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Expression of non-hepatic-type *S*-adenosylmethionine synthetase isozyme in rat hepatomas induced by 3′-methyl-4-dimethylaminoazobenzene

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It is known that a high incidence of hepatocellular carcinoma in rat liver can be induced by such azo dye carcinogens as 3′-methyl-4-dimethylaminoazobenzene (3′-MeDAB). Mammalian *S*-adenosylmethionine (AdoMet) synthetase exists as two isozymes, non-hepatic-type and liver-type enzymes, which are the products of two different genes. We have examined the expression of two AdoMet synthetase isozyme proteins and mRNAs in rat hepatomas induced by 3′-Me-DAB. The levels of non-hepatic-type enzyme protein and mRNA are clearly induced by 3′-Me-DAB feeding. On the other hand, the levels of liver-type enzyme protein and mRNA are nearly the same or slightly decreased during hepatocarcinogenesis. These results indicate that the expression of the non-hepatic-type isozyme gene is obviously influenced with the progression of carcinogenesis and that the non-hepatic-type isozyme is useful as a oncodevelopmental marker in the liver.

S-Adenosylmethionine synthetase; Isozyme; Carcinogenesis; Hepatocellular carcinoma

1. INTRODUCTION

S-Adenosylmethionine (AdoMet) synthetase (ATP:L-methionine *S*-adenosyltransferase (EC 2.5.1.6)), which Cantoni [1] first reported, is the enzyme responsible for the formation of AdoMet from methionine and ATP. AdoMet is an important methyl donor in most transmethylation reactions and is also the propylamino donor in the biosynthesis of polyamines.

In mammals, AdoMet synthetase exists as two isozymes, liver-type and non-hepatic-type enzymes, respectively. The liver-type isozyme is confined to the liver, whereas the non-hepatic-type isozyme is widely distributed in various tissues examined [2–6]. We have recently reported that the non-hepatic-type isozyme mRNA predominantly exists in fetal human liver and is replaced by the liver-type isozyme mRNA during development [7]. Therefore, it seems of particular interest to investigate whether the expression of non-hepatic-type isozyme during the hepatocarcinogenic process is a feature of cell proliferation and whether or not the non-hepatic-type enzyme is useful as a tumor marker for the detection of oncodevelopmental changes into hepatocellular carcinoma.

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In the present study, we have investigated the expression of the two AdoMet synthetase isozyme genes in the livers of rats treated with 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) as an experimental model for hepatocarcinogenesis. It is known that a high incidence of hepatocellular carcinoma in rat liver can be induced by such azo dye carcinogens as 3′-Me-DAB [8–12].

2. MATERIALS AND METHODS

2.1. *Materials*

[α-^{32}P]dCTP, Protein A-TSK gel, Hybond-C and Hybond-N were obtained from Amersham. The random-primed DNA labeling kit was from Takara (Kyoto, Japan). Alkaline phosphatase-conjugated goat anti-rabbit IgG was from Vector Laboratories. 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium chloride (NBT) were from Promega. Keyhole limpet hemocyanin was from Calbiochem. All other chemicals and reagents were of analytical grade and obtained from commercial suppliers.

2.2. *Animals*

Male Sprague Dawley rats 8–9 weeks of age were grown on a diet containing 0.06% 3′-Me-DAB and water freely, and were sacrificed on 0, 9, and 19 weeks.

2.3. *Northern blot analysis*

Total RNA was prepared by a single-step guanidium thiocyanate/phenol/chloroform extraction procedure described by Chomczynski et al. [13]. RNA (10 μg per lane) was denatured in glyoxal and dimethylsulfoxide and separated on a 1.0% agarose gel as described by Thomas [14], then transferred to nylon membranes. Prehybridization and hybridization of the membranes were performed as described [4].

*Eco*RI inserts of rat non-hepatic-type [4] and liver-type AdoMet synthetase cDNA [15] probes were labeled by the random-primed DNA labeling kit and [α-^{32}P]dCTP. RNA gels were stained with ethidium bromide and examined to ascertain that equivalent amounts of RNA were analyzed. Filters were washed twice for 15 min each in 2 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M Na-citrate, pH 7.0), 0.1% SDS at room temperature, then at 65°C for 2 × 15 min in 0.3 × SSC, 0.1% SDS. The final wash was for 1 h at 65°C in 0.1 × SSC, 0.1% SDS. Autoradiography was performed at −80°C for 1–4 days.

