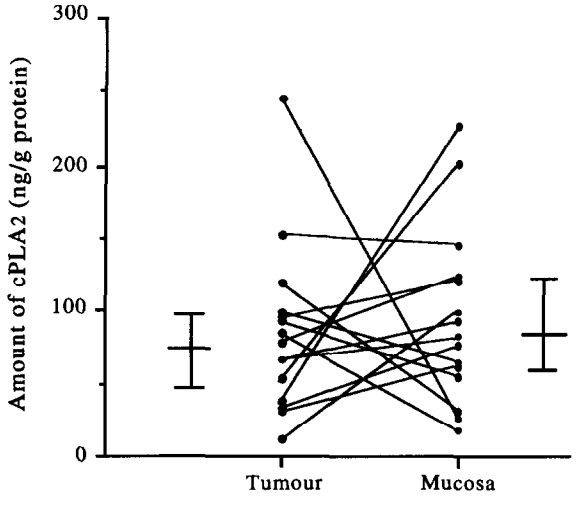

Figure 3. Total amounts of cPLA$_2$ from human colon tumours and associated normal mucosa/submucosa determined from immunoblots. Individual results, median and interquartile ranges are expressed as ng/mg protein: tumour 79(46–97); mucosa 82(57–122); n = 15. P = 0.570, Wilcoxon signed-rank test for paired data.

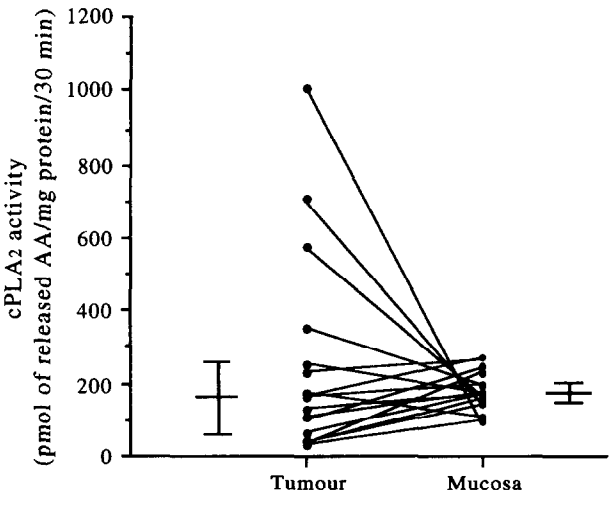

Figure 4. cPLA$_2$ activity of human colon tumours and associated normal mucosa/submucosa. Individual results, medians and interquartile ranges are expressed as pmol of released AA mg^{-1} protein 30 min^{-1} from [^{14}C]phosphatidylcholine: tumour 163(68–253); mucosa 171(147–200); n = 17. P = 0.906, Wilcoxon signed-rank test for paired data.

In our experiments, there was more total arachidonate in human colon tumours than in associated normal mucosa/submucosa, as found by Neoptolemos and colleagues [7] but not by Yazici and colleagues [8]. However, the methods and length of storage differed. Yazici and coworkers [8] studied only five specimens which had been stored at −70°C for 6–8 months; we found that during storage of human colon mucosa/submucosa at −70°C for 2 months, up to 20% of the arachidonate acid was lost. There was no correlation between the total arachidonate and cPLA$_2$ in

tumours or mucosa, but the amounts of tissue were sufficient to analyse both FAs and $cPLA_2$ in only eight specimens. A correlation between $cPLA_2$ and free arachidonate already released from phospholipid by $cPLA_2$ might have been expected, but this free arachidonate was too low to measure.

Increased membrane fluidity occurs in malignant transformation of cells [21], with a higher content of unsaturated FAs, which in turn increases the metabolic rate of many cellular enzymes [22]. The stearic:oleic acid ratio and the total saturated:unsaturated FA ratio reflect the fluidity of the membrane. In our study, the tumour stearic:oleic acid ratio was increased ($P<0.01$), in contrast to the findings in erythrocytes [13]. However, direct comparisons may not be appropriate since diet has a dominant effect on FA composition [8]. Furthermore, in our experiments the ratios of 20:3 to 20:4, and total saturated:unsaturated FAs were also higher in tumour compared with normal tissue ($P<0.01$) which may indicate reduced desaturation of FAs in human colon tumour tissue. If so, increased amounts of arachidonate would seem unlikely to occur via greater conversion from linoleic acid.

The rate of lipid peroxidation is often lower in tumours than in corresponding normal tissue [23], and the growth of cancer cells *in vitro* can be inhibited by stimulating lipid peroxidation [24]. Since long-chain polyunsaturated FAs are a source of lipid peroxides, if peroxidation is low, this might explain the increased amounts of arachidonate and docosahexaenoate in human colon tumours [25]. More arachidonate was also found in experimental colorectal cancers of rats [26, 27]. The mechanisms behind these changes are not yet fully understood, and the implications of these results for any therapeutic dietary manipulation still require further investigation.

In contrast, higher lipid peroxidation (determined by malondialdehyde), PLA_2 activity and myeloperoxidase activity (a marker of granulocyte neutrophils) was found in human colon tumours compared with associated normal mucosa using PLA_2 activity which differ from ours (e.g. substrate, incubation time) [9]. Others who found lowered lipid peroxidation either did not assay $cPLA_2$ activity or measured the total PLA_2 activity rather than just the high molecular weight fraction, whereas we excluded the interaction of low molecular weight PLA_2s by adding DTT to the assay buffer.

Other PLA_2s may also have a role in cancer. Recently, the Mom-1 locus (modifier of the multiple intestinal neoplasia) was identified as a modifier of tumour number in Min/+ mice bearing a mutation in the mouse homologue of the human *APC* gene [28]. MacPhee and colleagues [29] identified secretory type II phospholipase A_2 as the candidate for the Mom-1 locus, finding that mouse strains with the Mom-1 susceptible phenotype have mutations in this PLA_2 gene and are likely to be null for its enzyme activity. Thus, the presence of the wild-type secretory type II PLA_2 or normal levels of its enzyme activity seem to confer some tumour resistance in the Min/+ mouse. In contrast, Murata and coworkers [30] consistently found type II PLA_2 in human gastric and intestinal metaplasia, and especially in the invading zone of gastric tumours. Therefore, either type II PLA_2 plays a different role in man and the Min mouse, or there are other factors in man that have obscured the role of type II PLA_2 in tumorigenesis.

Bennett and colleagues [6] found that colonic tumours released more free arachidonate and usually produced more PGs compared with associated normal mucosal tissue. Increased PG production could be due to the increased arachidonate, which we found to be present in the phospholipid component of the tumours compared with associated normal tissue (mean increase 23%, $P<0.01$). The greater amounts and activity of $cPLA_2$ in only 6 of 17 specimens might also help to explain, at least in part, why not all tumours produce more PGs compared with normal tissues, but is unlikely to be the full explanation. Involvement of regulatory molecules such as lipocortins, growth factors, cytokines and phosphorylation of the enzyme are factors that remain to be determined in assessing $cPLA_2$ activity. Cyclo-oxygenase 1 and 2 activities are other factors that require investigation in human colonic tumours.

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