Inhibitory Effect of Acyclic Retinoid (Polyprenoic Acid) on the Secretion of α -Fetoprotein in CCl₄-Treated Rats

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Summary A study was conducted to examine the inhibitory effect of acyclic retinoid (polyprenoic acid) on the secretion of α -fetoprotein (AFP) in rats with chronic liver damage induced by CCl₄. Oral administration of the compound brought about a significant reduction of serum AFP levels at the time when liver cirrhosis was formed. Acyclic retinoid also decreased the activities of serum aminotransferases and ornithine carbamyl transferase, while it increased serum albumin levels, demonstrating the reduction of hepatic parenchymal damage. Significant negative correlation was observed between serum AFP and albumin levels. This cytoprotective effect of the retinoid on the parenchymal cell may well be related to the inhibition of the synthesis and/or secretion of AFP. No significant side effect was observed, despite a long-term administration of the compound. The present finding will provide a potential scope for the future use of acyclic retinoid for the treatment of chronic liver damage.

Key Words acyclic retinoid, α -fetoprotein, cytoprotection, carbon tetrachloride, cancer chemoprevention, transcriptional switch

Retinoids influence a variety of biological processes. One of the fundamental properties of retinoids is the regulation of the proliferation and differentiation of

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Abbreviations: AFP, α -fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; OCT, ornithine carbamyl transferase.

normal epithelial tissues. In addition, it has recently been established that the compounds also affect the differentiation and proliferation of preneoplastic as well as neoplastic cells (1): studies employing neoplastic cells have particularly provided important information regarding the mechanism(s) of retinoids in the control of cell differentiation on a molecular basis. These studies include reports of the preventive effect of acyclic retinoid (polyprenoic acid), a new synthetic compound, on hepatic fibrosis (2) and hepatocarcinogenesis (3, 4).

α -Fetoprotein (AFP) is one of the hepatocyte-synthesized macromolecules secreted into plasma. Since its emergence is contemporaneous with liver cell proliferation, AFP has been established as one of the most sensitive indicators for determining the hepatocyte regeneration and identifying the liver neoplasms (5). After partial hepatectomy or chemical injury, the protein appears at the time of liver regeneration, and then disappears with maturation and healing, suggesting that AFP production coincides with a 'step-down' state distinguished by the sacrifice of 'luxury' functions (6). Although the mechanism of AFP production in various liver injuries and its pathophysiological significance are not fully understood yet, much information is available on AFP with advances in recombinant DNA technology. One of the important studies is that the developmental transcriptional switch from AFP to albumin occurs just prior to birth, which is a specific marker of differentiated hepatocyte function (7-10). On the other hand, marked elevation of serum AFP levels was induced in rats by administration of various hepatotoxins including CCl_4 , thioacetamide, D-galactosamine, and others (6, 11), and the transcriptional switch from albumin to AFP has been reported to occur during liver regeneration induced by CCl_4 (12).

Here, we report the effects of acyclic retinoid on the secretion of AFP and albumin in CCl_4 -treated rats. Our results also demonstrate the cytoprotective effect of the retinoid on the liver parenchymal cells.

MATERIALS AND METHODS

Experimental animals. Male Wistar rats (Nihon Charles River, Atsugi, Japan), weighing 100 to 120 g at the initiation of the experiments, were used. The rats were maintained on the standard rat chow pellets (Oriental Yeast, Tokyo, Japan) and distilled water *ad libitum* throughout the experiment. The animals were divided into four groups: C-1 to C-4.

In group C-1, 20 rats received subcutaneous injections of CCl_4 (Wako Pure Chemical, Osaka, Japan) mixed with an equal volume of liquid paraffin, at the dose of 0.5 ml/kg of body weight twice a week. Rats also received oral administration of peanut oil (vitamin A-free), as a placebo, *via* a stomach tube. Five rats each were sacrificed at the end of the 8th and 12th week, respectively, and the remaining 10 rats at the end of the 16th week.

