Fate of Recombinant Human Renin Administered Exogenously to Anesthetized Monkeys

MASAHITO HIRUMA, SHOKEI KIM, FUMIHIKO IKEMOTO, KAZUO MURAKAMI, AND KENJIRO YAMAMOTO

SUMMARY Highly purified recombinant human renin (rh-renin), synthesized by Chinese hamster ovary cells, was labeled with iodine-125 and was given intravenously to pentobarbital-anesthetized common marmosets (Callithrix jacchus) to study the fate of the circulating renin. Specific anti-rh-renin antisera was used to identify the $^{125}$I-rh-renin. Plasma disappearance of the exogenously administered $^{125}$I-rh-renin in marmosets ($n = 6$) showed two exponential components, with a half-life of $12.1 \pm 1.9$ minutes for the rapid component and $120.3 \pm 16.4$ minutes for the slow component. The metabolic clearance rate was $1.17 \pm 0.26$ ml/min/kg. Thirty minutes after the injection of $^{125}$I-rh-renin, $43.1 \pm 0.9$ and $3.5 \pm 0.5$% of the injected dose had distributed to the liver and the kidneys, respectively. With time, the accumulated $^{125}$I-rh-renin in the liver and kidneys decreased. The accumulation of $^{125}$I-rh-renin was less than 1% of the dose injected in other organs such as lungs, heart, spleen, adrenal glands, testes, and ovaries. Analysis of liver and kidney extracts by high performance liquid chromatography at 30 and 120 minutes indicated that immunoreactive $^{125}$I-rh-renin decreased with time and was accompanied by an increase in nonimmunoreactive degradation products of a low molecular weight. The incubation of $^{125}$I-rh-renin with monkey or human plasma at 37 °C did not degrade the labeled renin. Therefore, rh-renin was rapidly cleared from the circulation by the liver and the kidney.

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KEY WORDS • recombinant human renin • renin clearance • monkey studies • liver • kidney

THE plasma level of renin, a key enzyme of the renin-angiotensin system, is regulated by the rate of release from the kidney and by plasma clearance. We studied the plasma clearance of exogenously administered mouse submaxillary renin in mice and rat renal renin in rats, both labeled with iodine-125 and found that mouse submaxillary renin is cleared mainly by the kidney, while rat renal renin is cleared by the liver as well as by the kidney. We suggested the possible contribution of glycosylation of renin to the hepatic distribution. However, there is a significant difference in the characteristics of the renin-angiotensin system between human renin and laboratory animal renins, including dog, hog, rat, and mouse renin. Specific antibodies against human renin only weakly cross-react with renin from these animal species. Human renin can catabolize angiotensinogen from other species, while laboratory animal renin cannot catabolize human angiotensinogen. Renin inhibitors specific for human renin are not potent against renin from various other species. Thus, the clearance of human renin should be studied using primates.

In the present study, highly purified active recombinant human renin (rh-renin), synthesized by Chinese hamster ovary cells transfected with a plasmid containing human renin complementary DNA (cDNA), was labeled with Iodine-125 and given intravenously to monkeys to study the fate of this renin.

Materials and Methods

Animals
Female cynomolgus monkeys (Macaca fascicularis; CLEA Japan, Tokyo, Japan), weighing 2.5 to 3.0 kg, were used to study the pressor activity of rh-renin. Common marmosets (Callithrix jacchus; CLEA Japan) of both sexes, weighing 250 to 350 g, were used to study the clearance of rh-renin given exogenously. The animals were kept in separate cages and at a constant temperature (23 °C). All
were fed a standard monkey diet (CMK-1; CLEA Japan) fruit, and tap water, ad libitum.

Preparation of Recombinant Human Renin

rh-Renin, expressed in Chinese hamster ovary cells transfected with a plasmid containing the human renin cDNA, and activated with trypsin for 2 hours at 24 °C, was purified to apparent homogeneity, as described by Poorman et al. and Murakami et al. Rh-Renin is similar to native human renal renin with regard to physiochemical characteristics.

