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Original Paper

Cytosolic Phospholipase A_2, Cyclo-oxygenases and Arachidonate in Human Stomach Tumours

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Human stomach tumours usually form more prostaglandins (PGs) than their associated normal mucosa/submucosa, but the mechanisms are not fully understood. The key enzymes are cytosolic phospholipase A_2 ($cPLA_2$, Mr 85 000) and the cyclo-oxygenases (COXs) which exist in constitutive (COX-1) and inducible forms (COX-2). In human stomach tumours and associated macroscopically normal tissues, we determined the fatty acid composition by gas chromatography, amounts of $cPLA_2$, COX-1 and COX-2 by immunoblotting with specific antibodies and $cPLA_2$ enzyme activity using a tritiated substrate. Although compared to normal mucosa there was less arachidonate in tumours ($P < 0.05$), the arachidonate/total fatty acid ratio was higher. Mean amounts of $cPLA_2$ and COX-1 and $cPLA_2$ activity were similar in tumours and normal mucosa. However, substantial amounts of COX-2 were found in the tumours but not in the mucosa, which may explain why many gastric tumours form increased amounts of PGs. © **1997 Elsevier Science Ltd.**

Key words: human stomach tumours, cancer, arachidonate, $cPLA_2$, COX-1, COX-2

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INTRODUCTION

MANY HUMAN tumours, including those from the stomach [1], colon [1–3], breast [4, 5] and lung [6, 7] form more prostaglandins (PGs) than their associated normal tissues. The mechanism(s) and implications of this increase are not fully understood, but PGs may act as tumour promoters, in tumour angiogenesis and in the supression of immune functions [8, 9]. The reason for the increased PG formation might be higher amounts of the fatty acid substrate, arachidonate, in the tumour phospholipids, more cytosolic phospholipase A2 ($cPLA_2$, *M*r 85 000) and/or more prostaglandin G/H synthase (cyclo-oxygenase, COX) enzyme activities.

COX, a key enzyme in PG biosynthesis, catalyses the conversion of arachidonate to PGG/H [10]. Recently, two isoforms of COX have been identified [11]. COX-1 is constitutively expressed in most tissues and has been proposed to generate PGs for normal physiological functions. The second isoform, COX-2, is involved in inflammation and has been shown to be induced by various stimuli including mitogens, hormones, cytokines and growth factors [11, 12].

$cPLA_2$ is involved in the release of fatty acids including arachidonate from the membrane phospholipid pools [13]. We have previously demonstrated $cPLA_2$ in human colon tumours [14] and in human gastric tumours and associated normal mucosa/submucosa [15]. In the present study we have compared human stomach tumours with associated normal mucosa/submucosa for the amounts of arachidonate in total lipid extracts, the high molecular weight $cPLA_2$, COX-1 and COX-2.

MATERIALS AND METHODS

Specimen collection

Human stomach tumours and associated macroscopically normal tissues from 17 patients were collected in glass bottles on ice immediately after surgical removal. The layer of normal mucosa/submucosa was cut away from the muscle,

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and the separate malignant and normal tissues were stored in liquid nitrogen until analysis (2–3 weeks).

Quantification of total arachidonate

Tissues were weighed (100 mg) and placed into 1 ml ice-cold 154 mM NaCl. After adding 100 µl internal standard (17:0, margaric acid), each tissue was cut into small pieces and homogenised using a Silverson homogeniser for 30 s. Chloroform (3.9 ml) was added, and the tisue homogenate was centrifuged at 2000 *g* for 10 min at 4°C. The lower lipid-containing phase (chloroform) was dried under oxygen-free nitrogen at 37°C, and the residue dissolved in 4 ml methanol/6 M HCl (5:1). Oxygen-free nitrogen was passed through the sample for 15 s, and the lid tightly sealed. Each sample was incubated at 90°C for 3 h, cooled in ice-cold water for 2.5 min, and then extracted with 4 ml 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% (SEM). For arachidonate, the intra- and inter-assay coefficients of variation were 5.9 and 6.8%, and the detection limit was 2 µg.

