Transferrin Is Not Involved in the Entry of ^{67}Ga into Hepatocytes from Regenerating Liver of Partially Hepatectomized Rats

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Ever since the first observation of ^{67}Ga accumulation in tumors and inflammatory lesions, ^{67}Ga has been used to detect various tumors and inflammations. The aims of this study were to clarify whether or not transferrin is involved in the uptake of ^{67}Ga by the regenerating liver after partial hepatectomy. The uptake of ^{67}Ga by the liver of rats reached a maximum 2 d after partial hepatectomy. In order to inhibit the binding of ^{67}Ga to transferrin in the blood, FeCl_3 was administered 5 min before the injection of ^{67}Ga . The administration of FeCl_3 decreased the uptake of ^{67}Ga by the liver of the partially hepatectomized rats, suggesting that transferrin is involved in the uptake by the liver. However, ^{67}Ga was taken up only slightly by hepatocytes obtained from the liver of these rats. We conclude that transferrin is involved in the uptake of ^{67}Ga by the liver tissue of partially hepatectomized rats but is not involved in its entry into the hepatocytes. Only a slight amount of gallium-67 enters the hepatocytes, and may accumulate primarily in the extracellular matrix of the liver tissue of these rats.

Key words gallium-67; ferric chloride; partial hepatectomy; hepatocyte; extracellular matrix

More than 30 years ago, Edwards and Hayes¹⁾ first described accumulation of ^{67}Ga in the lymph nodes of a patient with Hodgkin's disease. Subsequently, ^{67}Ga has been shown to localize in a variety of tumors.^{2,3)} In nuclear medicine, the utility of ^{67}Ga as a tumor-imaging agent is variable. Many types of tumors accumulate gallium poorly, while others, such as hepatomas and lymphomas, tend to be gallium avid but can be inconsistent in magnitude and uniformity of uptake. Nonetheless, no other gamma-emitting radiopharmaceutical, including monoclonal antibodies and receptor-avid agents, has surpassed ^{67}Ga in cost-effectiveness, availability and use for general tumor imaging.

Despite years of experience imaging this radiometal, the precise mechanism by which ^{67}Ga accumulates in normal tissues and in cancers remains controversial. It is well supported that gallium, as an iron analogue, binds to transferrin (Tf) and is concentrated by tumor cells enriched in the Tf receptor (TfR).^{4–6)} However, there is also evidence that Tf-independent mechanisms may be important determinants of ^{67}Ga uptake as well.^{7–9)} Sohn *et al.* suggest that the uptake of gallium by normal soft tissues is a Tf-dependent process and that ^{67}Ga uptake by tumors can be mediated by a completely different mechanism, one that is Tf independent.¹⁰⁾ The involvement of Tf in the uptake of ^{67}Ga by inflammatory tissue has not been conclusively demonstrated. Ohkubo *et al.* reported that Tf is not involved in the uptake of ^{67}Ga by rat granuloma tissue, that is, inflammatory tissue produced by turpentine oil,¹¹⁾ but is involved in the liver of normal mice.¹²⁾

Hammersley *et al.* reported the uptake of ^{67}Ga in regenerating rat liver after partial hepatectomy,¹³⁾ but the involvement of Tf in the uptake was not shown. In the present study, we investigated whether or not Tf is involved in ^{67}Ga uptake by the liver in partially hepatectomized rats.

MATERIALS AND METHODS

Animals Male Wistar rats weighing 180–250 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 tap water. This study was conducted in accordance with the standards established by the Guidelines for Animal Experimentation of Tohoku Pharmaceutical University.

Partial Hepatectomy A two-thirds partial hepatectomy was performed according to the technique described by Higgins and Anderson (1931).¹⁴⁾

Administration of ^{67}Ga Gallium-67 citrate (kindly supplied by Daiichi Radioisotope Laboratory, Ltd., Tokyo, Japan) was diluted with saline to 185 kBq/ml. Each rat was intravenously injected with ^{67}Ga at a dose of 37 kBq (200 μl).

Administration of FeCl_3 Each rat was intravenously injected with FeCl_3 (25 $\mu\text{mol}/\text{ml}$ saline) at a dose of 100 μl 5 min before the administration of ^{67}Ga .

