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Increased Choline Kinase Activity and Elevated Phosphocholine Levels in Human Colon Cancer†

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Nuclear magnetic resonance spectroscopy has detected elevated phosphocholine levels in human tumor tissues and cells, and in cells that were transformed with the activated Ha-*ras* gene and stimulated *in vitro* with growth-promoting factors such as platelet-derived growth factor, epidermal growth factor, and phorbol ester. However, the mechanism of the elevation and the function of the increased phosphocholine levels have not been clearly demonstrated. We studied phosphocholine levels enzymatically and analyzed the activity of choline kinase, which catalyzes the phosphorylation of choline to produce phosphocholine, in human colon cancer and adenoma. Both choline kinase activity and phosphocholine levels were increased in colon cancer and adenoma tissue. The activation of choline kinase and the increased levels of choline kinase α were partly responsible for the elevated phosphocholine levels. This study suggests that choline kinase might play a role in growth promotion or signal transduction in carcinogenesis.

Key words: Choline kinase — Phosphocholine — Human colon cancer

Cancer of the colon is one of the most common cancers in developed countries and its prevention is of great interest throughout the world. It is thought that the accumulation of certain mutated genes, including oncogenes, tumor suppressor genes, genes for DNA-repair enzymes, and invasion/metastasis-related genes, is necessary for the onset and progression of cancer. Mutation may cause further malignant changes in cellular proliferation, especially in enzymatic properties and activity.[1–3] Some of the changes in enzymatic properties and activity with proliferation may be advantageous to the cancer cells.[4–7] Studying the cellular properties of cancer cells improves our understanding of the mechanism of cellular growth control and sheds further light on cancer prevention and treatment.[8]

Choline kinase is the first enzyme in the cytidine 5′-diphosphate (CDP)-choline pathway for the synthesis of phosphatidylcholine, and phosphorylates choline to phosphocholine using adenosine 5′-triphosphate (ATP) as the phosphate donor.[9–11] *In vitro* studies of oncogenic ras proteins, and products and growth factors have shown that phosphocholine contributes to cellular growth regulation and intracellular signal transduction. Ras proteins play a pivotal role in cellular signal transduction, and help regulate cellular proliferation and terminal differentiation.[12–14] Microinjecting the oncogenic Ha-*ras* gene product p21^{ras} into *Xenopus* oocytes causes meiosis,[15] quickly activates choline kinase and elevates phosphocholine levels.[16] Transforming fibroblastic cells with oncogenic Ha-*ras* also activates choline kinase.[17–19] Growth factors essential for cellular growth also activate choline kinase, elevating the intracellular phosphocholine level. Prolactin is one such growth factor for Nb 2 rat node lymphoma cells.[20] It has been suggested that platelet-derived growth factor might use a choline kinase-phosphocholine route to promote cell growth in NIH3T3 fibroblasts.[21,22] In addition, phosphocholine has been shown to promote growth in NIH3T3 fibroblast cells.[22–24] Furthermore, nuclear magnetic resonance (NMR) spectroscopy has demonstrated higher concentrations of phosphocholine in human tumor tissues and growth-promoted cells.[25–29] These results suggest that phosphocholine and choline kinase may not only play a role in phospholipid synthesis, but also in regulating cellular growth in cancer cells. However, the role and mechanism of the increased phosphocholine level, and the function of choline kinase and its mechanism of activation are not yet clear. Moreover, the phosphocholine level and choline kinase activity in human cancer tissues have never been chemically analyzed.

In order to clarify the role of choline kinase in cancer growth, we measured the levels of choline kinase and phosphocholine in human colon cancer. In this study, we found increases in both choline kinase activity and phosphocholine levels. A western blot analysis using a choline

†Choline kinase α and β were previously designated as choline kinase R and P, respectively.

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kinase antibody showed that the increased enzyme activity was due, in part, to an increase in one of the isoforms of the protein.

MATERIALS AND METHODS

Patients and human tissue treatment Human colon cancer tissue and matched normal mucosa were obtained from surgically resected specimens taken from 48 patients immediately after resection. Two of these patients also had an adenoma. In addition, 11 adenomas resected from 9 patients during elective resection of the colon were studied. The mean age of the colon cancer patients was 65 years and ranged from 41 to 84. According to the Dukes' stage classification, 7, 13, 17, and 11 patients were Dukes' A, B, C, and D, respectively. Histologically, 14 patients had well differentiated adenocarcinoma, 28 had moderately differentiated adenocarcinoma, 4 had poorly differentiated adenocarcinoma, and 1 each had mucinous carcinoma and carcinoid tumor. The mean age of the adenoma patients was 68 years and ranged from 50 to 81. Histologically, 8 patients had tubular adenoma, 3 had tubulo-villous adenoma, and 2 had villous adenoma (Table I).

