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# Effect of retinoic acid on liver transglutaminase activity and carbon tetrachloride-induced liver damage in mice

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*Abstract.* Transglutaminase (TGase) activity in the cytosol fraction of the mouse liver increased following intraperitoneal injection of retinoic acid. Retinoic acid inhibited the carbon tetrachloride-induced increase in serum alanine transaminase activity. These findings suggest that TGase is involved in the effect of retinoic acid on carbon tetrachloride-induced liver damage.
*Key words.* Retinoic acid; transglutaminase; liver damage; carbon tetrachloride; serum alanine transaminase; $Ca^{2+}$ uptake.

Transglutaminase (TGase, EC 2.3.2.13) is a calcium-dependent enzyme that catalyzes the formation of covalent linkages between the gamma-carboxamide group of glutamine residues in some polypeptides and the amino groups of either peptide-bound lysine or primary amines[1-3]. Mammalian liver tissue is very rich in TGase[4,5], but its physiological role is almost unknown. Transglutaminase has been shown to cross-link hepatocellular cytokeratins in rat liver[6]. Cross-linking of cytokeratins is expected to increase the stability of the cytoskeletal network and influence the fluidity of the plasma membrane. Retinoic acid, a differentiation promoter[7], has been found to increase TGase activity in rat liver[8]. Recently, we reported that the degree of decrease in the TGase activity of the cytosol fraction induced by carbon tetrachloride treatment was closely related to an increase in serum alanine aminotransferase (ALAT) activity[9]. These findings suggest a relationship between TGase activity and resistance to hepatotoxins. In the present study we investigated whether an enhancement of TGase activity induced by retinoic acid treatment attenuates carbon tetrachloride-induced liver damage in mice.

## *Materials and methods*

*Animals.* Male *ddY* mice weighing 18–25 g (SLC, Hamamatsu, Japan) were kept under the following conditions: a 12-h light-dark cycle (light: 09.00 h–21.00 h), temperature 23 ± 1 °C and humidity 55 ± 5%, and free access to food (F2, Funabashi Farms, Funabashi, Japan) and tap water.

*Chemicals.* [1,4-$^{14}$C]putrescine dihydrochloride ($^{14}$C-PUT, 4.4 GBq/mmol) and $^{45}CaCl_2$ (1.26 GBq/mg) were from New England Nuclear, USA. All-trans retinoic acid and N,N-dimethylcasein were from Sigma, USA. Putrescine dihydrochloride was from Nacalai Tesque, Japan. All other reagents were of analytical grade.

*Retinoic acid treatment and tissue preparation for measurement of TGase.* In the experiment with normal mice, each mouse received 200 μmol/kg of retinoic acid suspended in olive oil intraperitoneally. The liver was removed after perfusion with cold physiological saline at the indicated times under sodium pentobarbital anesthesia (50 mg/kg, i.p.). Mice of the control group were injected with olive oil. In the experiments involving carbon tetrachloride damage to the liver, mice were injected s.c. with olive oil (control liver) or carbon tetrachloride (0.25 g/kg) 8 h after i.p. injection of olive oil or retinoic acid (200 μmol/kg). After 16 h, the liver was removed after perfusion with cold physiological saline under sodium pentobarbital anesthesia (50 mg/kg, i.p.). The liver was homogenized with 9 volumes of ice-cold buffer (0.25 M sucrose, 3 mM Tris, 1 mM EDTA, pH 7.4) by 10 strokes of a loose-fitting Dounce homogenizer. The homogenate was filtered through nylon mesh (No. 100) and subcellular fractionation was performed by the method described previously[9].

*Measurement of TGase activity.* Transglutaminase activity was measured by the method described previously[9], with the minor modification that the final pH in the assay mixture was 8.0. The enzyme activity was ex-

pressed as nmol PUT incorporated into dimethylcasein · $min^{-1}$ · mg $protein^{-1}$.

*Measurement of ALAT activity.* Blood was drawn from the inferior vena cava under sodium pentobarbital anesthesia and serum ALAT activity was measured by the method described previously[9].

*Liver uptake of $^{45}Ca$.* Mice were injected s.c. with olive oil or carbon tetrachloride (0.25 g/kg) 8 h after i.p. injection of olive oil or retinoic acid (200 µmol/kg). 16 h later the mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and injected i.v. with 0.1 ml of $^{45}CaCl_2$ (74 kBq) dissolved in pyrogen-free physiological saline containing 1 mM $CaCl_2$. The liver was perfused with cold physiological saline 10 min later, and immediately removed, weighed and minced. The liver tissue was then solubilized with tissue solubilizer (NCS, Amersham) for 2 h at 50 °C. Liquid scintillator (Cleasol I, Nacalai Tesque, Japan) was added and radioactivity counted in a liquid scintillation counter (LSC 7800, Beckman). Data were expressed as an uptake ratio, obtained from the equation: (sample radioactivity (dpm)/ sample weight (g)/ (injected radioactivity (dpm)/ body weight (g)).

