Sexual dimorphism in LEC rat liver: Suppression of carbonic anhydrase III by copper accumulation during hepatocarcinogenesis

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ABSTRACT

We examined age-related changes in the protein expression of carbonic anhydrase III (CAIII) in livers of Long-Evans with a cinnamon-like color (LEC) rats using an agouti color (LEA) rats as controls. The levels of the protein of CAIII in the liver of LEC male rats increased before 20 weeks of age, at the stage of acute hepatitis, and were decreased at 54 weeks of age, while those of CAIII in the liver of LEA male rats were highly expressed at all ages. In the normal LEA rats, CAIII showed sexual dimorphism. The level of CAIII in LEA male rat liver relative to female was four times higher. On the other hand, young LEC rat (at 4–12 weeks) showed a higher protein level of CAIII than LEA rats, and then decreased during development of hepatitis. CAIII mRNA also decreased in the LEC rat liver during hepatocarcinogenesis. The level of CAIII in the tumor region was lower than that in the tumor-free region. Immunohistochemical analysis showed that glutathione *S*-transferase P (GST-P) was positive and CAIII was negative in the precancerous region. The expression of CAIII was suppressed in cancerous lesions in hepatoma-bearing LEC rat liver compared to uninvolved surrounding tissues. These results indicated that suppression of CAIII accompanied hepatocarcinogenesis and it is a secondary consequence of the high copper levels in the liver.

Carbonic anhydrase (CA) [EC 4, 2, 1, 1] is a widely distributed enzyme in animals and plants. Numerous isoforms of CA have been identified and determined at both the protein level and the DNA level (3, 5, 14, 16). The expression patterns of CA are different in each tissue. CAI and CAII are predominant in erythrocytes (2, 8), while CAIII is expressed in the liver and muscle (12, 22, 24). Rat liver CAIII exhibits sexual dimorphism. Jeffery *et al.* (12) have shown that CAIII concentrations in males are correlated with the production of testosterone and pattern

of growth hormone release. No sexual dimorphism at 21 days was found, but from 50 to 400 days CAIII shows sexual difference. CAIII occurs in male rat liver at concentrations twenty times those in the female. Their group (11) has also shown that CAIII expression is highly sensitive to the endogenous growth hormone secretory pattern. Males release growth hormone episodically and have high concentrations of CAIII; however females produce growth hormone in a more continuous fashion and have lower CAIII levels.

CAIII is a major cytosolic protein accounting for approximately 8% of the protein in rat liver cytosol (28). The carbonic anhydrases catalyze the reversible reaction of carbon dioxide and water to form carbonic acid. In addition, CAIII was reported to play a role of an antioxidant that prevents H₂O₂-

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inducible apoptosis and a coplanar polychlorinated biphenyl, 3, 3', 4, 4', 5-pentachlorobiphenyl (PenCB) treatment on the rats results in reduced glutathione peroxidase-redox system and catalase. PenCB significantly suppresses the expression of rat liver CAIII (8). Oxidative stress is one of the toxicities caused by many polycyclic compounds.

LEC (Long-Evans with a cinnamon coat color) rats suffer from hereditary hepatitis and subsequent liver cancer at a high frequency (19, 20, 25, 37). Hepatitis suddenly appears approximately 12–16 weeks after birth and has a high lethality. The remaining animals survive more than one year and all develop hepatocellular carcinoma spontaneously, attributed to abnormal copper metabolism. This mutant strain of rat shows some clinical features that are similar to those of Wilson disease. Molecular studies have revealed that the copper transporting P-type ATPase (Atp7b) was defective in the LEC rat. These observations have confirmed that the LEC rat is a rodent model for Wilson disease. In addition, recent studies have suggested that the human ATP7B protein is involved in the intracellular transport of hepatic copper. The absence or diminution of Atp7B and ATP7B function results in abnormal copper metabolism in the LEC rat and in patients with Wilson disease (ATP7B), respectively. The gene responsible for Wilson disease, located on chromosome 13, was identified in 1993 by positional cloning and linkage disequilibrium analysis (23, 35) and by searching for genes with homology to ATP7A, the gene for the X-linked disorder of copper metabolism in Menkes disease.

There have been many reports about sexual dimorphism in LEC rats (7, 13, 27). We recently showed that the pattern of expressions of cytochrome P450 isoforms can be feminized during development of hepatitis and hepatoma (7). In the present study, we investigated sexual dimorphism of CAIII in LEC rat liver during hepatocarcinogenesis using normal LEA (Long-Evans with an agouti coat color) rat liver as a control, and we found that CAIII was suppressed by copper accumulation during hepatocarcinogenesis.

