Molecular and Cellular Biochemistry **162:** 97–103, 1996.

The modulation of choline phosphoglyceride metabolism in human colon cancer

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Received 8 November 1995; accepted 29 March 1996

Abstract

Colorectal cancer has a high incidence of morbidity and mortality in the North American population. Elevated levels of plasmalogens have been reported in some neoplastic tissues including colon tumors, but the mechanism for this increase has not been defined. Since changes in plasmalogen level are usually associated with changes in the other phospholipid subclasses, a general increase in all phospholipid subclasses may also be found in colonic neoplasms. In this study, the levels of the major phospholipids, including their plasmalogen and diacylphospholipid subclasses, were found to be elevated in human malignant colonic tissues. Since phosphatidylcholine is the most prominent type of phospholipid found in both malignant and control tissues, the mechanism for its accumulation during malignancy was investigated. Decreases in phospholipase C and D activities were observed in tumor samples, but an enhancement of the CTP: phosphocholine cytidylyltransferase activity was also detected. Immunoblotting analysis revealed that the elevated cytidylyltransferase activity was caused by a three-fold increase in the level of enzyme protein during tumor development. Based on these enzyme studies, we conclude that the high level of phosphatidylcholine in colon tumors resulted from a decrease in its turnover and an increase in its expression. (Mol Cell Biochem **162**: 97–103, 1996)

Key words: phospholipids, phosphatidylcholine, metabolism, cytidylyltransferase, colon cancer

Introduction

Colorectal cancer is a major cause of cancer death in North America, with a lifetime risk of 1:11 [1]. The incidence of colorectal cancer increases with age, and the risk is in the order of the sixth power between the fifth and eighth decade of life [1]. Interestingly, occurrence of this cancer is unusual under the age of 50. The incidence is similar in men and women, with slightly more women developing colon cancer, and slightly more men developing rectal cancer [2]. Diet is the most important environmental factor, and a fat intake of greater than 40% of total daily calories has been correlated with a high frequency of colorectal cancer [3]. A diet low in fiber and high in animal protein also contributes to its development [4]. Alternatively, a diet high in fiber is associated with a lower frequency of colon cancers, but the amount and type of fiber that offer protection remain unknown [3]. Hereditary factors are responsible for approximately 30% of colonic carcinomas. For example, an individual with a history of familial adenomatous polyposis, or Lynch syndrome I or II, has a higher risk of developing tumors at a much earlier age [3]. Other risk factors include familial or personal history of colonic polyps, previous colonic malignancy, inflammatory bowel disease such as ulcerative colitis, and a history of radiation treatment for gynecological cancers [1]. Although 90% of malignant tumors (carcinomas) of the colon and rectum arise from benign tumors (adenomas), only a small percentage of adenomas are actually malignant.

The phospholipids in all biological membranes, including membranes of tumor cells, are organized in a bilayer struc-

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ture [5]. Although phospholipid types are usually defined by the base group at the *sn*-3 position, membrane phospholipids can also be classified into subclasses according to the nature of the linkage at the *sn*-1 position [6]. The primary subclass is the 1,2diacylphospholipids which contain an ester bond at the *sn*-1 position. The most abundant types of phospholipid in this subclass are phosphatidylcholine and phosphatidylethanolamine Another subclass is the 1-alkenyl-2-acyl-glycerophospholipids which contain a vinyl ether bond at the *sn*-1 position. These lipids are also known as plasmalogens and are found in low quantities in mammalian organs except in electrically active tissues such as the brain and the heart. The prominent types of plasmalogens are plasmenylcholine and plasmenylethanolamine. The third subclass of phospholipids is the 1-alkyl-2-acyl-glycerophospholipids which have an ether bond at the *sn*-1 position. This subclass of phospholipid is found in very low amounts in mammalian tissues.

It is generally accepted that membrane characteristics are determined by the phospholipid components that comprise the membrane [5]. Hence, the content and composition of the membrane phospholipids are under stringent control. Elevated levels of plasmalogens have been reported in some neoplastic tissues [7–10] but the mechanism for this increase has not been defined. Since changes in plasmalogen levels are usually associated with changes in the other phospholipid subclasses, a higher diacylphospholipid may be found in colonic neoplasms when compared with the appropriate controls. However, information on changes in the phospholipid profiles of normal colonic tissue, benign and malignant colonic tumors is somewhat fragmented. In this study, the levels of total phospholipids, including plasmalogens and diacylphospholipids, were determined in control and malignant colonic tissues. Since phosphatidylcholine is the most prominent type of phospholipid found in these tissues [11], the mechanism responsible for its accumulation during malignancy was also investigated.

