Increased Choline Kinase Activity in 1,2-Dimethylhydrazine-induced Rat Colon Cancer

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Cancer cells acquire particular characteristics that benefit their proliferation. We previously reported that human colon cancers examined had increased choline kinase activity and phosphocholine levels. The elevated phosphocholine levels were in part due to both activation of choline kinase and increased choline kinase α protein levels. In this report, we analyzed choline kinase, which catalyzes the phosphorylation of choline to produce phosphocholine, in rat 1,2-dimethylhydrazine (DMH)-induced colon cancer. This study is the first to demonstrate increased choline kinase α enzymatic activity, protein levels, and mRNA levels in DMH-induced colon cancer as well as human colon cancer, although phosphocholine was not increased in DMH-induced rat cancer. The increase in the mRNA level was partly due to an increase in the transcription of the choline kinase α gene. The increased choline kinase activity may be a specific characteristic acquired by cancer cells that benefits their proliferation.

Key words: Choline kinase — DMH — 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 an accumulation of mutated genes, including oncogenes, tumor suppressor genes, DNA-repair enzyme genes, and invasion/metastasis-related genes, is necessary for the generation and progression of cancer. Mutation may cause further malignant changes in cellular proliferation, especially when enzymatic activity and properties are affected. Some of the changes that occur in enzymatic properties and activity with proliferation may favor the growth of cancer cells. Studying the cellular properties of cancer cells furthers our understanding of the mechanisms of cellular growth control and provides clues to strategies for cancer prevention and treatment.

1,2-Dimethylhydrazine (DMH) is widely used for experimental studies of specific colon carcinogenesis in rodents. In the body, the metabolic product of DMH modifies DNA, causes mutation, and leads to carcinogenesis. Choline kinase is the first enzyme in the CDP-choline pathway for the synthesis of phosphatidylcholine, and phosphorylates choline to phosphocholine using adenosine 5'-triphosphate (ATP) as the phosphate donor. Ras proteins play a pivotal role in cellular signal transduction, and help regulate cellular proliferation and terminal differentiation. Microinjection of the oncogenic Ha-ras gene product p21ras into Xenopus oocytes, which causes meiosis, quickly elevates the phosphocholine level and activates choline kinase. Transformation of fibroblastic cells with oncogenic Ha-ras activates choline kinase. Growth factors essential for cellular growth also activate choline kinase, elevating the intracellular phosphocholine level. It has been suggested that platelet-derived growth factor might use a choline kinase-phosphocholine route to promote cell growth in NIH3T3 fibroblast cells. We previously reported that human colon cancers examined had increased choline kinase activity and phosphocholine levels. The elevated phosphocholine levels were due in part to the activation of choline kinase and the increased choline kinase α protein levels. These results suggest that choline kinase and phosphocholine may play a role not only in phospholipid synthesis, but also in regulating cellular growth in cancer cells.

This study is the first to analyze choline kinase in DMH-induced rat colon cancer. We further analyzed the mechanism of activation and found that the activation involved increases in both the protein and mRNA levels. Transcription occurred at a specific transcription start site, which is not used in the liver or testis of normal adult rats, but is used in the liver of rats treated with 3-methylcholanthrene or carbon tetrachloride. The particular transcription factor or pathway that stimulates this choline kinase α gene may be activated in DMH-induced colon cancer.

MATERIALS AND METHODS

Materials Choline oxidase, horseradish peroxidase, bovine intestine alkaline phosphatase, and 4-aminoantipyrine.
rine were obtained from Wako Chemicals (Tokyo). DMH was purchased from Nacalai Tesque (Kyoto). The ECL western blotting detection reagent was purchased from Amersham International (Buckinghamshire, UK). Goat anti-rabbit IgG antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). [Methyl-14C]choline chloride (54 mCi/mmol), [α-32P]deoxyctydine 5′-triphosphate (3000 Ci/mmol), and [α-32P]uridine 5′-triphosphate (800 Ci/mmol) were purchased from Du Pont New England Nuclear (Boston, MA). Glutathione Sepharose 4B (800 Ci/mmol) were 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.

Animal and tissue treatment Male Wistar rats were obtained from the Institute of Experimental Animal Research, Gunma University School of Medicine, and housed under standard conditions with a 12-h light/12-h dark cycle and free access to rat chow and water. Ten-week-old rats were administered DMH dissolved in 1 mM EDTA, pH 6.5, subcutaneously in the right thigh at a dose of 20 mg/kg body weight once a week for 16 weeks. Control rats were treated with a saline-EDTA solution. Then the rats were kept without any treatment for an additional 10 weeks. The rats were starved for 12 h before being killed. The large intestine was resected and washed with cold buffer (20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5 mM 2-mercaptoethanol and 0.2 mM phenylmethylsulfonyl fluoride) containing 100 mM NaCl. Tumor and normal tissues were harvested, frozen in liquid nitrogen, and then stored at −80°C.

Preparation of cytosol Tissues were homogenized on ice in three volumes of homogenizing buffer (250 mM sucrose, 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 50 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml pepstatin A, and 2 µg/ml leupeptin). The homogenates were centrifuged at 4°C for 10 min at 800g and then for 1 h at 100,000g. The supernatants were used as enzyme sources.

Choline kinase assay Choline kinase activity was measured isotypically with [methyl-14C]choline as described previously.

Determination of choline and phosphocholine Choline and phosphocholine were measured spectrophotometrically using a combination of alkaline phosphatase and choline oxidase as described previously.

Affinity purification of anti-choline kinase α antibody Anti-choline kinase α antibody was purified as described previously.

Western blot analysis The cytosolic proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene 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 the ECL reagent as reported previously.