2.4. *Western blot analysis*

Antibody against rat liver-type AdoMet synthetase was prepared [16] and further purified by affinity chromatography as described in [17]. A hexadecapeptide with a sequence of NGQLNGFHEAFIEEGT (amino acid residues 2–17) near the NH_2 terminal segment of rat non-hepatic-type AdoMet synthetase [4] was selected as an immunogen based on its low homology with liver-type AdoMet synthetase [15]. The peptide was synthesized by using an Applied Biosystems model 430A peptide synthesizer and the product was confirmed by analytical HPLC and automated amino acid sequencing (Applied Biosystems model 470A gas phase sequencer) and coupled to keyhole limpet hemocyanin with glutaraldehyde [18]. The antibody was raised in rabbit and purified from the antiserum by the protein A-TSK gel.

Rats were killed by decapitation and their tissues were removed. Individual tissues were homogenized with a glass–Teflon homogenizer in 10 Vols. of 0.25 M sucrose containing 3.3 mM $MgCl_2$ and centrifuged at 3,000 × *g* for 10 min at 4°C to obtain postnuclear extracts or 105,000 × *g* for 60 min to prepare the cytosolic fractions. The samples were separated by SDS-PAGE according to Laemmli [19] with 10% acrylamide. Bovine serum albumin (67 kDa) and ovalbumin (43 kDa) were used as markers. Immunoblotting experiments were carried out as described in [20] except that the alkaline phosphatase-conjugated anti-rabbit IgG was used. The immunoreaction was visualized by staining with the BCIP/NBT detection kit. Protein concentration was determined by Bradford [21].

3. RESULTS AND DISCUSSION

It is difficult to accurately discriminate between the non-hepatic-type and liver-type AdoMet synthetases in cell extracts by determining the enzyme activities. Therefore, we have prepared the antibodies against each isozyme as described in section 2. To examine the specificity of each antibody against AdoMet synthetase isozymes, Western blot analysis was performed (Fig. 1). When the cytosolic extracts from various rat tissues were immunoblotted, the anti-rat liver-type AdoMet synthetase antibody specifically reacted with a 48-kDa band only in rat liver extract (Fig. 1A), but not in the extracts of rat brain, testis, and spleen. In contrast, the anti-rat non-hepatic-type AdoMet synthetase antibody also specifically detected the band of 48-kDa in rat brain, testis, and spleen extracts (Fig. 1B). Antibodies specific for each of the liver-type and non-hepatic-type enzymes reacted with the same molecular size, but distinct polypeptide species of 48 kDa, respectively. These results clearly show that these antibodies are highly specific.

Using these antibodies, we investigated the expression of the two AdoMet synthetase isozyme proteins in the liver extracts treated with 3′-Me-DAB as described in section 2 (Fig. 2). In the Western blot analysis shown in Fig. 2, the expression of the non-hepatic-type enzyme

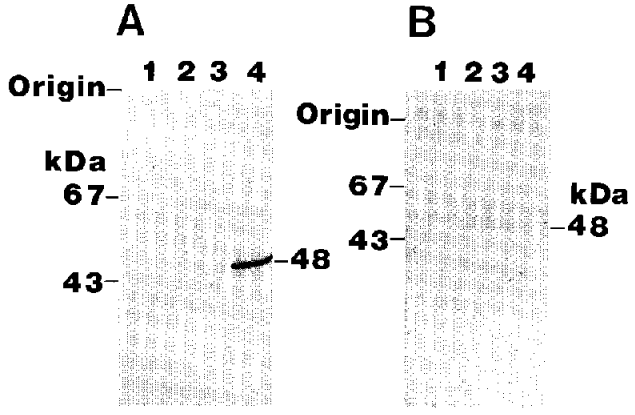

Fig. 1. Immunoblots of *S*-adenosylmethionine synthetase isozymes in various rat tissues. Cytosolic extracts of rat tissues (20 μg/lane) were subjected to SDS-PAGE followed by immunoblotting. (A) Anti-rat liver-type AdoMet synthetase antibody; (B) anti-rat non-hepatic-type AdoMet synthetase antibody; lane 1, spleen; lane 2, testis; lane 3, brain; lane 4, liver. Molecular mass markers used are bovine serum albumin (67 kDa) and ovalbumin (43 kDa).

protein (48 kDa) was clearly induced at 9 weeks after the onset of 3′-Me-DAB feeding and raised further at 19 weeks (Fig. 2B). On the other hand, the levels of liver-type isozyme protein were nearly the same during the period (Fig. 2A). These results have shown that the non-hepatic-type AdoMet synthetase protein is significantly induced in the liver by the 3′-Me-DAB feeding. At 19 weeks after the onset of 3′-Me-DAB feeding, a hepatocarcinoma was induced with a nearly 100% incidence.

