Uptake of Gallium-67 by the Hepatocytes during Liver Regeneration in Carbon Tetrachloride-Treated Mice

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Gallium-67 (^{67}Ga) has been used as a tumor or inflammation-imaging agent in nuclear medicine. We recently reported that the peak of serum alanine aminotransferase (ALT) level was 1 d after carbon tetrachloride (CCl_4) treatment in rats. However, the peak of ^{67}Ga uptake by the liver tissue and hepatocytes was 2 d after the treatment. If ^{67}Ga is taken in the hepatic disorder phase, the pattern of ^{67}Ga uptake by the hepatocytes should be consistent with that of the ALT level. In order to answer why it was not, we carried out a detailed examination. The lipid peroxidation of hepatocytes from CCl_4 -treated mice was greatly increased 1 d after CCl_4 treatment; serum ALT was also increased 1 d after the treatment. The uptake of ^{67}Ga by the liver tissue reached a maximum 1 and 2 d after the treatment, while maximum by the hepatocytes was achieved after 2 d. The incorporation of bromodeoxyuridine into the hepatocytes also reached maximum 2 d after the treatment. These results suggest that the uptake of ^{67}Ga by the hepatocytes is carried out during liver regeneration rather than during hepatic disorder by CCl_4 treatment.

Key words Gallium-67 (^{67}Ga); carbon tetrachloride (CCl_4); liver tissue; hepatocyte; lipid peroxidation; bromodeoxyuridine (BrdU)

Gallium-67 (^{67}Ga) has been used for the detection of various tumors^{1,2)} and acute and chronic inflammations,^{3,4)} since the first observation of its accumulation in tumors⁵⁾ and inflammatory lesions.^{6,7)} Moreover, in chronic hepatitis C, it was reported that there was more accumulation in the liver in patients with chronic persistent hepatitis than in those with cirrhosis.⁸⁾ Although many hypotheses concerning the mechanism of ^{67}Ga uptake by tumors and inflammatory lesions have been proposed, consensus has not been reached.

We recently reported that serum ALT level of carbon tetrachloride (CCl_4) treated rats reached a maximum within 1 d, whereas maximum uptake of ^{67}Ga by the liver tissue and hepatocytes was reached in 2 d.⁹⁾ Most hepatotoxic effects of CCl_4 are attributed to its metabolism by cytochrome P450, especially by P450 2E1, to yield toxic trichloromethyl radical (CCl_3^*).¹⁰⁾ This radical can react with oxygen to form the trichloromethylperoxy radical (CCl_3OO^*), a highly reactive species.¹¹⁾ The radical initiates the chain reaction of lipid peroxidation.^{12,13)} This affects the permeabilities of mitochondrial, endoplasmic reticulum, and plasma membranes, resulting in the loss of cellular calcium sequestration and homeostasis; this, in turn, can contribute heavily to subsequent cell damage.^{14–16)}

If ^{67}Ga is taken in the hepatic disorder phase, the pattern of uptake by the hepatocytes should be consistent with that of lipid peroxidation. A detailed examination was carried out to learn why this was apparently not so.

MATERIALS AND METHODS

Animals Male *ddY* mice weighing 24–34 g (SLC, Hamamatsu, Japan) were kept under conditions of a 12 h light–dark cycle, $23 \pm 1^\circ\text{C}$ and $55 \pm 5\%$ humidity, and had free access to food and water. Animals were cared following the standard procedures indicated in the “Guide for the Care and Use of Laboratory Animals” published by the Tohoku

Pharmaceutical University.

Administration of CCl_4 Mice were given a 10% suspension of CCl_4 in olive oil (10 ml/kg) orally, after being fasted for 16 h.

Determination of Serum Alanine Aminotransferase (ALT) Activity Serum alanine aminotransferase (L-alanine-oxoglutarate aminotransferase, EC 2.6.1.2) (ALT) activity was determined by the method of Reitman and Frankel,¹⁷⁾ and expressed as Karmen units (KU) per ml of serum.