Pressor Activity of Recombinant Human Renin

Experiments were performed on pentobarbital-anesthetized cynomolgus monkeys (25 mg/kg i.v.). A femoral artery was catheterized with a polyvinyl tube for the measurement of blood pressure and heart rate and for the collection of blood samples. A femoral vein was cannulated for the injection of substances. At specified times after the injection of rh-renin, arterial blood (3–5 ml) was collected for the measurement of angiotensins I and II.

Measurement of Plasma Angiotensins I and II

The levels of plasma angiotensins were routinely determined by the method of Kawamura et al. Arterial blood collected from cynomolgus monkeys into a chilled tube containing Na$_2$-EDTA powder was immediately centrifuged at 3000 rpm for 15 minutes at 4 °C. Then, 2 ml of plasma was applied to Sep-Pak cartridges (Waters Associates, Milford, MA, USA), and the bound angiotensins were eluted with 3 ml of methanol/water/trifluoroacetic acid (80:19.9:0.1, vol/vol/vol). The eluates were then applied to a reverse-phase column (TSK gel ODS-80TM, 4.6 × 150 mm; Toyo Soda, Yamaguchi, Japan) and eluted with a 15-minute linear gradient of 10 to 50% methanol at a flow rate of 0.8 ml/min to separate angiotensins I, II, and III. The retention time of angiotensin I, II, and III standards was 27.3, 24.4, and 23.6 minutes, respectively. Radioimmunoassay (RIA) for angiotensin II was performed using specific antiserum against angiotensin II, a gift from Dr. K. Shimamoto. Sensitivity of the assay was 0.2 pg of angiotensin II. RIA for angiotensin I was performed using specific antiserum against angiotensin I, as reported.

Iodination of Recombinant Human Renin

rh-Renin was labeled with Iodine-125 by the chloramine-T method, as reported. First, 1 mCi of Na$^{125}$I and 15 μg of rh-renin were allowed to react with 100 μg of chloramine-T in 140 μl of 0.1 M sodium phosphate buffer (pH 7.4) for 15 seconds at room temperature (20–25 °C). Then, 200 μg of sodium metabisulfite in 50 μl of 0.1 M sodium phosphate buffer (pH 7.4) was added to halt the reaction. The reaction mixture was then applied to a Sephadex G-25 column equilibrated with 50 mM sodium phosphate buffer (pH 7.4) containing 0.1 M NaCl and 0.3% bovine serum albumin (BSA) at a flow rate of 0.5 ml/hr, with fractions of 0.8 ml being collected. $^{125}$I-labeled rh-renin, eluted in the void volume from this column, was then applied to a concanavalin A–Sepharose column (7 × 20 mm) equilibrated with 20 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl at room temperature. The column was exhaustively washed with the equilibration buffer and then eluted with 50 mM α-methyl-D-mannoside in the equilibration buffer, with 0.5-ml fractions being collected. This $^{125}$I-rh-renin was further purified by gel permeation high performance liquid chromatography (HPLC) on a G 3000 SW column (Toyo Soda) equilibrated with 20 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl.

Determination of Immunoreactive $^{125}$I-rh-Renin

Rabbit-specific anti-rh-renin antiserum was used to detect $^{125}$I-rh-renin in samples of plasma, urine, and liver and kidney extracts. This antiserum completely cross-reacted with native human kidney and plasma renin. The 50% inhibitory concentrations of this antiserum for pure native human renal renin and plasma renin were 7.5 × 10$^{-4}$ and 2 × 10$^{-4}$, respectively. Nonimmunized rabbit serum was used as the control. For plasma samples, 25 μl of plasma was added to 475 μl of the antiserum, diluted with 50 mM phosphate buffer (pH 7.4) containing 0.1 M NaCl and 0.3% BSA (1:2500) and was incubated for 48 hours at 4 °C. Then, 100 μl of goat antirabbit Immunoglobulin G antiserum diluted with the same buffer (1:200) was added and the preparation further incubated for 24 hours at 4 °C, followed by centrifugation at 3000 rpm for 20 minutes at 4 °C, for the separation of antibody-bound and free radioactivities. Both precipitate and supernatant were counted for $^{125}$I and $^{131}$I radioactivities.