MATERIALS AND METHODS

Animals. The LEC rats were maintained under normal conditions at the Institute of Experimental Animal Sciences, Osaka University Graduate School of Medicine and Akita University Graduate School of Medicine. All research procedures complied with the ethical standards set in the Helsinki Declaration

of 1975. Detailed information on the origin, maintenance, and breeding history of the LEC rats has been previously described (25, 37). Long-Evans Agouti (LEA) rats, which are a sibling line of LEC rats and do not develop hepatitis and hepatocellular carcinoma, were used as a control. The LEC and LEA rats used in this study were at the 50th and 49th generations of brother-sister mating, respectively. We used male and female LEC rats from aged 4 weeks to 16 weeks that had not yet developed acute hepatitis, aged 8 months with chronic hepatitis, and after those ages with hepatocellular carcinoma.

Sample collection. Animals were anesthetized with diethyl ether and the liver was perfused with cold physiological saline (0.86% NaCl) and homogenized in five volumes of cold 0.25 M sucrose containing 5 mM Tris-HCl (pH 7.4) and 1 mM EDTA with a Potter-Elvehjem homogenizer. The homogenates were centrifuged at $9,000 \times g$ for 15 min, and the resulting postmitochondrial supernatant fraction was centrifuged at $105,000 \times g$ in a Hitachi RP-55T rotor for 1 h. The cytosolic fractions were stored at -80°C for up to one month until analysis. Since hyperplastic nodules formed the tumors with a white/reddish smooth surface, it can be excised visually. The hyperplastic nodules and the surrounding normal tissues were excised carefully under a stereoscopic microscope from the three livers of each male and female LEC rats at 52 weeks.

Western blotting. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted in 15% gel according to the method of Laemmli (15). Cells were lysed in cell lysis buffer [50 mM Tris-HCl (pH 7.4), 0.1% SDS, 1% NP40, 0.5% deoxycholic acid sodium salt, and 150 mM NaCl]. Fifty μg of protein per lane were then separated by SDS-PAGE and blotted onto PVDF membranes, which were subsequently saturated with 10% skimmed milk in Tris-buffered saline containing 0.5% Tween 20. The membrane was then incubated overnight at 4°C in a sealed plastic bag with 75 mL of buffer A (PBS, 0.05% Tween 20) containing the rabbit antibody to equine CA III purified from equine liver cytosol (22). The membranes were washed four times with buffer A for 10 min at room temperature and then incubated in a sealed bag with 75 mL of buffer A containing 1000-fold diluted peroxidase-conjugated goat anti-rabbit IgG (Dako Japan, Kyoto, Japan) for 3 h at room temperature. The membrane was again washed three times with buffer A for 10 min at room temperature and once with

PBS. The membrane was then incubated in 4 mM 4-chloro-1-naphthol and 0.01% H_2O_2 in PBS.

Immunohistochemical staining for CAIII and GST-P. Glutathione *S*-transferase P (GST-P) was observed in the hyperplastic foci and nodules induced by chemical carcinogens (23, 24). Hyperplastic nodules were observed in all of the 52-week old LEC rats. Hyperplastic nodules and the surrounding normal tissues were excised carefully under a stereoscopic microscope from the livers of the 52-week old rats. Liver tissues were fixed in 4% paraformaldehyde. Specimens were embedded in paraffin. Then 5 μ m sections were cut and deparaffinized. Liver sections were treated sequentially with skim milk, rabbit anti-GST-P (BIOPRE, Dublin, Ireland; 1000-fold dilution), biotin-labeled goat anti-rabbit IgG (Dako Japan, Kyoto, Japan; 1,000-fold dilution) and avidin-biotin-peroxidase (LSAB Kit; Dako Japan) was used to demonstrate GST-P positive liver cells. Sections are then counter-stained with hematoxylin for microscopic examination. The cytological images observed with a microscope (BH2; Olympus, Tokyo Japan) via a TV camera (Ikegami ITC-350M, Tokyo Japan).

Assay of testosterone concentration. Testosterone in LEC and LEA serum was assayed by RIA by Otsuka Assay Laboratories.

RESULTS

Expression of CAIII in LEC and LEA rats

CAIII levels varied significantly during development and aging of male rats as shown in Fig. 1 and Fig. 2. Expression of CAIII in LEA male rats increased rapidly from the age of 4 weeks to 24 weeks and after this period it gradually decreased. In LEA female rats, CAIII levels increased in a similar pattern to that of LEA males; however, the level was a third to a half of that of LEA males.

On the other hand, expression of CAIII in LEC male rats increased sharply from the age of 4 weeks and decreased with development of hepatitis. The expression of CAIII in LEC male rats during the immature period of 4 to 8 weeks was higher than that of LEA rats. LEC female rat liver expressed CAIII two times higher than LEA females, during the immature period, and then a sharp decrease was seen at the age of 12 weeks and it became constant.