Materials Choline oxidase, horseradish peroxidase, bovine intestinal alkaline phosphatase, and 4-aminoantipyrine were obtained from Wako Pure Chemicals (Tokyo). The ECL western blotting detection reagent was purchased from Amersham International (Buckinghamshire, UK). The polysulfone membrane filter (New Steradisc 25-Kurabo) was purchased from Kurabo Industry Ltd. (Osaka). Goat anti-rabbit IgG antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). [*Methyl*-^{14}C]choline chloride (54 mCi/mmol) was purchased from Du Pont New England Nuclear (Boston, MA). Glutathione Sepharose and formyl-Cellulofine were obtained from Pharmacia Biotech (Uppsala, Sweden) and Seikagaku Kogyo (Tokyo), respectively.

Table I. Characteristics of Patients

	Cancer		Adenoma	
No. of patients	48		13	
Mean age (range) yrs.	65 (41–84)		68 (50–81)	
Sex (female/male)	22/26		1/12	
Stage	Dukes' A	7		
	B	13		
	C	17		
	D	11		
Histology	well	14	tubular adenoma	8
	mod	28	tubulo-villous adenoma	3
	poorly	4	villous adenoma	2
	muc	1		
	carcinoid	1		

Well, well differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; poorly, poorly differentiated adenocarcinoma; muc, mucinous carcinoma; carcinoid, carcinoid tumor.

Preparation of cytosol Tissues were homogenized on ice in 3 volumes of homogenizing buffer (250 m*M* sucrose, 10 m*M* Tris-HCl (pH 7.5), 5 m*M* EDTA, 50 m*M* NaCl, 1 m*M* phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, and 2 μg/ml leupeptin). The homogenates were centrifuged at 4°C for 10 min at 800*g* and then for 1 h at 100,000*g*. The supernatants were used for the choline kinase assay.[30]

Choline kinase assay Choline kinase activity was measured isotopically as described previously.[31]

Determination of choline and phosphocholine levels Frozen tissue (200–300 mg) was homogenized in methanol at room temperature. The homogenates were centrifuged at 4°C for 10 min at 15,000*g*. The supernatant was evaporated *in vacuo* and the residue was dissolved in 1.5 ml of water by sonication. The solution was filtered through a polysulfone membrane filter (New Steradisc 25-Kurabo).

To determine the choline level, an aliquot (1 ml) was incubated at 37°C for 15 min in 100 m*M* Tris-HCl, pH 8.0, 0.5% Triton X-100, 1.5 m*M* phenol, 0.15 m*M* 4-aminoantipyrine, 1 U/ml choline oxidase, and 1 U/ml peroxidase (total volume 2 ml). The developed color was measured at 500 nm. To determine the amount of phosphocholine plus choline, 2 U/ml alkaline phosphatase was added to the above reaction mixture. The amount of phosphocholine was calculated by subtracting the amount of choline from the amount of phosphocholine plus choline.

Affinity purification of anti-choline kinase α antibody Glutathione-*S*-transferase (GST)-Taq choline kinase α, the fusion protein of choline kinase α with GST, containing the entire deduced amino acid sequence of rat choline kinase α, expressed in *Escherichia coli*,[32] was partially purified with a glutathione-Sepharose column according to the manufacturer's protocol. The eluate from the column treated with glutathione (crude GST-Taq choline kinase α) was used to prepare an affinity-column without further purification. One milliliter of crude GST-Taq choline kinase α (5 mg of protein) was dialyzed against 1 liter of 200 m*M* sodium phosphate buffer (pH 9.0) containing 150 m*M* NaCl and then coupled to 1 ml of formyl-Cellulofine with 4 mg/ml sodium cyanoborohydride in 5 ml of the dialyzing buffer at room temperature for 16 h. The remaining formyl groups were blocked with 0.2 *M* ethanolamine and sodium cyanoborohydride.

An antiserum to choline kinase α was prepared by immunizing rabbits with choline kinase α that was expressed in *E. coli*[33] and purified.[32, 34] Anti-choline kinase α antibody was purified with the GST-Taq choline kinase α-coupled affinity matrix, which was pre-equili-

brated with buffer A (20 m*M* Tris-HCl (pH 7.5), 1 m*M* EDTA, 5 m*M* 2-mercaptoethanol and 0.2 m*M* phenylmethylsulfonyl fluoride) containing 100 m*M* NaCl. The antiserum was loaded on the column, which was washed successively with buffer A, a mixture of 3 *M* NaCl and 20 m*M* $NaPO_4$ (pH 7.0) and buffer A, then eluted with 0.1 *M* Na citrate (pH 3.0). The eluate was neutralized with 1 *M* Tris-HCl (pH 8.0) containing 5% bovine serum albumin.