Sample preparation for protein studies

During experiments the samples were kept in glass bottles either on ice or at 4°C. Tissues were weighed accurately (approximately 150 mg) and placed into 1 ml homogenisation buffer. For cPLA$_2$ studies the buffer was 140 mM NaCl, 5 mM KCl, 25 mM Tris pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 100 µM leupeptin, 50 µM pepstatin-A, 1 mM aminoethylbenzenesulphonyl fluoride, 1000 kallikrein inhibitor units (aprotinin) and 25 mU α2-macroglobulin. For the COX studies the homogenisation buffer was 50 mM Tris pH 7.4, 100 mM NaCl, 2 mM EDTA 0.4 mM phenylmethylsulphonyl fluoride (PMSF), 60 µg/ml soya bean trypsin inhibitor, 2 µg/ml leupeptin, 2 µg/ml pepstatin and 2 µg/ml aprotinin. Tissues were cut into small pieces, homogenised for 1 min (3 min for the COX studies) using a glass manual homogeniser and then centrifuged (900 *g*, 15 min) to remove the cell debris. The supernatant was then ultracentrifuged (100 000 *g*, 1 h) to obtain the cytosolic and mitochondrial/microsomal fractions. Each pellet was 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 (100 000 *g*, 1 h) to obtain the supernatant. For the COX studies, the method was as described above, except that pellets obtained by centrifugation were resuspended in 150 µl of the COX homogenisation buffer and ultracentrifuged without further homogenisation. Total protein was measured by a Bio-Rad protein assay kit using bovine serum albumin (BSA) as the standard.

SDS–PAGE and Western blotting

Separate gels were prepared for cPLA$_2$, COX-1 and COX-2. Proteins in the samples were separated by electrophoresis using SDS–polyacrylamide slab gels [16]. The separation and stacking gels for cPLA$_2$ were 7.5 and 6% acrylamide, respectively, and those for COX were 10 and 3%, respectively. Protein standards were purified recombinant human cPLA$_2$ (Merck Frosst, Canada) and purified COX-1 and COX-2 (Cayman, Ann Arbor, Michigan, U.S.A.). Samples for analysis were diluted 1:3 in loading buffer (63 mM Tris/10% glycerol/2% SDS/5% 2-mercaptoethanol/0.001% bromophenl blue) and denatured at 60°C for 15 min. Samples for COX analysis were diluted 1:1 in loading buffer (25 mM Tris/2% SDS/5% glycerol/8% 2-mercaptoethanol/0.001% bromophenol blue). They were then electrophoresed for 45 min at 200 V. The gels were equilibrated in 25 mM Tris/192 mM glycine/20% methanol for 15 min. Proteins were electroblotted either to nitrocellulose membranes (cPLA$_2$ analysis) or polvinylidene difluoride membrances (PVDF, COX analysis) at 100 V for 1 h or 35 V/200 mA overnight at 4°C.

Immunoblotting

Rabbit polyclonal antibodies raised specifically for cPLA$_2$ (MF140, Merck Frosst) [17], COX-1 and COX-2 (MF241 and MF243, Merck Frosst) [18, 19] were characterised for specificity using a method as previously described [14, 17]. Each immunoblot gave several bands, one of which was located at the same level as the purified standards (apparent molecular weight of 85 kDa for cPLA$_2$, 72 kDA for both COX-1 and COX-2). Using antibody pretreated with excess standard, it was shown that the band at the same location as the standard shared homology with the standard, while other bands were primarily due to non-specific binding. With cPLA$_2$, one lower band was probably a degradation product as previously described [14]. COX-1 and COX-2 are also sensitive to degradation, with COX-1 giving rise to a 60 kDa band and COX-2 to a 59 kDa band [18, 19].