In groups C-2 to C-4, 15 rats in each group received the same dosage of CCl_4 and also received oral administration of acyclic retinoid (3,7,11,15-tetramethyl

2,4,6,10,14-hexadecapentaenoic acid (13), Eisai, Tokyo, Japan) in peanut oil through a stomach tube, from the 9th week at the dose of 20 mg/kg in C-2, 40 in C-3, or 80 in C-4, five times a week. Five rats in each group were sacrificed at the end of the 12th week, and 10 rats at the end of the 16th week. Six control rats were maintained without any treatment and sacrificed at the end of the 16th week.

At the time of sacrifice, animals were anesthetized by intravenous injection of sodium pentobarbital, and blood was drawn from abdominal aorta. Sera were kept at -80°C until needed.

Determination of serum AFP. Serum AFP was measured by an enzyme-linked immunosorbent assay (ELISA). The assay was based on a two-step sandwich method using specific sheep anti-rat AFP IgG and HRP labeled Fab'.

IgG was purified from specific sheep antisera against rat AFP (Nordic Immunological Laboratories, Tilburg, The Netherlands) by a sequence of affinity chromatography on Protein G (Pharmacia Fine Chemicals, Uppsala, Sweden), ion-exchange chromatography on DEAE-cellulose (QA 52) (Whatman, Kent, UK), and gel filtration on Sephacryl S-200 (Pharmacia). Specific anti-rat AFP IgG was obtained from the purified IgG by affinity chromatography on rat AFP-coupled Sepharose (CNBr-Sepharose was from Pharmacia) and by passing through a column of normal rat sera-coupled Sepharose. HRP labeled Fab' was prepared from the purified specific IgG by the method of Hashida *et al.* (14), using *N*-(ϵ -maleimido-caproyloxy)-succinamide (Wako Pure Chemicals, Osaka, Japan).

Rat AFP was purified from rat amniotic fluids and from weaning rat liver homogenates, for the standard substance in ELISA and for preparation of affinity chromatography. Purification steps were; i) ion-exchange chromatography on DEAE-Sepharose (Pharmacia); ii) affinity chromatography on purified anti-human AFP IgG coupled Sepharose (anti-human AFP IgG was from Cosmo Bio, Tokyo, Japan); and iii) gel filtration on Sephacryl S-200. Step ii) was based on the partial cross-reactivity between rat and human AFP (15). Purity was approximately 90%, assessed by SDS-polyacrylamide gel electrophoresis.

The assay was performed using 96-well microplates by successive procedures as follows: i) 100 μl of 50 mM sodium bicarbonate buffer, pH 9.0, containing 1 μg of affinity purified sheep anti-rat AFP IgG for 18 h at 4°C ; ii) 100 μl of standard substance or diluted samples (1 : 20 for serum) for 2 h at room temperature; iii) 100 μl of 1 $\mu\text{g}/\text{ml}$ HRP-Fab' anti-rat AFP in phosphate-buffered saline, containing 0.05% Tween 20, 1% bovine serum albumin, and 0.1% thimerosal (PBS-Tween) for 2 h at room temperature; iv) 100 μl of 0.04% ortho-phenylenediamine, 0.003% H_2O_2 in 50 mM phosphate-citrate buffer, pH 5.0, for 1 h at room temperature. Between each step, wells were washed with PBS-Tween. After the last step, reaction was stopped by adding 100 μl of 0.5 M H_2SO_4 and absorbance at 492 nm was measured.

Other procedures. Activities of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and ornithine carbamyl transferase (OCT), and serum levels of albumin and total bilirubin (T.Bili.) were determined with a Hitachi

736-40 automatic analyzer.

Statistical analysis. Comparison of group means was performed by Dunnett's *t*-test. Correlation coefficients (*r*) among serum AFP, AST (or ALT), and albumin were examined using the Spearman correlation analysis.