Western blot analysis The cytosolic proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis,[35] and transferred onto polyvinylidine difluoride (PVDF) membranes (Millipore Corp., Bedford, MA). Choline kinase α was detected with the affinity-purified anti-choline kinase α antibody and goat anti-rabbit IgG, using ECL as reported previously.[32]

Protein assays Protein concentrations were determined using Bio-Rad protein assay dye reagent (Bio-Rad) with bovine serum albumin as the reference standard.[36]

Statistical analyses The results are expressed as the mean±the standard error of the mean (SEM). The statistical differences between groups were examined with the Kruskal-Wallis test and Student's *t* test. A *P* value less than 0.05 was considered to be significant.

Ethics This study conformed to the ethical guidelines of Gunma University School of Medicine.

RESULTS

Choline and phosphocholine levels The phosphocholine levels were measured in 30 cancer specimens and matched normal tissue from the same patients. The level in human colon cancer was 1.5 times higher than in normal colon tissue ($P<0.01$) (Fig. 1). The phosphocholine level was not correlated with age, sex, histologic differentiation, or Dukes' stage. On the other hand, the choline levels in human colon cancer were not significantly different from those in normal colon tissue (data not shown).

Increased choline kinase activity Subsequently, we measured the choline kinase activity in 28 cancer specimens and matched normal tissue, since the phosphocholine levels were increased in these tumor tissues. The choline kinase activity in human colon cancer and adenomas was 3.7 and 3.2 times higher than in normal colon tissue, respectively (Fig. 2). Both increases were significant ($P<0.01$). However, there was no significant difference in choline kinase activity between human colon adenoma and colon cancer (Fig. 2). The choline kinase activity was not correlated with age, sex, histologic differentiation, or Dukes' stage (Fig. 3). Equal amounts of protein from human colon cancer and normal colon tissue were mixed and assayed for choline kinase to exclude the presence of an activator in cancer or an inhibitor in the normal tissue. The activity was the average of the activity of the cancer and the normal tissue. This result rules out the presence of an activator in cancer or an inhibitor in the normal tissue.

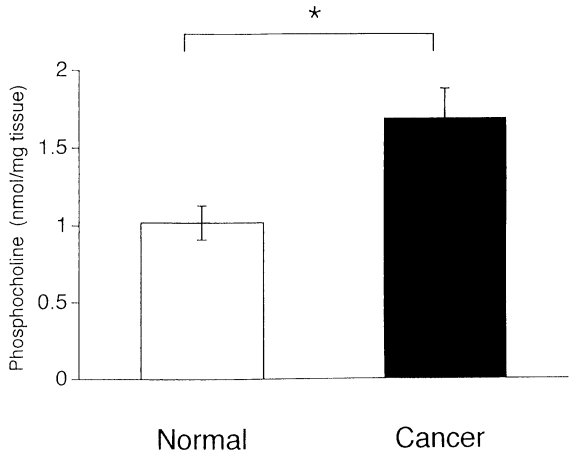

Fig. 1. Phosphocholine levels in human colon cancers and normal colon tissues. The phosphocholine assay is described in "Materials and Methods." Values are mean±SE (n=30: 5, 5, 13, and 7 cases of Dukes' A, B, C, and D, respectively). Phosphocholine levels in human colon cancers were about 1.5 times higher than in normal colon tissue. The difference was significant (∗ $P<0.01$).

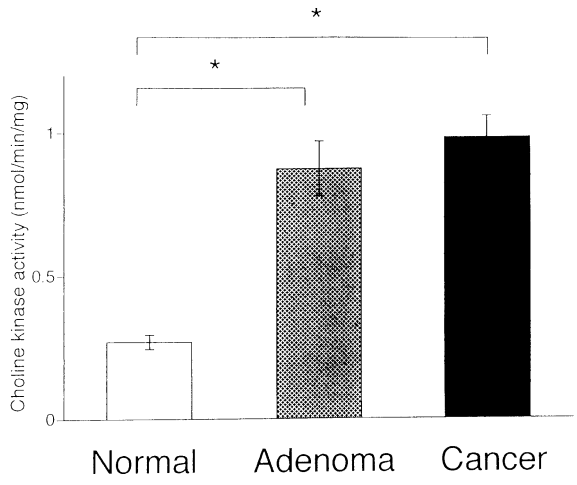

Fig. 2. Choline kinase activity in human colon cancer, adenoma, and normal colon tissue. The method of choline kinase activity measurement is described in "Materials and Methods." The values are the mean±SE. In human colon cancers (n=28), the choline kinase activity was 3.7 times higher than in normal colon tissues and the difference was significant ($P<0.01$). In human colon adenomas (n=13), the choline kinase activity was 3.2 times higher than in normal colon tissues, which was also significant ($P<0.01$). There was no difference between the choline kinase activities in human colon cancers and adenoma. ∗ $P<0.01$.