Removal of Liver The rats were anesthetized with urethane (1.5 g/kg, i.p.) 4 h after the administration of ^{67}Ga and blood was obtained from the inferior vena cava and then immediately perfused with cold saline. The whole liver and spleen were then removed.

Preparation of Rat Hepatocytes Rat hepatocytes were prepared essentially according to Katoh *et al.*¹⁵⁾ as follows. Rat liver was perfused *in situ* at 37°C via the portal vein with 300 ml of calcium-free Hanks' balanced salt solution containing 5 mM EGTA and 10 mM *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (Hepes, pH 7.4). The perfusion was continued with 200 ml of Hanks' balanced salt solution containing 5 mM CaCl_2 and 0.05% (w/v) collagenase. After the perfusion, the digested liver was suspended in 20 ml of Ca^{2+} -free Hanks' buffer and filtered through coarse gauze. The filtrate was centrifuged for 3 min at $50 \times g$ at 4°C and the resulting pellet used to determine the radioactivity with a well-type NaI-scintillation counter (ARC-300, Aloka, Japan).

Determination of Radioactivity The amount of radioactive ^{67}Ga was determined with this scintillation counter. The uptake ratios of ^{67}Ga in the blood, urine, liver, hepatocyte were expressed using the following formula:

$$\text{Uptake ratio} = A/B$$

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

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

Extraction and Crude Acid Glycosaminoglycans The liver tissues obtained from partially hepatectomized rats were mixed and minced in acetone using a homogenizer and then dried under a vacuum to constant weight in a desiccator. Acid glycosaminoglycans (AGAGs) were extracted according to the method described by Ohkubo *et al.*¹⁶⁾ with a slight modification. The liver tissue was extracted with 0.5 mol/l NaOH at 4 °C for 4 h. The extracted solution was dialyzed against running tapwater overnight followed by distilled water for an additional 24 h, and the lyophilized materials were then digested with pronase-E (Kaken Kagaku Co., Tokyo, Japan)/phosphate buffer, pH 7.4, at 37 °C for 24 h followed by an additional 24 h at 37 °C. Trichloroacetic acid (TCA) was added to the solution to give a final concentration of 10% at 4 °C. The precipitated materials were removed by centrifugation. The supernatant solution was dialyzed and the lyophilized materials dissolved in a 1% cetylpyridinium chloride (CPC)/0.04 M NaCl solution. The precipitate was collected by centrifugation and washed twice with a 0.1% CPC/0.03 M NaCl solution. The crude AGAG fraction was extracted with a 2.1 M NaCl solution from the CPC precipitated fraction.

Electrophoresis The electrophoretic identification of AGAGs was carried out by the method described by Seno *et al.*¹⁷⁾ Electrophoresis was run at a constant current of 1.0 mA/cm in 0.3 M calcium acetate buffer, pH 7.25, for 3 h. At the end of each run, cellulose acetate strips (Joko Sangyo Co., Tokyo, Japan) were stained in 0.5% *o*-toluidine blue/1% acetic acid, and rinsed with 1% acetic acid.

RESULTS

Time Course of the Changes in Liver Weight after Partial Hepatectomy After 70% partial hepatectomy, the remaining lobes of the liver rapidly increased in weight and doubled during the first 2 to 4 d (Fig. 1). After this initial acute phase of growth, the liver continued to increase in size

Fig. 1. Time Course of Liver Weight after Partial Hepatectomy (PH)

Each point represents the mean and SE of 5–7 rats.

more slowly.

Time Course of the Uptake of ^{67}Ga by Blood and Urine and the Effect of FeCl_3 on Uptake after Partial Hepatectomy The uptake of ^{67}Ga by the blood and urine changed slightly (Figs. 2, 3). In order to inhibit the binding of ^{67}Ga to transferrin in the blood, FeCl_3 was administered 5 min before the injection of ^{67}Ga . The administration of FeCl_3 decreased the uptake of ^{67}Ga by the blood (Fig. 2) and increased urinary uptake (Fig. 3).