RESULTS

Determination of AFP (Fig. 1)

Figure 1 shows the standard curve of the ELISA, and also shows the curves obtained with serial dilutions of CCl₄-treated rat sera and rat amniotic fluid, exhibiting the identity with the standard curve. The most sensitive portion of the standard curve corresponded to the AFP levels of 1–50 ng/ml of the protein. Thus, the ELISA appears to be specific and sensitive to rat AFP.

Growth curves

No significant growth retardation was observed among C-1 and retinoid-treated groups (body weight: 339.7 ± 20.9 g in C-1 at the 16th week ($M \pm SD$); 334.1 ± 16.1 in C-2; 347.9 ± 21.7 in C-3; and 344.7 ± 24.4 in C-4; liver weight: 4.60 ± 0.22 g/100 g of body weight in C-1; 4.38 ± 0.19 in C-2; 4.57 ± 0.35 in C-3; and 4.56 ± 0.31 in C-4) and also no significant difference was observed in total food

Fig. 1. Standard curve of the ELISA for rat AFP. Identical curve was obtained with serial dilutions of CCl₄-treated rat sera and rat amniotic fluids.

intake during the whole experimental period among the experimental groups (22.2 g/day/rat in C-1; 20.8 in C-2; 20.8 in C-3; and 20.5 in C-4).

Liver function tests (Fig. 2)

Activities of serum AST and ALT in group C-1 gradually elevated until the 12th week and decreased at the 16th week. Both activities were significantly lower in C-3 and C-4 at the 12th week than those in C-1. Thereafter, these activities remained at almost the same levels in retinoid-treated groups. Activities of serum OCT represented similar time courses to those of AST or ALT.

Serum albumin levels gradually decreased throughout the experimental period in C-1. Significantly higher level was observed in C-4 than that in C-1 at the 16th week.

Serum T. Bili. levels in group C-1 gradually increased until the 12th week and declined at the 16th week. Significantly lower level was observed in C-4 at the 16th week as compared with that in C-1.

Serum levels of AFP (Fig. 3)

In group C-1, serum levels of AFP (sAFP) gradually increased until the 12th

Fig. 2. Comparison of liver function tests in each group at the given time point. Each column and bar represent mean and standard error, respectively. Rats in group C-1 through C-4 received subcutaneous injections of CCl_4 mixed with an equal volume of liquid paraffin at the dose of 0.5 ml/kg of body weight twice a week. In parallel, C-2, C-3, and C-4 were orally administered the acyclic retinoid at doses of 20, 40, and 80 mg/kg, respectively, five times a week, while C-1 received the vehicle (peanut oil) alone. C represents the control rats without administrations of CCl_4 and the acyclic retinoid. $n=6$ in C at 16th week. $n=5$ in C-1 at 8th week and, in C-1 through C-4 at 12th week. $n=10$ in C-1 through C-4 at 16th week. * $p<0.05$, ** $p<0.01$.

Fig. 3. Serum levels of AFP. Each column and bar represent mean and standard error, respectively. Rats in groups C-1 through C-4 received subcutaneous injections of CCl_4 mixed with an equal volume of liquid paraffin at the dose of 0.5 ml/kg of body weight twice a week. In parallel, C-2, C-3, and C-4 were orally administered the acyclic retinoid at doses of 20, 40, and 80 mg/kg, respectively, five times a week, while C-1 received the vehicle (peanut oil) alone. C represents the control rats without administrations of CCl_4 and the acyclic retinoid. $n=6$ in C at 16th week. $n=5$ in C-1 at 8th week and, in C-1 through C-4 at 12th week. $n=10$ in C-1 through C-4 at 16th week. * $p<0.05$, ** $p<0.01$.

Fig. 4. Correlations between serum AFP levels and serum ALT activities (a), and between serum AFP and albumin levels (b).

week as compared with control rats, and remained at the same level at the 16th week. In retinoid-treated groups, sAFP levels were significantly lower at the 16th week.