Materials and methods

Materials

[*methyl*-^{3}H]Choline, phospho-[*methyl*-^{14}C]choline and CDP-[*methyl*-^{14}C]choline were obtained from New England Nuclear (Mississauga, Ontario, Canada). Non-radioactive choline, phosphocholine and CDP-choline were purchased from Sigma Chemical Company (St. Louis, MO). All lipid standards were obtained from Serdary (London, Ontario, Canada). Thin layer chromatographic plates (sil-G25) were the product of Macherey-Nagel (Duren, Germany) and obtained through Brinkman (Rexdale, Ontario, Canada). Rabbit serum containing anti-rat liver CTP: phosphocholine cytidylyltransferase antibodies was a generous gift from Dr. D.E. Vance, University of Alberta. All other chemicals and solvents (reagent or HPLC grade) were obtained from the Canlab division of Baxter Co (Mississauga, Ontario, Canada).

Lipid analysis of tissue samples

Samples of human colonic mucosa (0.5–1.0 g wet weight) were obtained from patients undergoing elective colonic resection for carcinoma at the Health Sciences Centre, Winnipeg. Lipids were extracted from the sample by the procedure of Folch *et al.* [12]. The lipid extract was analyzed for phospholipid content and composition by thin-layer chromatography [13]. Briefly, an aliquot of the lipid extract was applied to a thin-layer chromatographic plate, and then developed in a solvent system containing chloroform/ methanol/ water/ acetic acid (70:30:4:2, v/v). The silica gel containing each phospholipid fraction was removed from the chromatographic plate, and the phospholipid was eluted from the gel. The amount of phospholipid in each fraction was determined by the method of Bartlett [14]. Since both diacyl- and plasmalogen forms were present in the choline and ethanolamine phosphoglyceride fractions, each phosphoglyceride fraction was exposed to HCl fumes. This treatment destroyed the plasmalogens but not the diacylphospholipids [15, 16]. The plasmalogen content in the choline or ethanolamine phosphoglyceride was estimated by the difference between the total phosphoglyceride content and the value obtained after the lipid sample was exposed to HCl fumes [16].

Preparation of subcellular fractions

Tissue samples were blotted dry, weighed, and homogenized with a polytron homogenizer in a buffer containing 20 mM Tris (pH 8.0), 100 mM NaCl, 2 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 0.2 mM sodium orthovanadate, 10 μg/ml leupeptin, and 20 μg/ml aprotinin [17]. The homogenate was centrifuged at 1,000 g for 2 min to remove unbroken cells and other debris. The resulting supernatant was recentrifuged at 15,000 g for 6 min to obtain the postmitochondrial fraction which contained both cytosol and microsomes. This fraction was used for all subsequent enzyme assays.

Enzyme assays

Choline kinase activity was assayed by determining the conversion of [*methyl*-^{3}H]choline to phosphocholine as previously described [18]. The labelled product was separated from the substrate by thin-layer chromatography with a sol-

vent system containing methanol/ 0.6% sodium chloride/ ammonium hydroxide (50:50:5, vlvlv). CTP: phosphocholine cytidylyltransferase activity was assayed by the conversion of phospho[*methyl*-^{14}C]choline to labelled CDP-choline. The reaction mixture contained 80 mM Tris-succinate (pH 6.5), 6 mM magnesium acetate, 0.5 mM CTP, 1 mM phospho-[*methyl*-^{14}C]choline (2 μCi/μmol), and 0.1 mg enzyme protein in a final volume of 0.1 ml. The reaction mixture was incubated at 37°C for 15 min, and the reaction was terminated by boiling the mixture for 2 min [19]. The CDP-choline formed was separated from the substrate by thin-layer chromatography with a solvent system containing methanol/0.6% sodium chloride/ammonium hydroxide (50:50:5; vlvlv). The CDP-choline: 1,2-diacylglycerol cholinephosphotransferase activity was determined by the conversion of CDP-[*methyl*-^{14}C]choline to phosphatidylcholine as previously described [20]. The reaction was incubated at 37°C for 15 min, and the reaction was terminated by the addition of 2 ml chloroform. The labelled lipid was separated from the substrate by a two-phase extraction. Phospholipase A (A_1 and A_2) activity was determined by the production of labelled lysophosphatidylcholine from l-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phospho-[*methyl*-^{14}C]choline using the postnuclear fraction of the colonic mucosa as the enzyme source. The reaction mixture (0.1 ml) contained 10 mM Tris-HCl, pH 8.0, 1 mM calcium chloride, 1 μM labelled substrate (sonicated), and the enzyme preparation containing approximately 0.1 mg protein, as previously described [21]. After incubation for 30 min at 37°C, the labelled lysophosphatidylcholine was separated from the substrate by thin-layer chromatography. The activities of phospholipases C and D were determined with l-palmitoyl-2arachidonoyl-*sn*-glycero-3-phospho-[*methyl*-^{14}C] choline as substrate under the same incubation conditions as the assay for phospholipase A. The labelled choline or phosphocholine produced by the reaction was isolated by thin-layer chromatography as previously described [21].