Time Course of the Uptake of ^{67}Ga by the Liver and the Effect of FeCl_3 on Uptake after Partial Hepatectomy The uptake of ^{67}Ga by the liver of partially hepatectomized rats reached a maximum 2 d after partial hepatectomy (Fig. 4), but the uptake was greatly decreased by the administration of FeCl_3 . The administration of FeCl_3 greatly decreased this uptake.

Fig. 2. Time Course of the Uptake of ^{67}Ga by the Blood and the Effect of FeCl_3 on Uptake after PH

Each rat was intravenously preinjected with saline or FeCl_3 5 min before administration of ^{67}Ga . Each point represents the mean and SE of 5–7 rats.

Fig. 3. Time Course of the Urinary Uptake of ^{67}Ga and the Effect of FeCl_3 on Uptake after PH

Each rat was intravenously preinjected with saline or FeCl_3 5 min before administration of ^{67}Ga . Each point represents the mean and SE of 5–7 rats.

Fig. 4. Time Course of the Uptake of ⁶⁷Ga by the Liver and the Effect of FeCl₃ on Uptake after PH

Each rat was intravenously preinjected with saline or FeCl₃ 5 min before administration of ⁶⁷Ga. Each point represents the mean and SE of 5–7 rats.

Fig. 5. Time Course of the Uptake of ⁶⁷Ga by Hepatocytes Isolated from Liver of Partially Hepatectomized Rat and the Effect of FeCl₃

Each point represents the mean and SE of 5 rats.

Time Course of the Uptake of ⁶⁷Ga by Hepatocytes Isolated from the Liver of Partially Hepatectomized Rats and the Effect of FeCl₃ The uptake of ⁶⁷Ga by hepatocytes isolated from partially hepatectomized rat liver was studied to ascertain whether or not this uptake was carried out by transferrin receptor-mediated endocytosis (Fig. 5). Compared with the uptake of ⁶⁷Ga by the liver tissue, only a slight amount was taken up by hepatocytes isolated from partially hepatectomized rat liver. The administration of FeCl₃ greatly decreased the uptake of ⁶⁷Ga by hepatocytes isolated from partially hepatectomized rat liver.

Identification and Composition of Crude AGAGs Obtained from the Liver of Partially Hepatectomized Rats Since ⁶⁷Ga was only slightly taken up by hepatocytes isolated from partially hepatectomized rats, it was possible that ⁶⁷Ga accumulated in the extracellular matrix of the liver tissue. Therefore, we analyzed crude AGAGs obtained from the

Fig. 6. Electrophoretogram of Crude AGAG Fraction Obtained from the Liver of Partially Hepatectomized Rats on a Cellulose Acetate Strip in 0.3 M Calcium Acetate (pH 7.25) at 1.0 mA/cm for 3 h

HS; heparan sulfate, HA; hyaluronic acid.

liver of these animals. The electrophoretic pattern of crude AGAGs is shown in Fig. 6. The crude AGAG fraction was extracted with a 2.1 M NaCl solution from the CPC precipitated fraction. This AGAG fraction contained at least two types of AGAG, *i.e.*, heparan sulfate (HS) and hyaluronic acid (HA). HS and HA increased gradually, reached a maximum 2 d after partial hepatectomy, and then decreased.