Following electroblotting, the nitrocellulose membranes were blocked with 1% BSA and 0.1% Tween-20 in phosphate-buffered saline (PBS) pH 7.4 (T-PBS) for 2 h while the PVDF membranes were blocked for 1 h with 50 mM Tris pH 7.5/250 mM NaCl/0.1% Tween 20 (TBS) containing 5% dried milk protein. For cPLA$_2$, the nitrocellulose membranes were incubated for 1.5 h at room temperature with MF 140 diluted 1:1000 in T-PBS. For COX-1 and COX-2, the PVDF membranes were incubated for 1 h at room temperature with MF241 and MF243 diluted 1:5000 in TBS. Both membrane types were incubated at room temperature for 1 h with horseradish peroxidase conjugated goat anti-rabbit IgG as the secondary antibody (1:3000 dilution). Immunodetection was by enhanced chemiluminescence (ECL, Amersham) according to the manufacturers instructions. The images were recorded on Amersham Hyperfilm-ECL, and the autoradiographs scanned using a laser scanning densitometer. Bands were quantified by comparison with a calibration curve of cPLA$_2$, COX-1 or COX-2 loaded in the same gel as the samples. The intra-assay coefficient of variation was 7.9% and the detection limit was 1 ng.

cPLA$_2$ activity assay

cPLA$_2$ activity was assayed using 1-stearoyl-2-[1-^{14}C]-arachidonyl-L-3-phosphatidylcholine, 200 mg protein from the sample and 5 mM Ca^{2+}. Each sample was pretreated with 3 mM dithiothreitol for 15 min at 37°C. After addition of substrate (0.025 µCi), incubation was continued for a further 30 min at 37°C. The reaction was stopped and released arachidonate was extracted using 1.25 ml of 1N H_2SO_4/heptane/isopropyl alcohol 0.05:1.4. Following

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

Fatty acid	Tumour ($n = 11$)	Mucosa ($n = 11$)
Arachidonate	1080 ± 135	1440 ± 115†
$n-3$ FAs	380 ± 81	518 ± 70*
$n-6$ FAs	2980 ± 355	5280 ± 414†
Saturated FAs	3660 ± 792	6700 ± 1000†
Unsaturated FAs	6400 ± 1380	13 400 ± 2020†
Total FAs	10 100 ± 2170	20 100 ± 3000†

Results are expressed as μg/g tissue, means ± SEM given to three significant figures. *$P < 0.05$, †$P < 0.01$, tumour versus mucosa, *t*-test for paired data.

extraction, the upper organic phase was passed through a 100 mg silica column previously equilibrated with 0.5 ml heptane. Released arachidonate was eluted with 1 ml diethyl ether and the radioactivity was measured. The intra-assay coefficient of variation for the assay was 5.6%.

Statistics

The results were compared using Student's *t*-test for paired data (two-tailed).

RESULTS

The patients presenting with human stomach tumours in our studies were all elderly. For the measurements of $cPLA_2$ and arachidonate in tumour and normal associated mucosa/submucosa, the patients' age range was 58–84 years and 10 of the 11 patients were male. In 10 cases the lymph nodes were infiltrated (Dukes' grade C). Histopathology showed that all 11 were adenocarcinoma (7 poorly differentiated, 4 moderate to poor). For the COX study, tumour and normal tissues were taken from a further 6 elderly patients (age range 64–76 years, 4/6 male) with gastric carcinoma. In 4 cases there was lymph node infiltration (Dukes' grade C). Histopathology showed that all 6 were adenocarcinomas (2 poorly differentiated, 1 moderate to poor and 3 moderate). Tumour and normal tissues were all taken from the body of the stomach.

The fatty acids (FAs) measured in the lipid extracts of stomach tumours and associated normal mucosa/submucosa

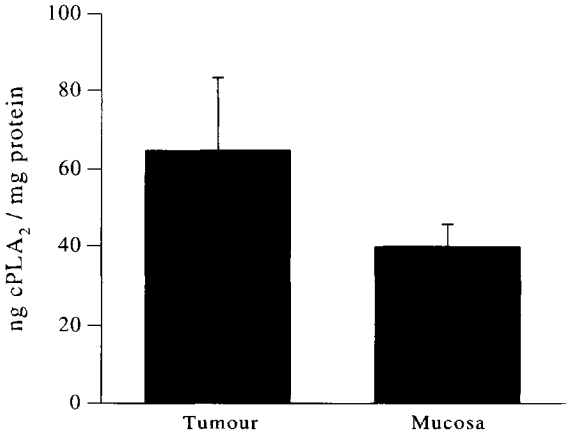

Figure 1. Total amounts of high molecular weight $cPLA_2$ from human stomach tumours and associated macroscopically normal mucosa/submucosa. Results are expressed as ng/mg protein means ± SEM: tumour 64.1 ± 19.3; mucosa 39.0 ± 6.1; $n = 7$. $P = 0.68$, *t*-test for paired data.