Quantitation of CTP: phosphocholine cytidylyltransferase protein by immunoblotting

Homogenates of the mucosa were analyzed by electrophoresis using a 10% polyacrylamide gel containing sodium dodecyl sulfate. The protein bands in the polyacrylamide gel were transferred to a nitrocellulose membrane by a semi-dry blotting system (Biorad). The nitrocellulose membrane was incubated in 3% BSA in TBS (50 mM Tris-HCl, pH 7.5, 100 mM NaCl) containing 0.5% Tween 20 (v/v) and further incubated with a 1:200 dilution of rabbit serum containing polyclonal anti-rat liver cytidylyltransferase antibodies which have been shown to cross-react with the enzyme from the colonic mucosa. After extensive washing in TBS containing Tween 20, the nitrocellulose membrane was placed in a solution containing a 1:500 dilution of a peroxidase conjugated goat anti-rabbit IgG antibodies in 1% BSA and TBS. The cytidylyltransferase protein band was detected by incubation of the membrane in 50 mg% 4chloro-1-napthol, 50 μl% H_2O_2 in methanol/TBS (1:5, v/v) for 15 min, and the resulting color development was quantitated with a densitometer [22]. The protein in each sample was quantitated by the method of Lowry [23].

Statistical analysis

The means ± standard error of the mean (S.E.M.) were used in all data computations. The paired *t*-test was used for the statistical analysis of some enzyme activities. Student's t-test was employed for all other analyses. The level of significance was defined as $p < 0.05$ in all cases.

Results

Histological examination

Samples of human colon were obtained from patients undergoing elective surgery for colonic carcinoma at the Health Sciences Centre, Winnipeg. Mucosa from the tumor site and normal mucosa remote from the tumor (control) were removed for histological examination. All tumor samples used in this study were at the B1, B2 or C2 stage according to Duke's classification [1]. Typical examples of the tumor and control tissues used for this study are depicted in Fig. 1.

Phospholipid analysis

Total phospholipid contents in colon cancer and control samples were determined with respect to their wet weight and dry weight (Fig. 2). Since the ratio of wet weight to dry weight were quite similar (± 5%) between tumor and control samples, the wet weight was used throughout the study. Total phospholipids in tumors were found to be increased by 30–40% when compared with controls. Analysis of the individual phospholipids after separation by thin-layer chromatography revealed that phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol contents were significantly increased in malignant samples (Fig. 3A). The plasmalogen contents in colonic tissues were not uniform between samples, but a significant increase was detected in tumor tissues (Fig. 3B). The choline plasmalogen (plasmenylcholine) level was increased by 45%, while the ethanolamine plasmalogen (plasmenylethanolamine) level was elevated by 58% in malignant samples.