Administration of ^{67}Ga Gallium-67 citrate (Daichi Radioisotope Laboratory, Ltd., Tokyo, Japan) was diluted with saline to 74 kBq/ml. Each mouse was injected subcutaneously with ^{67}Ga at a dose of 7.4 kBq (100 μl).

Removal of Liver The mice were administered ^{67}Ga under urethane anesthetization (1.5 g/kg, i.p.). After 4 h they were immediately perfused with 0.9% NaCl solution. The whole liver was then removed. The amount of radioactive ^{67}Ga was determined with a well-type NaI-scintillation counter (ARC-300, Aloka, Japan). The uptake ratio of ^{67}Ga in the liver was expressed using the following formula:

Uptake ratio = A/B

A = sample activity (cpm)/sample weight (g)

B = total activity administered (cpm)/rat body weight (g).

Preparation of Hepatocytes Hepatocytes were isolated from mice as described previously¹⁸⁾; briefly, hepatocytes were isolated by a method in which the liver was perfused with collagenase (Nitta Gelatin, Osaka, Japan). The hepatocytes were counted with a well-type NaI-scintillation counter and the results expressed as cpm/ 1×10^6 cells.

Determination of Hepatic Lipid Peroxidation The extent of lipid peroxidation was estimated in hepatocyte homogenates by determining malondialdehyde (MDA) formation using the thiobarbituric acid method.¹⁹⁾ Hepatocyte samples were homogenized with 1.15% KCl. A fraction from the homogenate was combined with thiobarbituric reagent [15%

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(w/v) trichloroacetic acid, 0.375% (w/v) thiobarbituric acid dissolved in 0.25 M of hydrochloric acid], the mixture was heated for 15 min in a boiling water bath. After cooling, the precipitate was removed by centrifugation at $2000\times g$ for 10 min. Following decanting of the supernatant, the absorbance was determined at 535 nm against a blank that contained all the reagents minus sample. The MDA concentration from the samples was calculated using an extinction coefficient of $1.56\times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$. The results were expressed as the concentration of MDA/mg protein.

Flow Cytometric Analysis of Cell Cycle Cell cycle was analyzed using a BrdU Flow Kit (Becton Dickinson, San Diego, CA, U.S.A.). BrdU was dissolved in phosphate buffered saline (PBS) and was given intravenously (10 mg/kg) 2 h before killing. Incorporation of BrdU into hepatocytes was detected by FACScan (Becton Dickinson).

RESULTS

Changes in the Lipid Peroxidation of Hepatocytes from CCl_4 -Treated Mice To ascertain the degree of disorder in the hepatocytes, we determined the lipid peroxidation by thiobarbituric acid assay (Fig. 1). The peroxidation increased after CCl_4 treatment and showed the highest value (7.2 times higher than that of normal mice) 1 d after the treatment.

Changes in Serum ALT Level after CCl_4 Treatment Serum ALT level also increased after CCl_4 treatment and reached a maximum in 1 d (Fig. 2). Two days after the treatment the value rapidly decreased.

Uptake of ^{67}Ga by Liver and Hepatocytes after CCl_4 Treatment The uptake of ^{67}Ga by the liver tissue increased 1 and 2 d after CCl_4 treatment (Fig. 3), while the uptake of ^{67}Ga by the hepatocytes showed the highest value (8.5 times higher than that of normal mice) 2 d after treatment (Fig. 4).

Changes in the Incorporation of Bromodeoxyuridine into Hepatocytes after CCl_4 Treatment To confirm DNA synthesis of hepatocytes during liver regeneration after CCl_4 -induced hepatic disorder, we determined their incorporation of BrdU (Fig. 5). Incorporation reached a maximum 2 d after CCl_4 treatment.