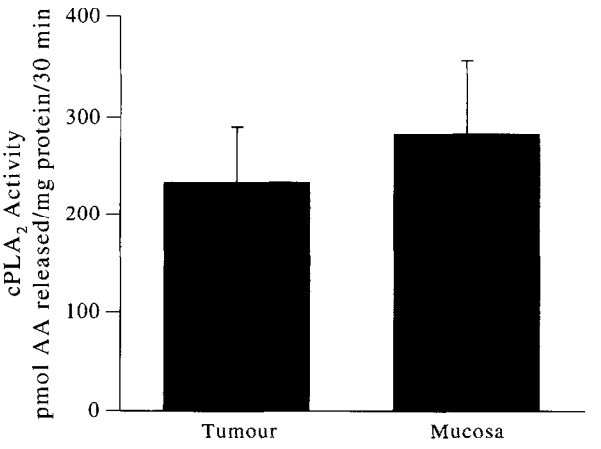

Figure 2. $cPLA_2$ activity from human stomach tumours and associated macroscopically normal mucosa/submucosa. Results are expressed as pmol of released arachidonate/mg protein during 30 min incubation, means ± SEM: tumour 229 ± 63.8; mucosa 276 ± 81.7; $n = 7$, $P = 0.344$, *t*-test for paired data.

($n = 11$) are shown in Table 1. Compared with normal mucosa/submucosa, stomach tumours had less total amounts of FAs (50%, $P < 0.01$), as well as less arachidonate (25%, $P < 0.01$), total n-3 FAs (27%, $P < 0.05$), n-6 Fas (44% $P < 0.01$), saturated FAs (45% $P < 0.01$) and unsaturated FAs (52%, $P < 0.01$). However, when the amount of arachidonate was compared as a percentage of the total FAs present, tumour tissue contained a higher percentage of arachidonate (12.4 ± 1.5% versus 8.9 ± 1.4%, $P < 0.05$) compared with normal mucosa/submucosa.

For high molecular weight $cPLA_2$, both the cytosolic and pellet fractions were assayed since some was found in the pellet even when high amounts of calcium-chelating agents were used (54.8 ± 25.0% and 66.4 ± 4% in the tumour and normal mucosa pellet fractions, respectively). Amounts of $cPLA_2$ extracted from the tumours ($n = 7$), analysed by immunoblotting and laser scanning densitometry, did not differ significantly from those in associated normal mucosa/submucosa (64.1 ± 19.3 versus 39.0 ± 6.2 ng/mg protein respectively, $P = 0.68$; Figure 1). Similarly, the mean $cPLA_2$ activity also did not differ significantly (tumours versus normal mucosa/submucosa, 229 ± 63.8 versus 276 ± 81.7 pmol released arachidonate/mg protein in 30 min, $P = 0.34$;

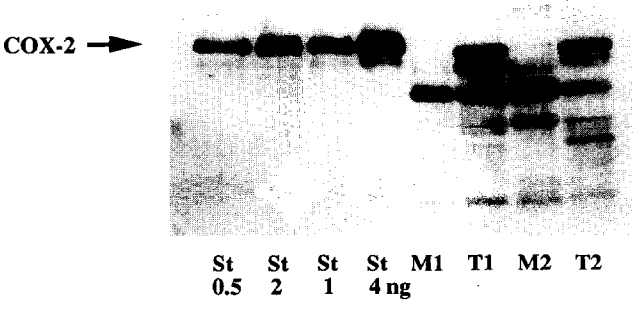

Figure 3. Typical immunoblot of the microsomal pellets from two human stomach tumours and associated normal mucosa/submucosa following treatment with COX-2 antibody (MF 243). St, standard; M1, mucosa/submucosa 1; T1, tumour 1; M2, mucosa/submucosa 2; T2, tumour 2. 40 μg of protein loaded. Bands below the standard are due primarily to non-specific binding.