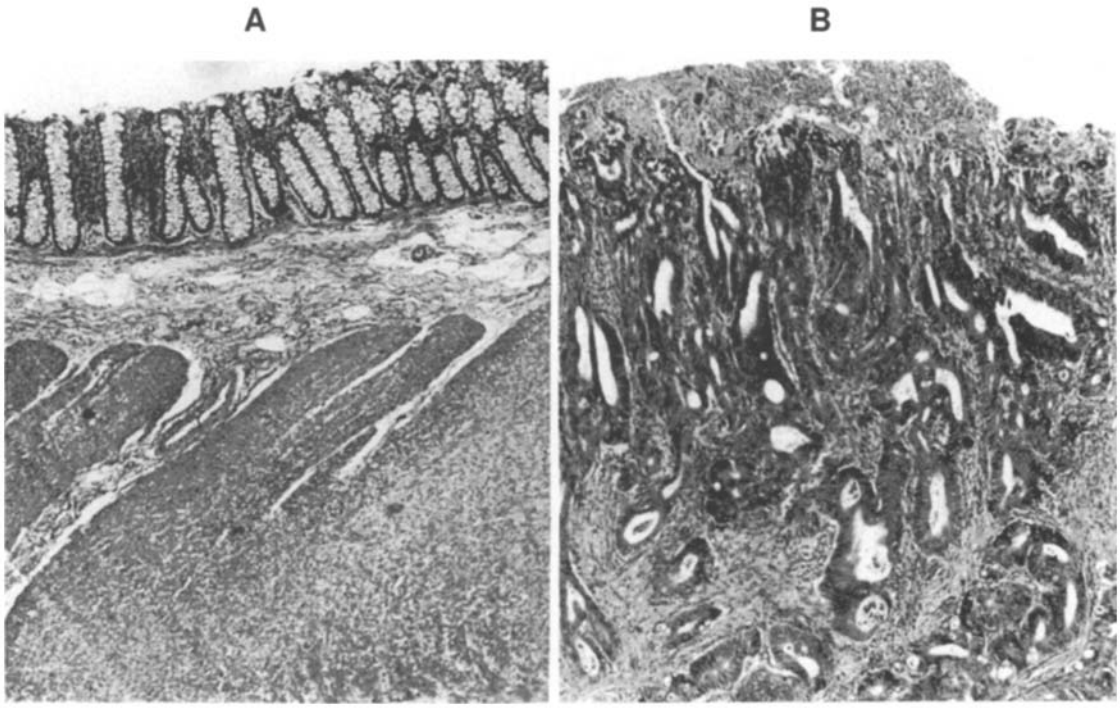

Fig. 1. Histological sections of normal and malignant colonic mucosa. The structure of normal (control) colonic mucosa is depicted in (A). Note the orderly arrangement of glands with underlying submuscosa and muscular wall. Invasive colonic adenocarcinoma is depicted in (B). Note the surface ulceration and malignant glands infiltrating through the submucosa and muscular wall. The photomicrographs were taken at a magnification of 50X in both cases.

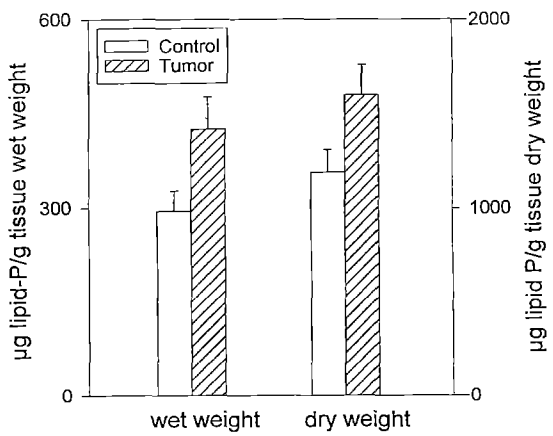

Fig. 2. Total phospholipid contents in control and malignant colonic mucosa. The total phospholipid content in each sample was determined and expressed in the dry and wet weight of the tissue. Each set of data represents the mean ± S.E.M. of at least six separate experiments.

Determination of enzyme activities

The mechanism for the elevation of phospholipid levels in the colon tumor was explored. In general, alterations in phospholipid levels can be caused by changes in the rate of synthesis and/or degradation of phospholipids. Hence, the activities of the enzymes responsible for the metabolism of phosphatidylcholine, the major phospholipid in the colon, were determined. As depicted in Table 1, no change in phospholipase A (A_1 and A_2) was detected in colon tumors when labelled phosphatidylcholine was used as the substrate. However, the activities of phospholipases C and D were markedly decreased in the tumor tissue. Our results suggest that the hydrolysis of phosphatidylcholine was attenuated during tumor development. The synthesis of phosphatidylcholine in the tumor tissue was also examined. Since the CDP-choline pathway is the major pathway for phosphatidylcholine biosynthesis, the activities of the enzymes in this pathway were determined in tumors and compared with the values obtained from the controls. As depicted in Table 2, no significant changes in the activities of choline kinase or CDP-choline: 1,2-diacylglycerol cholinephosphotransferase were detected. However, there was a high degree of variability in the activity of the CTP: phosphocholine cytidylyltransferase in both the control and tumor samples. Nevertheless, application of the paired *t*-test revealed that the enzyme activities in malignant samples were significantly elevated when compared with the appropriate controls. The enhancement of the cytidylyltransferase activity might be an important factor for the increase in phosphatidylcholine production which might lead to its accumulation in tumor tissues.