For liver extract and urine and for kidney extract, 25- and 15-μl aliquots, respectively, were incubated with specific antiserum or nonimmunized serum, as described. After incubation for 24 hours at 4 °C, 100 μl of 1.6% rabbit γ-globulin (final concentration, 0.1%) and 1 ml of 20% polyethylene glycol 6000 (final concentration, 12.5%) were added to the incubation mixture, followed by centrifugation at 3000 rpm for 15 minutes at 4 °C, and the radioactivity in both supernatant and precipitate was measured. In all assays, the $^{125}$I radioactivity precipitable with specific antiserum was corrected for that seen with nonimmunized rabbit serum. Nonspecific binding with nonimmunized rabbit serum was only 2 to 5% throughout the assays. Thus, $^{125}$I-rh-renin in each sample could be specifically measured using this immunoprecipitation method.

Plasma Clearance of $^{125}$I-rh-Renin in Marmosets

Marmosets were anesthetized with ketamine (10 mg/kg i.m.), and polyethylene tubes were inserted into the left femoral artery and vein for blood sampling and for the injection of $^{125}$I-rh-renin, respectively. The pentobarbital-anesthetized animals (20 mg/kg i.v.) were given a bolus injection of 7.7 to 9.0
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\( \times 10^6 \text{cpm of } ^{125}\text{I-rh-renin (86–100 ng)} \) and \( 2.0 \times 10^5 \text{cpm of } ^{131}\text{I-labeled human serum albumin for the determination of plasma volume, in 500 } \mu\text{l of phosphate-buffered saline (PBS) containing 0.3% BSA.} \)

Then, 5, 10, 15, 20, 30, 60, 90, and 120 minutes after the injection, 300 } \mu\text{l of arterial blood was collected from the cannulated femoral artery into chilled tubes containing Na$_2$-EDTA powder, and the volume of blood withdrawn was replaced each time with the same volume of heparinized saline (20 U/ml). Immediately after blood sampling at 120 minutes, the animals were killed by a bolus injection of pentobarbital and organ distributions of \(^{125}\text{I-rh-renin at 120 minutes were studied.} \)

The plasma was separated from the whole blood by centrifugation at 3000 rpm for 20 minutes at 4 °C. An aliquot of 25 } \mu\text{l of each plasma sample was used for the determination of immunoreactive \(^{125}\text{I-rh-renin, as described.} \)

Organ Distribution of \(^{125}\text{I-rh-Renin in Marmosets} \)

Organ distribution of \(^{125}\text{I-rh-renin was studied 30 and 120 minutes after the intravenous injection of \(^{125}\text{I-rh-renin (7.7–9.0 } \times 10^6 \text{ cpm and } ^{131}\text{I-labeled human serum albumin (2.0 } \times 10^5 \text{ cpm). Immediately after the blood sampling at 30 or 120 minutes, the animals were killed by a bolus injection of pentobarbital. The urine in the bladder was collected with a syringe, and heart, lung, liver, kidneys, adrenal glands, testes or ovaries, and spleen were removed. Each organ was rinsed with cold saline, weighed, and counted for \(^{125}\text{I and } ^{131}\text{I radioactivities.} \)

Liver and kidneys were homogenized with cold phosphate buffer (pH 7.4) using a glass homogenizer (1 : 10, wt/vol). \(^{125}\text{I-rh-renin was not degraded by the homogenization procedure, as demonstrated in preliminary experiments in which the } ^{125}\text{I-rh-renin was not catabolized when incubated with liver or kidney homogenates at pH 7.4 for 3 hours at 37 °C or for 72 hours at 4 °C. The supernatant was separated from the whole homogenate by centrifugation at 105,000 g for 60 minutes at 4 °C.} \)