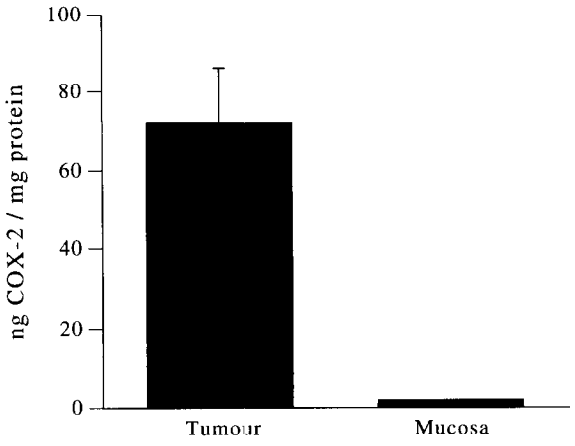

Figure 4. Total amount of COX-2 from human stomach tumours and associated macroscopically normal mucosa/submucosa. Results are expressed as ng/mg protein, means ± SEM: tumour, 72.6 ± 14.3; mucosa, 0; $n = 6$.

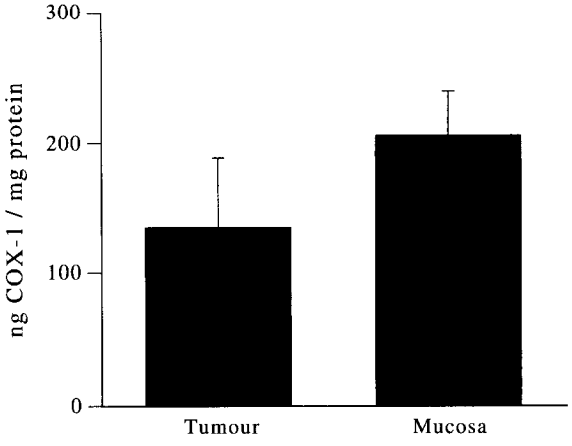

Figure 6. Total amounts of COX-1 from human stomach tumours and associated macroscopically normal mucosa/submucosa. Results are expressed as ng/mg protein, means ± SEM: tumours, 134 ± 54.2; mucosa, 204 ± 33.8; $n = 6$. $P = 0.14$, t-test for paired data.

Figure 2). Results using the two methods were consistent with each other since the amount and activity of cPLA$_2$ were greater in the same specimens. In these 7 samples, where both FA and cPLA$_2$ analysis was carried out, there was no significant correlation between the total amount of arachidonate and the amount and activity of cPLA$_2$ in tumour or mucosa ($P > 0.1$).

The amount of tumour COX-2 ($n = 6$), analysed by immunoblotting (Figure 3) and laser scanning densitometry, was 72.6 ± 14.3 ng/mg protein, whereas none was detected in normal mucosa/submucosa (Figure 4). In contrast, COX-1 immunoreactive protein was detected both in tumours and normal mucosa/submucosa (Figure 5). However, the amount of COX-1 in tumour and in normal mucosa/submucosa were not significantly different (134 ± 54.4 versus 204 ± 33.8 ng/mg protein respectively, $n = 6$, $P = 0.14$; Figure 6).

DISCUSSION

The present study suggests that human stomach tumours usually form more PGs than their associated normal tissues partly because of the higher percentage of arachidonate in the tumour fatty acids. Thus, more arachidonate may be released following activation of cPLA$_2$, making more substrate available for conversion to PGs. The mean amounts and activity of cPLA$_2$ were not significantly different in the tumours compared to the associated normal tissues, making it unlikely that increased amounts and/or activity of this enzyme would in general account for the elevation of tumour PG formation. In our previous study of human colon tumours the median amount and the activities of cPLA$_2$ were also not significantly different compared with associated normal tissue [14].