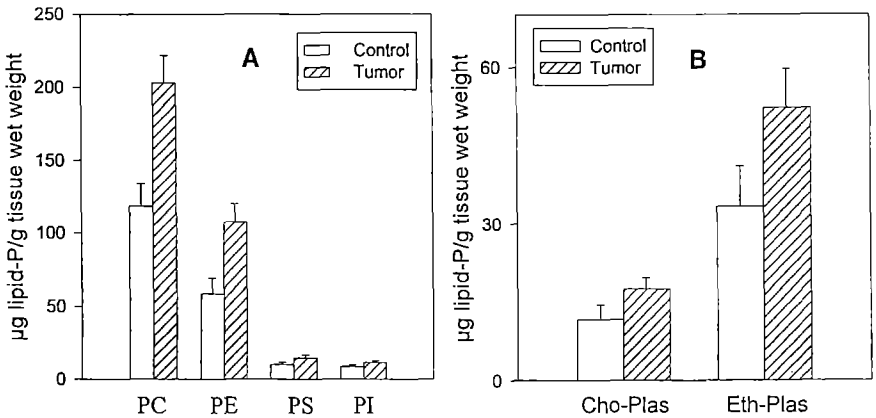

Fig. 3. Contents of diacylphospholipid and plasmalogen groups in control and malignant colonic mucosa. Contents of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) in control and tumor samples are depicted in (A). Contents of choline plasmalogen (Cho-Plas) and ethanolamine plasmalogen (Eth-Plas) in control and tumor samples are depicted in (B). Each set of data represents the mean ± S.E.M. of at least six sparate experiments.

Table 1. Activities of Phospholipases in controls and colonic tumors

Enyme	Control	Tumor
	(pmol product/h/mg protein)	
Phospholipase A (A_1 and A_2)	2.93 ± 0.84	3.48 ± 1.05
Phospholipase C	3.75 ± 1.03	0.84 ± 0.41*
Phospholipase D	0.88 ± 0.21	0.28 ± 0.10*

Each set of data represents the mean ± S.E.M. of four separate experiments. *$p < 0.05$ when compared with the control.

Table 2. Activities of enzymes in CDP-choline pathway for phosphatidylcholine biosynthesis in controls and colonic tumors

Enzyme	Control	Tumor
	(nmol product/min/mg protein)	
Choline kinase	0.64 ± 0.09	0.57 ± 0.07
CDP-choline: diacylglycerol cholinephosphotransferase	1.10 ± 0.04	1.01 ± 0.09
CTP: phosphocholine cytidylyltransferase	0.64	1.15
	0.45	0.71
	0.26	0.30
	0.35	1.02
	0.37	0.42
	0.14	0.27

Each set of data represents the mean ± S.E.M. of six separate experiments, except for CTP: Phosphocholine cytidylyltransferase, in which case the value obtained from each assay is given.

Immunological quantitation of the cytidylyltransferase

The enhancement of the cytidylyltransferase activity in tumor tissues might arise from activation of the enzyme by translocation from the cytosol to microsomal membranes. Alternatively, the higher enzyme activity might be caused by a net increase in enzyme protein. In order to differentiate between these two possibilities, polyclonal antibodies to the cytidylyltransferase were used to quantify the amount of enzyme present in tumor and control samples. Aliquots of tissue homogenates containing identical amounts of protein were analyzed by sodium dodecyl sulfate/ polyacrylamide gel electrophoresis, and the protein bands in the polyacrylamide gel were transferred to a nitrocellulose membrane. The membrane was treated with rabbit anticytidylyltransferase antibodies, and the membrane was subsequently treated with goat anti-rabbit IgG antibodies coupled to horse radish peroxidase. The cytidylyltransferase band was visualized using 1-chloro-4-napthol as the substrate for the peroxidase reaction. A typical Western Blot is depicted in Fig. 4. An average 3.1-fold increase in the level of the enzyme protein was detected in malignant tissues in four separate experiments.