Preparation of total RNA RNA was extracted by the acid guanidine isothiocyanate phenol method, and further purified by ultracentrifugation. The RNA thus purified was used as total RNA in the experiments.

Northern blot analysis Total RNA was fractionated by electrophoresis in 1.2% agarose containing formaldehyde, and transferred onto nylon membranes (BioDyne A; Pall Biosupport, Glen Cove, NY). Hybridization was carried out with rat choline kinase α1 cDNA labelled by a random priming method with [α-32P]deoxyctydine 5′-triphosphate. Choline kinase α mRNA was detected using a Bio-Imaging Analyser BAS2000 (Fuji Photo Film Co., Ltd., Minamiasahi, Japan).

Ribbonuclease protection assay Ribonuclease protection assays were carried out to examine spliced transcripts of choline kinase α and transcription start sites. Antisense RNA probes labelled with [α-32P]uridine 5′-triphosphate were prepared by subcloning the EcoRI/BssHII (positions −220 to +47) fragment into pBluescript II SK+ at the Smal site to determine the transcription start site, and by subcloning the EcoRI/BamHI (positions 237 to 429) fragment with cDNA to pBluescript II SK+ between the EcoRI and BamHI sites to analyze isoform expression. Protected fragments were separated in polyacrylamide gels and exposed to X-ray films and phosphoimaging plates.

Protein assays Protein concentrations were determined using BioRad protein assay dye reagent with bovine serum albumin as the reference standard.

Statistical analyses The results are expressed as the mean±standard error of the mean (SEM). The statistical significance of 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 and the UKCCCR ‘Guidelines for the Welfare of Animals in Experimental Neoplasia’ were helpful in this regard.

RESULTS

General observations The control rats appeared healthy and bore no cancers. On the other hand, all the DMH-treated rats had colon cancers without metastasis on both macroscopic and microscopic examination. In this report, cancerous tumors were compared with those of age-matched control rats.

Choline kinase activity increased by carcinogenesis The cancer tissues studied in this report were typically around 10 mm in diameter. The choline kinase activity was compared in control normal colon, non-tumor colon of cancer-bearing rats, and colon cancer tissues (Fig. 1). Cancer tissues, 0.97±0.072 nmol/min/mg (mean±SE; n=6), had almost twice the activity of the normal colon of
controls and non-tumorous colon of cancer-bearing rats, 0.46±0.048, and 0.57±0.059, respectively.

The addition of cytosol from cancerous tissue to normal cytosol only resulted in an additive increase in the choline kinase activity. Therefore, the increased choline kinase activity was not due to an increase in intracellular choline kinase activators.

Choline and phosphocholine levels The elevation of choline kinase activity in cancer tissues should lead to an increase in the phosphocholine level. Therefore, the concentrations of phosphocholine were determined along with those of choline. There were no differences in the choline and phosphocholine levels between the cancer tissues, control normal colon, and non-tumor colon of cancer-bearing rats.

Increase in the choline kinase α protein level In order to examine the mechanism of the increased choline kinase activity in cancer tissues, we conducted a western blot analysis. The result shows that the increased activity was in part due to an increase in the choline kinase α protein level (Fig. 2).

Increase in the choline kinase α mRNA level with carcinogenesis In order to determine whether the increase in the choline kinase α protein level is due to an increase in the choline kinase α mRNA level, we next carried out a northern blot analysis. Fig. 3 shows that there was a higher level of choline kinase α transcripts in cancer tissues. This increase in mRNA was further confirmed by means of a ribonuclease protection assay (Fig. 4).

A previous report<sup>27</sup> found that choline kinase α isoforms α1 and α2 were derived by alternative splicing in...
Fig. 5. Ribonuclease protection analysis for the transcription start sites of the choline kinase α gene. Total RNA (20 µg of each sample) was annealed with a 32P-RNA probe, digested with RNases T1/A, and then analyzed by polyacrylamide gel electrophoresis. The gel was exposed to an X-ray film and the protected fragments were quantitatively analyzed with a Phospho Imager. In cancer, the transcription start sites were increased in positions 100–140. Control: protected fragments from the normal colon of control rats. Cancer: protected fragments from colon cancer tissue.

The transcription from this site is controlled through some cis-acting element. Sequences similar to the xenobi-
otic responsive element (XRE)\(^5,43\) and the antioxidant responsive element (ARE) core sequences\(^46\) are located nearby. The XRE was identified in the cytochrome P-450 gene,\(^43,45\) which regulates phase I metabolizing enzymes,\(^6,7\) and the ARE is contained in NAD(P)H:quinone reductase and glutathione S-transferase \(Y\) subunit genes, whose transcription is activated by phenolic antioxidants and metabolizable planar aromatic compounds. Increased expression of the choline kinase \(\alpha\) gene is induced by 3-methylcholanthrene in rat liver.\(^26,27\) The increase of choline kinase in Hepa 1c1c7 cells is produced by an XRE-dependent inducer, \(\beta\)-naphthoflavone, but not by an XRE-dependent inducer, benzo[a]pyrene or tetrachlorodibenzo-p-dioxin,\(^45\) which suggests a role of the ARE. In addition, the ARE is also responsive to hydrogen peroxide.\(^46\) Bile acids can induce an increase in reactive oxygen species.\(^42\)

Of course, it is not known whether transcription from this site is similar or different in drug-treated liver and colon mucosa. This problem must be resolved to understand the function of choline kinase in cancer growth and the mechanism of the increased choline kinase activity. The increase in the choline kinase \(\alpha\) mRNA cannot be explained by the elevation of transcription alone. Other mechanisms such as transcription stoppage\(^21\) may also function. The roles of different isozymes, such as choline kinase \(\beta\), in carcinogenesis of colon mucosa also represent an interesting future problem.

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