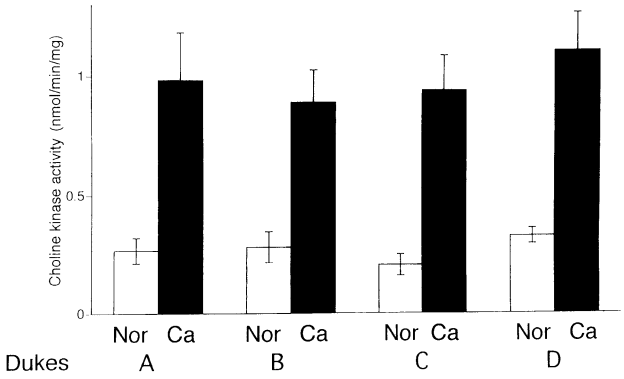

Fig. 3. Choline kinase activity in colon cancer and Dukes' stage. The choline kinase activity measurements are described in "Materials and Methods." Values are the mean±SE. There were 5, 7, 8, and 8 patients with Dukes' stage A, B, C, and D, respectively. Choline kinase activity was not correlated with Dukes' stage. □ normal tissue, ■ cancer.

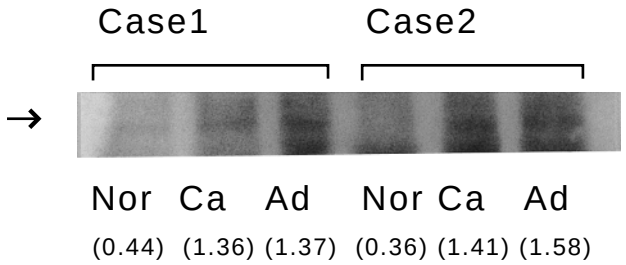

Fig. 4. Western blotting of choline kinase α in human colon cancer, adenoma, and normal tissue. The techniques of the western blot analysis are described in "Materials and Methods." The 52 kDa band (shown with an arrow) in human colon cancer and adenoma was denser than that of normal colon tissue. The values in parentheses indicate choline kinase activity (nmol/min/mg). Nor, normal tissue; Ca, cancer; Ad, adenoma.

Increase in the level of choline kinase α Western blot analysis was used to examine the mechanism of the elevated choline kinase activity in cancer tissue. Fig. 4 clearly shows increased amounts of choline kinase α protein in colon cancer and adenoma. Each of the two cases presented represents tissue from colon cancer and adenoma in the same resected specimen.

DISCUSSION

The accumulation of certain gene mutations is necessary for the development and progression of cancer,[4] although changes in enzymatic properties or activity that benefit the cancer cells also commonly occur with proliferation.[1–3] Choline kinase may be one enzyme that is so altered. Its role in growth control and signal transduction in transformed cells[8] suggests that choline kinase may be one of the targets modified by carcinogenesis and that the increased activity may favor the growth of cancer cells.

Phosphocholine serves as a precursor for the synthesis of other phospholipids,[11] phosphatidylserine,[37] and sphingomyelin.[38] Phosphatidylserine is a substrate for the synthesis of phosphatidylethanolamine. Therefore, regulating choline kinase is crucial for the synthesis of most cellular phospholipids. These phospholipids are the major components of biological membranes and are also precursors for signal transduction.[39] The activation of choline kinase is necessary for building membranes, cell growth, and cell proliferation, and for rebuilding phospholipids that are degraded in the process of signal transduction. Accordingly, choline kinase and phosphocholine have an essential role in growth control and signal transduction.

This study found elevated phosphocholine levels in human colon cancer enzymatically. Our results support NMR spectroscopy-based reports of higher concentrations of phosphocholine in human tumor tissue and growth-promoted cells.[25–29] Furthermore, this study is the first to show that choline kinase activity and choline kinase α levels are increased in both human colon cancer and adenoma. These results support the idea that this enzyme may play a role in growth promotion or signal transduction in carcinogenesis. However, neither the phosphocholine level nor the choline kinase activity was associated with the progression or invasion of the cancer, since neither was correlated with the Dukes' stage. Studies on the prevention of carcinogenesis and cancer therapy will focus on choline kinase in the near future.[8]

The elevated phosphocholine level was in part due to the elevated choline kinase activity, which in turn may, in part, be due to the elevated choline kinase α, although the elevated choline kinase activity and choline kinase α level were not always correlated. Choline kinase β, another isozyme that remains to be purified, may be involved in the increased choline kinase activity. Further examination using animal models must be conducted to study the function of choline kinase in carcinogenesis and cancer progression.

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