DISCUSSION

It is well known that Tf is a carrier glycoprotein for iron in the blood. A major pathway for cellular iron uptake is through the internalization of the complex of iron-bound Tf and the Tf receptor.¹⁸⁾ Many researchers have expressed confidence that ⁶⁷Ga exclusively binds to Tf and is transported to various tissues by Tf in the blood. Hayes *et al.* reported that the uptake of ⁶⁷Ga by normal tissues such as the liver occurred to a major extent by endocytosis in which Tf was involved, and that the initial entry of ⁶⁷Ga into inflammatory lesions might occur in the same way as to that of normal soft tissues.¹⁹⁾ On the other hand, Ohkubo *et al.* reported that the uptake of ⁶⁷Ga by inflammatory tissues such as granuloma occurs in Tf-unbound form, while the uptake by normal liver occurs in Tf-bound form.¹²⁾ Vallabhajosula *et al.* reported that the uptake of ⁶⁷Ga by tumors was reduced by Tf, as compared with ⁶⁷Ga-citrate alone, *i.e.*, the uptake of ⁶⁷Ga by tumors occurred in an unbound form.²⁰⁾ Kumagai *et al.* reported that Tf receptors increased in hepatocytes during rat liver regeneration.²¹⁾ There is, however, no report concerning the involvement of Tf in the ⁶⁷Ga uptake by regenerating liver after partial hepatectomy. Hara reported that the binding affinity of iron for Tf was stronger than that of gallium,²²⁾ *i.e.*, Fe³⁺ can inhibit the binding of ⁶⁷Ga to Tf. The results of the present study indicate that the retention of ⁶⁷Ga in the blood is greatly reduced by the administration of FeCl₃ 5 min before the injection of ⁶⁷Ga, and that the latter is extensively excreted in the urine because FeCl₃ inhibits its binding to Tf in the blood after partial hepatectomy. Additionally, FeCl₃ reduced the uptake of ⁶⁷Ga by the liver of partially hepatectomized rats. These results suggest that an unbound form of ⁶⁷Ga is only slightly taken up by the liver after partial hepatectomy, and that Tf is involved in the uptake of this substance by partially hepatectomized rats. We expected that the entry

of ^{67}Ga into the hepatocytes from the liver of partially hepatectomized rats might occur to a major extent by Tf receptor-mediated endocytosis. To confirm this, we isolated hepatocytes from the liver of the rats after the administration of ^{67}Ga . Contrary to our expectation, ^{67}Ga was only slightly taken up by the hepatocytes isolated from partially hepatectomized rats. We speculate that ^{67}Ga binds to Tf-receptors on the hepatocyte surface in a Tf-bound form but that the Tf- ^{67}Ga complex is not internalized into the hepatocytes by endocytosis, i.e., Tf is involved in the uptake of ^{67}Ga by liver tissue but not in the entry of ^{67}Ga into the hepatocytes. A major pathway for cellular iron uptake is through internalization of the Tf-iron complex and Tf-receptors.¹⁸⁾ The Tf- ^{67}Ga complex also binds to Tf-receptors on the hepatocyte surface, but the complex may not be internalized into hepatocytes. There are two differences between iron and gallium: (i) iron is essential for DNA synthesis²³⁾ while gallium is not, and (ii) the binding affinity of gallium for Tf is weaker than that of iron.²²⁾ We believe that the Tf- ^{67}Ga complex easily dissociates on the hepatocyte surface and the Tf-unbound form (free ^{67}Ga) is only slightly taken up by the hepatocytes. Kojima and Kubodera reported that HS, an AGAG which is present in the extracellular matrix, plays an important role in the mechanism of ^{67}Ga binding to tumor cells.²⁴⁾ The results from the present study suggest that the extracellular matrix may also be involved in the uptake of ^{67}Ga by the liver of partially hepatectomized rats. Thus, we extracted AGAGs from the liver of these rats and identified them by electrophoretic identification. The results showed that the period during which the uptake of ^{67}Ga by the liver was elevated after partial hepatectomy was in good accord with that of increasing HS content. These results suggest that ^{67}Ga in Tf-bound form is dissociated on the surface of hepatocytes of partially hepatectomized rat liver, and immediately after the dissociation free ^{67}Ga binds to HS in the extracellular matrix. Vallabhajosula *et al.* reported that acidic pH at the tumor site might be one of the factors involved in ^{67}Ga localization in tumors.^{25,26)} Moreover, a reduction in pH to 6.8–7.0, below that of normal tissue (pH 7.4), might be due to the accumulation of lactic acid produced by anaerobic glycolysis.²⁷⁾ In the partially hepatectomized rats, the Tf- ^{67}Ga complex may be most likely dissociated on the surface of hepatocytes under conditions of low pH in a similar manner as above, or another exchange mechanism of the Tf- ^{67}Ga complex for the free ^{67}Ga may be involved on the hepatocyte surface.

Based on our data, we conclude that Tf is involved in the

uptake of ^{67}Ga by the liver tissue of partially hepatectomized rats but is not involved in its entry into the hepatocytes.

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