A significant positive correlation was observed between serum AFP levels and serum AST (or ALT) activities (Fig 4a), and a significant negative correlation was observed between serum AFP and albumin levels (Fig. 4b).

DISCUSSION

In the present study, serum AFP levels moderately increased following a long-term administration of CCl₄ in C-1 group, while the levels were markedly decreased in retinoid-treated groups at the time when liver cirrhosis was formed. We have previously reported that the retinoid inhibited hepatic parenchymal damage and also hepatic fibrosis in CCl₄-treated rats (2). The cytoprotective effect of the retinoid on the hepatocytes may well contribute to the reduction of AFP synthesis. Significant positive relationship between serum AFP levels and serum AST activities supports this possibility.

As to cell types involved in the production of AFP during liver damage and regeneration induced by CCl₄, AFP mRNA has been reported to appear in nonparenchymal cells but seemingly not in hepatocytes (16). However, nonparenchymal epithelial cell population, which also proliferate at the early stages of carcinogenesis (commonly known as 'oval cells'), are likely to be heterogeneous and contain cells with diverse developmental potentials (17,18). It has been suggested that some of these cells may be the source of new hepatocytes, because nonparenchymal cells proliferate extensively under the conditions where hepatocyte replication is inhibited (19,20). Moreover, a study that examined the expression of albumin and AFP genes employing *in situ* hybridization in preneoplastic liver lesions induced by the Solt-Farber method (21) reported that AFP was expressed in oval cells in the early stage following the initiation, and thereafter the expression became weak in the cells at later time points and neoplastic nodules were always negative for AFP, whereas albumin-positive cells appeared inside the neoplastic nodules (22). This observation also suggests the presence of primitive 'stem-cell'-like oval cells in the liver. Since the neoplastic transformation of the liver was inhibited by the acyclic retinoid used in this study (3), it is likely that the retinoid influenced the proliferation and/or maturation of such stem-cells which produce AFP.

On the other hand, it may also be possible that the retinoid directly influenced the genomic expression (23). Several studies have reported that the single administration of a large dose of CCl₄ induced marked elevation of serum AFP levels (6,11) and mRNA levels of AFP in the liver (12), whereas albumin transcription rate was observed to be decreased. Albumin gene transcription was reported to be sharply increased thereafter and AFP transcription returned to normal within a few days. Since this transcriptional change preceded the increase of DNA synthesis in

the liver, the change appeared to be associated with the liver regenerative response (12). Significant negative relationship between serum AFP and albumin levels observed in the present study suggests that the retinoid inhibits the transcriptional switching from albumin to AFP in the damaged liver. Although it has not been demonstrated yet that the nuclear receptors for retinoic acid (retinoic acid receptor, RAR) (24) bind to the gene regions coding AFP or albumin, the genomic expression of RAR β , one of the RAR family, in hepatocytes seems to be closely related to the hepatocarcinogenesis (25). Since we have previously reported the inhibitory effect of the retinoid in the promotion phase of hepatocarcinogenesis in experimental models (3, 4), the anti-promoter action of the compound may well be related to the inhibition of the initial elevation of AFP which represents one phase of a specific sequence of gene alteration associated with carcinogenic evolution (22). In fact, recent study in our laboratory demonstrated that the retinoid inhibited the secretion of AFP while it enhanced that of albumin by human hepatoma-delivered cell line (PLC/PRF/5) (26), supporting this observation.

We are now engaged in a clinical study aimed at chemoprevention of hepatocarcinogenesis in cirrhotic patients. The present data provides the important information that the acyclic retinoid has a cytoprotective effect on the hepatocyte and inhibits the synthesis and/or secretion of AFP, as well as that the retinoids present no significant side effects, despite a long-term administration, in the chronic liver-damaged model. These findings suggest that the retinoid is also a potential candidate for the treatment of chronic liver damage.

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