DISCUSSION

We recently reported that serum ALT level of CCl_4 -treated rat reached a maximum 1 d after the treatment, whereas the uptake of ^{67}Ga by the liver tissue and hepatocytes was highest 2 d after treatment.⁹⁾

In the present study, we confirmed the precise index of disordered hepatocytes by the degree of lipid peroxidation (Fig. 1). The lipid peroxidation of hepatocytes from CCl_4 -treated mice was greatly increased within 1 d of the treatment. Furthermore, serum ALT (a conventional indicator of hepatic disorder) was also increased within 1 d (Fig. 2). This result was consistent with that of CCl_4 -treated rats,⁹⁾ and indicated that hepatocyte destruction occurred within 1 d after CCl_4 treatment.

The uptake of ^{67}Ga by the liver tissue increased 1 and 2 d after the treatment (Fig. 3). However, uptake by the hepatocytes reached a maximum after 2 d (Fig. 4). Moreover, little ^{67}Ga was present in the nonparenchymal cells (data not shown). Therefore, we theorized that a large amount of ^{67}Ga

Fig. 1. Time Course of the Thiobarbituric Acid-Reactive Substances (TBARS) Production in Whole Homogenates of Hepatocytes after the Treatment of Carbon Tetrachloride (CCl_4)

Each point represents the mean $\pm$ S.E. of 4–5 mice. The data were analyzed by Student's *t*-test. * $p < 0.01$ compared with normal mice.

Fig. 2. Time Course of Serum Alanine Aminotransferase (L-Alanine-Oxoglutarate Aminotransferase, EC 2.6.1.2) Levels after the Treatment of Carbon Tetrachloride (CCl_4)

Each point represents the mean $\pm$ S.E. of 4–5 mice. The data were analyzed by Student's *t*-test. * $p < 0.01$ compared with normal mice.

Fig. 3. Time Course of the Uptake Ratio of ^{67}Ga by the Liver Tissue after the Treatment of Carbon Tetrachloride (CCl_4)

Each point represents the mean $\pm$ S.E. of 4–5 mice. The data were analyzed by Student's *t*-test. * $p < 0.01$ compared with normal mice.

Fig. 4. Time Course of the Uptake of ⁶⁷Ga by the Hepatocytes after the Treatment of Carbon Tetrachloride (CCl₄)

Each point represents the mean ± S.E. of 4–5 mice. The data were analyzed by Student's *t*-test. **p* < 0.01 compared with normal mice.

Fig. 5. Time Course of the Incorporation of Bromodeoxyuridine into Hepatocytes after the Treatment of Carbon Tetrachloride (CCl₄)

Each point represents the mean ± S.E. of 4–5 mice. The data were analyzed by Student's *t*-test. **p* < 0.01 and ***p* < 0.05 compared with normal mice.

taken by liver tissue may already exist extracellular matrix. This speculation is supported by the report that ⁶⁷Ga bound to heparan sulfate²⁰ and that CCl₄ treatment caused an increase of this substance in rat.^{21–23}

The pattern of ⁶⁷Ga uptake by the hepatocytes was not consistent with the peak of serum ALT and lipid peroxidation, suggesting that the uptake was not carried out during hepatic disorder. Therefore, we suspected that ⁶⁷Ga uptake by hepatocytes might correlate with hepatocyte proliferation during liver regeneration after hepatic disorder caused by

CCl₄ treatment.

To ascertain the relationship between ⁶⁷Ga uptake and cell proliferation, we confirmed DNA synthesis of the regenerating hepatocytes after the CCl₄ treatment. The incorporation of BrdU into the hepatocytes reached a maximum 2 d after the treatment (Fig. 5). This pattern of hepatic DNA synthesis was consistent with that of ⁶⁷Ga uptake by the hepatocytes.

In conclusion, our data demonstrated that the uptake of ⁶⁷Ga by the hepatocyte is carried out during hepatocytes proliferation rather than hepatic disorder following CCl₄ treatment.

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