To examine if the expression of the non-hepatic-type AdoMet synthetase gene was associated with the induction of its enzyme protein, we have performed a Northern blot analysis using both rat non-hepatic-type and

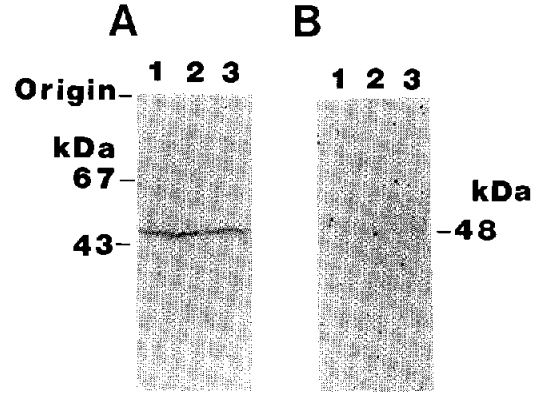

Fig. 2. Immunoblots of *S*-adenosylmethionine synthetase isozymes in liver extracts of rats treated by 3′-Me-DAB feeding. Rat liver postnuclear extracts (50 μg/lane) at various times after the onset of 3′-Me-DAB feeding were subjected to SDS-PAGE followed by immunoblotting. (A) Anti-rat liver-type AdoMet synthetase antibody; (B) anti-rat non-hepatic-type AdoMet synthetase; lane 1, normal control; lane 2, 9 weeks; lane 3, 19 weeks. Molecular mass markers used are bovine serum albumin (67 kDa) and ovalbumin (43 kDa).

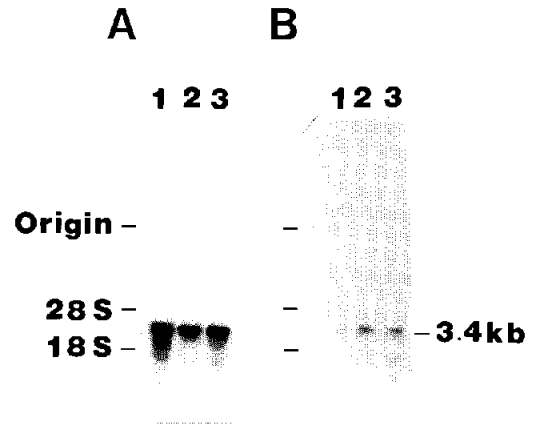

Fig. 3. RNA blots of *S*-adenosylmethionine synthetase isozymes in liver extracts of rats treated by 3′-Me-DAB feeding. Total RNAs (10 μg/lane) from normal control (lane 1), at 9 weeks (lane 2), and at 19 weeks (lane 3) after the onset of 3′-Me-DAB feeding were subjected to RNA blot analysis using the *Eco*RI inserts of cDNAs for rat liver-type AdoMet synthetase (A) and rat non-hepatic-type AdoMet synthetase (B). Molecular size markers used are 28 S and 18 S rRNAs.

liver-type AdoMet synthetase cDNAs as probes (Fig. 3). When hybridization is performed at high stringency, we can discriminate between the two isozyme mRNAs as described in [4]. The levels of the liver-type AdoMet synthetase mRNA (3.4 kb) are nearly the same during the progress of carcinogenesis (Fig. 3A). This result is consistent with Mato's group's findings that no significant differences in the liver-type AdoMet synthetase mRNA levels were observed when samples obtained from biopsies of patients with alcoholic cirrhosis and hepatocellular carcinoma were compared with the corresponding controls [22]. In their study, however, the levels of non-hepatic-type isozyme mRNA was not examined. Therefore, we have investigated the expression of non-hepatic-type isozyme mRNA. Contrary to the liver-type isozyme, the non-hepatic-type AdoMet synthetase cDNA probe detected a very faint band of 3.4-kb mRNA in the normal liver (0 week), and then its expression was induced strikingly at 9 and 19 weeks after administration of 3′-Me-DAB. The amount of RNA loaded was approximately equal as demonstrated by the ethidium bromide-stained 28 S and 18 S ribosomal RNA bands (data not shown). The expression of the non-hepatic-type AdoMet synthetase mRNA correlates quite well with the levels of this enzyme protein. The above observation has clearly shown that the expression of non-hepatic-type AdoMet synthetase gene is induced by 3′-Me-DAB treatment. In this respect, the relationship between the expression of non-hepatic-type AdoMet synthetase and the progression of hepatomas seems to be correlated.

Mato's group has reported that AdoMet synthetase activity is greatly reduced in human cirrhosis, however, the cirrhotic liver presents normal levels of liver-type AdoMet synthetase mRNA compared with the controls [22,23]. We have recently reported that the non-hepatic-type AdoMet synthetase protein and mRNA were predominantly expressed in the fetal liver [7,24]. These findings strongly suggest that the induction of non-hepatic-type AdoMet synthetase may be one of the cancerous signal in the liver. The physiological significance of this observation remains to be clarified. The mechanisms underlying the control of non-hepatic-type AdoMet synthetase may be important to understand the progression of hepatocarcinogenesis. We are at present continuing work to investigate whether or not any differences exist between the fetal and hepatoma tissues.

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