The level of \(^{131}\text{I radioactivity in an organ was assumed to reflect contamination with blood of the injected albumin. With } ^{131}\text{I-labeled albumin as a plasma marker, the amount of } ^{125}\text{I radioactivity in the plasma in each organ was calculated, and this value was subtracted from the total amount of } ^{125}\text{I radioactivity in each organ to give the distribution of } ^{125}\text{I radioactivity.} \)

Incubation of \(^{125}\text{I-rh-Renin with Plasma} \)

The labeled renin (\( 2 \times 10^5 \text{cpm} \)) was incubated with 1 ml of monkey or human plasma for 1, 3, or 7 hours at 37 °C. The incubated sample (500 } \mu\text{l) was applied to a G 3000 SW column at a flow rate of 0.5 ml/min at room temperature, with fractions of 0.5 ml being collected. Total radioactivity and radioactivity precipitable with specific antirenin antibody in each fraction were counted.

Molecular Weight Determination

The molecular weight of radioactive substances in plasma, liver and kidney extracts, and urine was measured by HPLC on a G 3000 SW column equilibrated with PBS at a flow rate of 0.5 ml/min at room temperature. \(^{125}\text{I and } ^{131}\text{I radioactivities were counted using the immunoprecipitation technique described. } \)

Measurement of Radioactivity

\(^{125}\text{I and } ^{131}\text{I radioactivities were assayed with a two-channel } y\text{-counter (Packard Model Auto Gamma 500 C Counting System, Rockville, MD, USA).} \)

Corrections were made for isotope spillover, decay, and counting efficiency. Spillover of \(^{131}\text{I into the } ^{125}\text{I channel was } 15\%. \) There was no spillover of \(^{125}\text{I into the } ^{131}\text{I channel.} \)

Results

Pressor Activity of Recombinant Human Renin

As shown in Figure 1, the intravenous administration of rh-renin (1 } \mu\text{g/kg) to an anesthetized
Iodination of Recombinant Human Renin

As shown in Figure 2A, most of the $^{125}$I-rh-renin applied was adsorbed to a concanavalin A column and eluted with 50 mM α-methyl-D-mannoside, thereby indicating the glycosylation of this rh-renin. Rechromatography of this purified $^{125}$I-rh-renin on a G 3000 SW column showed a single symmetrical peak with a retention time of 39.0 minutes, corresponding to a molecular weight of 42,000 (Figure 2B). $^{125}$I-rh-renin retained the immunoreactivity and pressor activity, thereby indicating little iodination damage. $^{125}$I-rh-renin was stored in PBS containing 0.5% BSA at 4 °C and used within 1 week after iodination, during which time the immunoreactivity and pressor activity remained unchanged. In addition, BSA did not bind to this labeled renin.

Plasma Disappearance of $^{125}$I-rh-Renin

As shown in Figure 3, the disappearance of immunoreactive $^{125}$I-rh-renin from the circulation could be resolved into two exponential components, the half-life for the rapid phase and for the slow phase being 12.1 ± 1.9 and 120.3 ± 16.4 minutes (means ± SE), respectively, and the metabolic clearance rate being 1.17 ± 0.26 ml/min/kg ($n = 6$).

The HPLC profile of the plasma, as shown in Figure 4, represented two major peaks, including the immunoreactive $^{125}$I-rh-renin and nonimmunoreactive degradation products, with a retention time of 39.0 and 47.0 minutes, respectively. With time, immunoreactive $^{125}$I-rh-renin decreased while the degradation products with a low molecular weight increased.

In Vitro Incubation of $^{125}$I-rh-Renin with Plasma

As shown by the HPLC profile in Figure 5, incubation of $^{125}$I-rh-renin with monkey plasma for 3 hours at 37 °C resulted in no degradation of the labeled renin and no loss of its immunoreactivity. Similar results were observed during the incubation with human plasma.