Discussion

It has been demonstrated in previous studies that neoplastic tissues contain elevated levels of plasmalogen. For example, plasmalogen levels in the ethanolamine- and cholinephosphoglyceride fractions have been found to be 4.5-fold higher in human brain tumors than in normal tissues [7]. Snyder and Wood [8] have shown higher quantities of ether-linked neutral glycerides, particularly the alkyl type, and ether-linked phosphoglycerides in a variety of human neoplastic tissues, such as liver and adipose tissue. Rat hepatomas also contain higher levels of both alkyl and alkenyl moieties of glycerides compared with non-malignant tissue [9]. Based on these findings, Howard *et al.* [9] suggested that a correlation exists between the rate of tumor growth and the glyceryl-ether content in phospholipids. The levels of ether-linked lipids may become higher in fast growing tumors than in slow growing tumors. In this study, we have confirmed that the level of

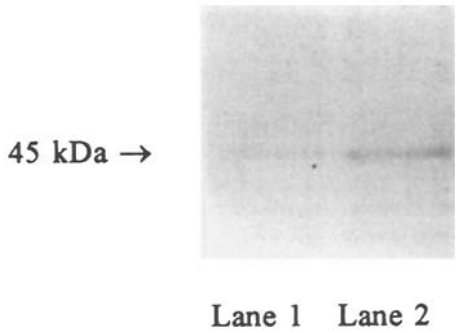

Fig. 4. The determination of CTP: Phosphocholine cytidylyltransferase content in control and malignant colonic mucosa by Western blot. Each lane contained 0.12 mg protein from the tissue homogenate. Lane 1 represents the control sample and lane 2 represents the tumor sample.

plasmalogens is elevated in colon tumors. In addition, we have documented that the level of total phospholipids, including the diacylphospholipids, was significantly increased during neoplastic formation. This finding is not at all surprising since a higher amount of membrane phospholipids is usually required for the rapid cellular growth during tumor development. This notion is supported by the fact that the higher phospholipid content in tumor cells was not confined to a particular phospholipid group.

The mechanism for the increase in phospholipid content was examined. The increase in phosphatidylcholine was employed as a model for this study since it is the major phospholipid in both control and tumor samples. In addition, its major metabolic pathways in mammalian tissues are well established. It is clear from our study that the turnover of phosphatidylcholine via phospholipase A was not changed in tumors, but hydrolysis via phospholipases C and D was markedly decreased in these tissues. The lack of change in phospholipase A activity during cancer development has been reported by other investigators [24]. Although the importance of phospholipases C and D for the turnover of phosphatidylcholine remains undefined, the severe attenuation of their activities suggests that phosphatidylcholine turnover must be reduced during neoplastic formation.

In the intestinal mucosa, the CDP-choline pathway is the major pathway for phosphatidylcholine biosynthesis [25]. In this pathway, choline is rapidly phosphorylated into phosphocholine which is converted to CDP-choline for subsequent condensation with diacylglycerol to form phosphatidylcholine. The rate-limiting step in this pathway is the conversion of phosphocholine to CDP-choline which is catalyzed by CTP: phosphocholine cytidylyltransferase [26]. Hence, an increase in the cytidylyltransferase activity in colonic cells may increase the rate of phosphatidylcholine biosynthesis. It is important to point out that the activity of the cytidylyltransferase was found to vary greatly from one sample to another, even within the control groups. The fluctuation of enzyme activities in controls was probably caused by enzyme activation during sample storage, a phenomenon that has been well documented in several studies [19, 27]. Although great care was taken to duplicate the conditions of tissue storage prior to analysis, factors such as the exact time of excision and the time lapse between excision and cold storage could not be rigidly defined. Nevertheless, elevated enzyme activity was confirmed in tumor samples in every case when both the tumor and control were treated under identical conditions.

In mammalian tissues, the cytidylyltransferase is located in at least two cellular compartments, with the less active form in the cytosol and the more active form associated with the endoplasmic reticulum [28]. The enhancement of enzyme activity by translocation from the cytosol to microsomes is promoted by certain phospholipids, and the process is regarded as an important mechanism for the regulation of the cytidylyltransferase activity *in vivo*. Alternatively, an increase in cytidylyltransferase activity in tumors may result from an increase in enzyme protein. In order to delineate the mechanism for the activation of the cytidylyltransferase, total enzyme protein in the tumor and control samples were estimated by a Western Blotting technique. A dramatic increase in enzyme protein was detected in tumor samples, suggesting that there was a general increase in the production of the key enzyme for the synthesis of phosphatidylcholine. Since the cytidylyltransferase is the rate limiting component we postulate that the increase in enzyme level must precede the increase in phosphatidylcholine biosynthesis in order to produce an elevated phosphatidylcholine level in the tumor tissue. Hence, the elevated cytidylyltransferase level may serve as a potential marker for the early detection of colon cancer.

Acknowledgement

This study was supported by the Thorlakson Foundation. Part of the study was taken from the thesis submitted by Dorie-Anna Dueck in partial fulfilment of the B.Sc.(med) degree at the University of Manitoba.

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