Testosterone levels in LEC and LEA rat sera

Testosterone in 4-week-old LEC rats was un-detect-

A. LEC male

B. LEA male

Fig. 1 Expression of carbonic anhydrase III in the liver of male LEA and LEC rats at various ages. Each sample (40 μ g of cytosol fraction) was electrophoresed on 15% SDS-polyacrylamide gel and analyzed by Western blotting, as described in "MATERIALS AND METHODS."

Fig. 2 A age-related changes in the contents of carbonic anhydrase III of liver cytosols in LEC and LEA rats. Each samples (40 μ g of protein of cytosol fraction) was electrophoresed on 15% SDS-polyacrylamide gel and analyzed by Western blotting, as described in "MATERIALS AND METHODS." The amount of proteins measured the density of a band by the densitometer. The amount of proteins in male LEC rats at 4 weeks was set to 1, and the concentration of each protein was calculated as the relative value.

able but little is present in LEA rats of the same age (Fig. 3). The levels of testosterone in both LEC and LEA male rats increased during maturation, but there was no significant difference in testosterone level between LEC and LEA rats. From this result, it can be concluded that the level of testosterone may not be responsible for the expression of CAIII in LEC and LEA male rats.

Fig. 3 Developmental changes in serum testosterone concentrations of male LEC and LEA rats. Each point represents the mean values obtained from the rats ($n = 5$). Standard deviations are shown using bars. The n.d. means "not detectable".

CAIII levels in hepatic hyperplastic nodules of LEC rats

Fig. 4 shows that surrounding tissue had higher level of CAIII than hyperplastic nodules and CAIII was almost absent in female LEC rat nodules. The levels of CAIII in the surrounding tissues of male rats were four times higher relative to female rats. The levels of CAIII in hyperplastic nodules of female rats were almost absent but present in males (about a half of that in surrounding tissue). From these results it is concluded that hyperplastic nodules may suppress the expression of CAIII.

Immunohistochemical detection of CAIII

Hyperplastic nodules were larger in size than hepatic lobules and compressed the surrounding hepatocytes. These hyperplastic nodules became much larger and developed into hepatomas. Hepatomas were of a well-differentiated type with a trabecular structure of tumorous hepatocytes containing round nuclei with large nucleoli. Surrounding hepatocytes were apparently normal. The results of immunohistochemical staining for CAIII as well as GST-P in hyperplastic nodular liver of LEC rats are shown in Fig. 5. GST-P foci could be clearly distinguished from surrounding hepatocytes on the basis of GST-P staining. CAIII was predominantly present in the surrounding hepatocytes, with little or none being detected in the hyperplastic nodules.

DISCUSSION

Age-related CAIII expressions in LEA and LEC

In this study, we demonstrated that the expression pattern of CAIII in both LEA and LEC rat livers

Fig. 4 Carbonic anhydrase III levels in hepatic hyperplastic nodules of male or female LEC rats determined by Western blot analysis. Hyperplastic nodules and surrounding tissues were excised carefully from the three livers of each male and female LEC rats at 52 weeks. * $P < 0.05$, ** $P < 0.025$.

was dimorphic, and CAIII was much more suppressed in LEC rats than in LEA rats of the corresponding sexes. Age-related expressions are shown in Fig. 2. Before puberty, CAIII levels of both sexes were higher in the LEC rats than corresponding LEA rats, but a rapid decrease was seen with the onset of hepatitis around 12 weeks. After recovery of hepatitis, CAIII levels declined in senescent LEC males. Therefore, it is possible that accelerated aging occurred in the LEC rats.

Effect of testosterone on CAIII expression

CAIII content in the liver is known to be affected by the levels of testosterone or estradiol (1). In neonatal male rats, testosterone is the predominant sex hormone, but it then declines in the prepubertal stage (up to day 30). The levels of testosterone increase at puberty and peaks at about day 70, after which fertility declines. It is known that castration of male rats leads to a marked reduction in liver CAIII concentrations that can be partially restored to control levels by testosterone replacement (1). Kasai *et al.* (13) examined the effects of sex hormones on fulminant hepatitis by administering exogenous sex hormones to ovariectomized or orchidectomized LEC rats. Estradiol did not cure the hepatitis but sup-

Fig. 5 Immunostaining of serial sections of LEC rat livers at 52 weeks. Serial thin sections were immunostained for CAIII and GST-P by the biotin-streptavidin method. **A**, GST-P immunohistochemistry; hyperplastic nodule showing intense staining for GST-P. **B**, CAIII immunohistochemistry; surrounding normal hepatocytes showing low intensity staining of CAIII and the virtual absence of staining in hyperplastic nodules. $\times 250$.

pressed the deterioration and delayed the occurrence of fulminant hepatitis. In contrast, testosterone did not affect the onset of hepatitis but enabled the animal to survive the disease.