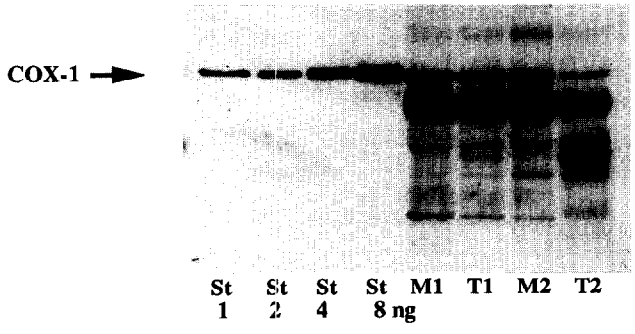

Figure 5. Typical immunoblot of the microsomal pellets from two human stomach tumours and associated normal mucosa/submucosa following treatment with COX-1 antibody (MF 241). St, standard; M1, mucosa/submucosa 1; T1, tumour 1; M2, mucosa/submucosa 2; T2, tumour 2; 40 µg of protein loaded. Bands below the standard are due primarily to non-specific binding.

The higher amounts of total FAs, $n-3$ FAs, $n-6$ FAs, unsaturated FAs and saturated FAs in normal gastric tissues compared to tumours might result from different types of cells and stroma present in the tumours. Tissues destroyed by tumour are replaced by a fibrous stroma which may be dense and acellular, as in scirrhous cancers, or infiltrated with lymphocytes, plasma cells and even polymorphs and eosinophils.

Amounts of constitutively expressed COX-1 were not significantly different between tumours and normal tissues. In contrast, tumours expressed COX-2 immunoreactivity whereas there was little or none in the normal mucosa. COX-2 therefore probably plays a role in the reported greater PG formation. Similarly, Kargman and colleagues [18] detected COX-2 protein expression in tissue from colon carcinomas but not in a small sample of premalignant polyps or control colon tissue from non-cancer patients. Sano and colleagues [20], using immunohistochemistry, found that although COX-1 expression was weak in both normal colon and tumour tissue, colon tumours showed enhanced COX-2 expression.

At least some of the COX-2 may come from invading host cells. Maxwell and associates [21] reported that the primary source of PGE$_2$ in human colon tumours may be host cells such as tissue-fixed macrophages rather than tumour cells. However, Sano and colleagues [20] found more COX-2 immunoreactivity in the colon cancer cells themselves but also in inflammatory cells, vascular endothelium and fibroblasts of the lesional tissues compared with the non-lesional and normal colon tissues. Perhaps the involvement of host and/or tumour tissues in COX-2 induction depends on the magnitude of the local inflammatory re-

sponse and the amounts of cytokines and growth factors generated. For example, TNFα increased the amounts of mRNA for COX-2 but not COX-1 in cultured intestinal epithelial cells [22]. Similarly hepatocyte growth factor, a protein with pleiotropic biological activity with effects on cell growth and motility, markedly increased PG production (mainly PGE_2) in the human gastric carcinoma cell line THK-1 by enhancing both cytosolic PLA_2 and cyclo-oxygenase activities [23]. Croxtall and associates [24] proposed that epidermal growth factor induced G protein-dependent release of arachidonic acid from A549 human lung adenocarcinoma cells (which require PGs for their growth) via kinase-dependent activation of cytosolic PLA_2.

In breast cancer, however, the elevated PG formation [4, 5] may not involve COX-2 since Kargman and colleagues [18] reported no COX-2 protein expression in a small number of human breast tumours. Perhaps COX-1 protein expression or COX-1 activity are responsible for the elevation in PGs, and/or there is an increase in the content and/or release of membrane phospholipids as occurs with lysophosphatidylcholine, phosphatidylcholine and phosphatidylethanolamine in colon cancers [25].

To summarise, our present results are the first finding of elevated COX-2 protein expression in human stomach cancers. This observation may help to explain why human stomach cancers often form more PGs than their associated normal tissues. Further studies are necessary to determine the exact cell type(s) in which tumour COX-2 expression occurs, and whether differences in the tumour membrane phospholipids help explain elevated PG formation.

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