*Measurement of cytochrome P-450 and $b_5$ levels.* Mice were injected i.p. with olive oil (control group) or 200 µmol/kg retinoic acid. 24 h later, the liver was perfused with physiological saline and homogenized with 4 volumes of ice-cold 1.15% KCl by 10 strokes of a loose-fitting Dounce homogenizer. The homogenate was filtered through nylon mesh (No. 100) and centrifuged at 9000 × g for 20 min at 4 °C. The supernatant fractions were centrifuged at 105,000 × g for 60 min at 4 °C. The precipitate was washed once with the same volume of the homogenization buffer and recentrifuged at 105,000 × g for 60 min at 4 °C. The precipitate (microsomal fraction) was suspended in a Potter-Elvehjem homogenizer with 80 volumes of 0.1 M phosphate buffer containing 20% glycerol. The concentrations of cytochromes P-450 and $b_5$ were determined by the method of Omura and Sato[10], using extinction coefficients of 91 and 171 $cm^{-1}$ · $mM^{-1}$, respectively.

*Protein assay.* The protein content was determined according to the method of Bradford[11] with bovine serum albumin as a standard.

*Statistics.* Statistical analysis was performed by Mann-Whitney's U-test (two-tailed).

## Results

*Effect of retinoic acid on TGase activity in the normal mouse liver.* Changes in TGase activity in each fraction of the mouse liver after retinoic acid treatment (200 µmol/kg, i.p.) are given in figure 1. Transglutaminase activity in the cytosol fraction increased significantly (170% of the normal liver) at 12 h after retinoic acid treatment, and this higher activity was maintained for a further 12 h. This increase in TGase activity due to retinoic acid was dose-dependent (data not shown). Retinol (200 µmol/kg) did not significantly elevate the TGase activity (data not shown).

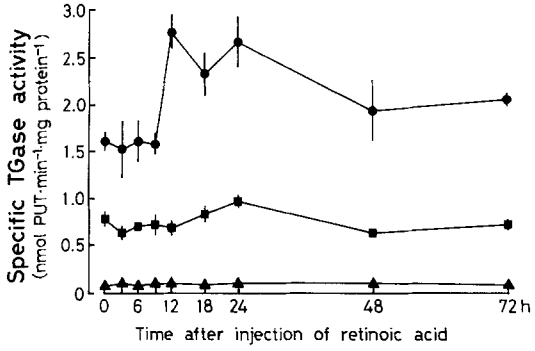


Figure 1. Changes in specific TGase activity in the cytosol (●), cytoplasmic particulate (▲) and nuclear (■) fractions of mouse liver after retinoic acid treatment. The values at 0 h indicate the activity of the normal liver. Each point is the mean ± SE of 4–6 experiments.

*Effect of retinoic acid on serum ALAT activity, liver TGase activity, and liver uptake of $^{45}Ca$ in carbon tetrachloride-treated mice.* Serum ALAT activity began to increase at 12 h after carbon tetrachloride treatment (0.25 g/kg, s.c.), reached the maximum value (4058 Karmen units) at 16 h and subsequently decreased (data not shown). This increase in serum ALAT activity induced by carbon tetrachloride was significantly attenuated (56% of the retinoic acid (−) group) by retinoic acid treatment (200 µmol/kg) (fig. 2A). Retinoic acid did not affect the serum ALAT activity in the control group. Retinol (200 µmol/kg) did not affect the increase in serum ALAT activity in carbon tetrachloride-treated mice (data not shown).

There was a slight decrease in the TGase activity in the cytosol fraction from the carbon tetrachloride-damaged liver. The TGase activity in the carbon tetrachloride-damaged liver was significantly increased (163% of the retinoic acid (−) group) by retinoic acid treatment. (fig. 2B). Retinol (200 µmol/kg) did not increase cytosolic TGase activity in carbon tetrachloride-damaged liver (data not shown).

$^{45}Ca$ uptake into liver tissue significantly increased (700% of the control liver) after carbon tetrachloride treatment, and was significantly inhibited (45% decrease) by retinoic acid treatment (200 µmol/kg, i.p.) (fig. 2C). Retinoic acid did not exert any effect on the uptake of $^{45}Ca$ in the control liver.

*Effect of retinoic acid on cytochrome P-450 and cytochrome $b_5$ levels.* There were no significant changes in cytochrome P-450 and cytochrome $b_5$ levels after retinoic acid treatment (fig. 2D). Retinol did not significantly change these levels either (data not shown).