In the present study, the levels of testosterone in both LEC and LEA male rats increased during maturation, and there was no significant difference in testosterone level between LEC and LEA rats, as shown in Fig. 3. From these results, it can be concluded that the level of testosterone may be responsible for the expression of CAIII in LEA male rats. On the other hand, the expression patterns of CAIII in the LEC rats were different from those observed in the normal LEA rats.

The sexual dimorphism during hepatocarcinogenesis

Sexual dimorphism has been extensively studied in LEC rats (7, 13, 27). A sex difference in the growth of carcinoma has been observed in LEC rats, with the size of hepatocellular carcinomas being larger in males than in females (20), and preneoplastic foci appeared more frequently in livers of male LEC rats than those of females (34). On the other hand, cholangiofibrosis was more extensive in females than in males (20). The inducibilities of chromosomal aberrations and sister chromatid exchanges in bone marrow cells by cyclophosphamide were lower in females than in males of both strains, and there were strain differences between LEC rats and LEA rats of the same sex (the frequency was lower in LEC rats) (18).

We have investigated the expression of 14 forms

of cytochrome P450 in the liver as well as changes in the testosterone hydroxylation activities of hepatic microsomes during the development of hepatitis in LEC rats (7). During the immature period at 4 to 8 weeks, the levels of the sex-specific forms of cytochrome P450 such as SYP2C11 and SYP3A2 were higher in LEC rats than in LEA rats, but in mature rats the levels of several sex-specific forms of P450 decreased markedly with aging or during development of hepatitis. These results suggest that the sexual dimorphism observed in cytochrome P450 expression may be involved in the inducibilities of hepatoma.

We compared to CAIII from the livers of 4-, 16- and 52-week old LEC rats predisposed to hepatitis and hepatoma with that of LEA rats by two dimensional gel electrophoresis (21). In the present study, we confirmed that CAIII was dramatically greater in 4 week-old LEC rats than in 4 week-old LEA rats and that CAIII was lower in the LEC rats as compared with the LEA rats during aging, as shown in Figs. 1 and 2.

Expression of enzyme markers during the hepatocarcinogenesis

Currently, several enzyme markers, such as ATPase, glucose-6-phosphatase, γ -GTP, and aldehyde dehydrogenase, are frequently used to identify preneoplastic and neoplastic lesions (27). We have previously shown that the Phase I enzymes in drug metabolism of the liver from LEC rats are markedly decreased, whereas those of Phase II are selectively increased (30, 31). It is also reported that LEC rats are particularly susceptible to hepatocarcinogens (29, 33, 34).

It is well known that liver specific isozymes—glucokinase and pyruvate kinase—are decreased during hepatocarcinogenesis. We showed that hexokinase may be used as an enzyme marker of the early stages of liver carcinogenesis (9). Hexokinase activity showed two phases; an increase at 4 weeks and a decrease at 10 weeks followed by an increase peaking at 20 weeks. These two phase patterns were shown for Mn-superoxide dismutase (32), γ -glutamyl transpeptidase and cytochrome P450 isozymes (10). These enzyme profiles in hepatocarcinoma showed a general tendency toward approaching the undifferentiated patterns in LEC rats. Fig. 5 shows that preneoplastic nodules could be clearly distinguished from surrounding hepatocytes as GST-P-positive and CAIII-negative. Therefore, CAIII is available for one of the negative markers such as glucose-6-phosphatase and ATPase.

Effect of copper concentration on CAIII expression

The defective Atp7B protein in LEC rats is not able to incorporate cytosolic copper into the Golgi apparatus. This leads to the accumulation of copper in liver cytosol. Li *et al.* (17) reported that liver copper concentrations of 3- and 8-month-old LEC rats were over 50 and 40 times higher, respectively than those of age- and sex-matched LEA rats. Furthermore, Grimes *et al.* (4) demonstrated the decreased CAIII levels in the liver of the mouse mutant 'toxic milk' (tx) due to copper accumulation. In this study, expression of CAIII in both LEC male and female rats sharply decreased from the age of 18 weeks with development of hepatitis caused by copper accumulation as shown in Fig. 2.

In conclusion, it is most likely that the decrease in CAIII in the LEC mutant is a secondary consequence of copper accumulation, which developed into hereditary hepatitis and hepatoma with aging. Marked changes in liver CAIII content were also observed in developing and aging male rats. The expression patterns of CAIII in the LEC rats were different from those observed in the normal LEA rats. It will be of interest to examine further the molecular basis of the effect of copper on CAIII and to determine whether CAIII play any role in copper transport.

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