## Discussion

In vivo treatment with retinoic acid increased TGase activity only in the cytosol fraction of the mouse liver. This increase in TGase activity is almost certainly due to

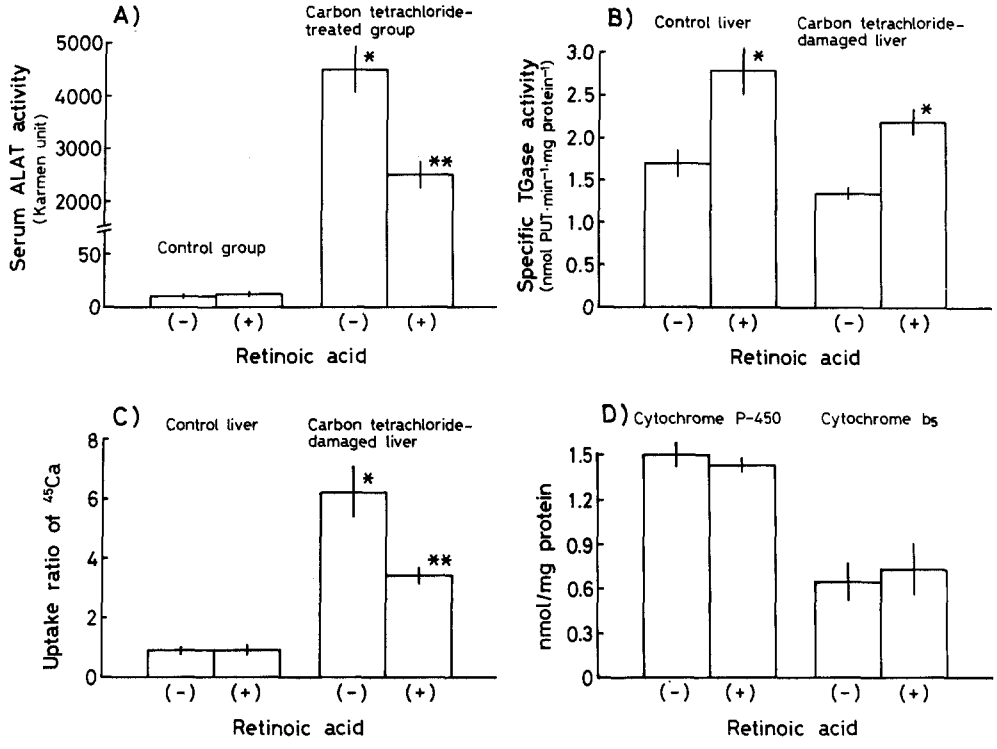


Figure 2. *A* Effect of retinoic acid on serum ALAT activity of the control and carbon tetrachloride-treated groups. *B* Effect of retinoic acid on the specific TGase activity of the cytosol fraction in control and carbon tetrachloride-damaged liver. *C* Effect of retinoic acid on $^{45}Ca$ uptake into control and carbon tetrachloride-damaged liver. *D* Effect of retinoic acid on cytochrome P-450 and $b_5$ levels of control liver. Each column is the mean ± SE of 5–6 experiments. In *A* and *C*, * significantly different from the retinoic acid (−) group of the control group, $p < 0.01$ and ** significantly different from the retinoic acid (−) group, $p < 0.01$, in *B*, * significantly different from the retinoic acid (−) group, $p < 0.01$, Mann-Whitney's U-test (two-tailed).

an increase in the amount of the enzyme, since retinoic acid has been shown to augment the expression of TGase-mRNA levels[12], and Piacentini et al. also observed an increase in the amount of TGase protein in retinoic acid-treated rat liver[8].

It has been reported that a critical step in cellular damage is an increase in the $Ca^{2+}$ influx into cells[13]. Retinoic acid significantly diminished the carbon tetrachloride-induced increase in $^{45}Ca$ uptake. The attenuation of carbon tetrachloride-induced liver damage by retinoic acid treatment may be mainly due to an inhibitory effect on $Ca^{2+}$ influx.

It is recognized that the hepatotoxicity of carbon tetrachloride is due to lipid peroxidation, which is brought about by a highly reactive free radical intermediate resulting from the metabolism of carbon tetrachloride by cytochrome P-450 and associated mixed-function oxidases. Therefore, we investigated the effect of retinoic acid on the levels of cytochromes P-450 and $b_5$. Retinoic acid had no effect on the levels of these cytochromes in mouse liver. These results suggest that the anti-hepatotoxic effect of retinoic acid is not due to an inhibitory effect on the metabolism of carbon tetrachloride.

A reciprocal relationship between the decrease in serum ALAT activity and the increase in TGase activity in the cytosol fraction of the liver was observed following retinoic acid treatment in carbon tetrachloride-treated mice. In a previous report, we also observed a close relationship between the elevation of serum ALAT levels and the reduction of TGase activity in the cytosol fraction of the liver in the carbon tetrachloride-treated rat[9]. These findings suggest that a TGase-mediated increase in membrane stability[14] is involved in retinoic acid-induced attenuation of liver damage, although the detailed mechanism of the antihepatotoxic effect of retinoic acid remains unclear.

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