Organ Distribution of $^{125}$I-rh-Renin

As shown in Table 1, thirty minutes after the injection, 43.1 ± 0.9 and 3.5 ± 0.5% of the injected dose accumulated in the liver and kidney, respectively, and 52.3 ± 2.6 and 45.4 ± 4.0%, respectively, of the accumulated radioactivities were immunoreactive $^{125}$I-rh-renin ($n = 5$). In the other organs.
TABLE 1. Distribution in Monkeys of Intravenously Administered \textsuperscript{125}I-Labeled Recombinant Human Renin

<table>
<thead>
<tr>
<th>Organ or fluid</th>
<th>Percentage of dose injected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min (n = 5)</td>
</tr>
<tr>
<td>Plasma</td>
<td>29.7 ± 2.5 (80.5 ± 1.2)</td>
</tr>
<tr>
<td>Heart</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>Lung</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Liver</td>
<td>43.1 ± 0.9 (52.3 ± 2.6)</td>
</tr>
<tr>
<td>Kidney</td>
<td>3.5 ± 0.5 (45.4 ± 4.0)</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Testis or ovary</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Total</td>
<td>78.7 ± 2.6</td>
</tr>
</tbody>
</table>

Values represent means ± SE for each time point. Values in parentheses indicate the percentage of immunoreactive \textsuperscript{123}I-renin to total \textsuperscript{125}I.

Discussion

rh-Renin was prepared by trypsin activation of prorenin synthesized by Chinese hamster ovary cells. The rh-renin\textsuperscript{14, 15} has physicochemical characteristics similar to those of native human renal renin,\textsuperscript{17} including compositional or N-terminal amino acid sequence, molecular weight, isoelectric point, specific enzymatic activity, and optimum pH against hog angiotensinogen. In addition, as shown in Figure 1, rh-renin had as potent a pressor activity as the native human renin has.\textsuperscript{23} In the present study, we used as a probe highly purified rh-renin labeled with \textsuperscript{125}I and specific antiserum. Clearance of exogenously administered rh-renin was studied in the monkey, a species with characteristics of the renin-angiotensin system similar to those in humans. This seems to be a useful model for studying the human renin-angiotensin system.\textsuperscript{25} In studies of the clearance of circulating renin,\textsuperscript{26-28} renin was detected in the form of its enzymatic activity. Hence, renin activity is not specific for renin itself;\textsuperscript{29} it is affected by various factors in the plasma\textsuperscript{30, 31}; and, therefore, is probably an inadequate method for evaluating the clearance of this enzyme.

Plasma disappearance of exogenously administered rh-renin in monkeys showed two exponential components, in agreement with data on the disap-
Fer's cells, and endothelial cells, possess various grades of glycoproteins and participate in the uptake of glycoproteins. In rats, hepatocytes possess receptors specific for galactose-terminated glycoproteins, while Kupffer's cells and endothelial cells have receptors specific for mannose or N-acetylglucosamine-terminated glycoproteins. The rate of uptake of glycoproteins by these liver cells and their subsequent intracellular processing depend on the carbohydrate structure. In the present study, the binding of rh-renin to concanavalin A indicates glycosylation of this rh-renin, a finding consistent with the data on native renal renin of various species, including that of humans, hogs, and rats. However, cells of different animal species and different types of cells glycosylate proteins in a different manner. It is highly likely that the carbohydrate structure of rh-renin differs from that of native human renin; therefore, the clearance of rh-renin may differ from that of native renin. In addition, a significant difference between rh-renin and rat renin in metabolic clearance rate (1.17 vs 11.4 ml/min/kg) may be partly due to differences in the carbohydrate structures.

In the present study, 125I-rh-renin was distributed in the kidney to a small extent and was subsequently catabolized in this organ. Light and electron microscopic autoradiography of exogenously administered 125I-labeled rat renal renin demonstrated that this renin is filtered through glomerular capillaries, reabsorbed by cells in the proximal tubules, and thereafter is distributed in the lysosomes to be degraded. Immunohistochemical findings with specific human renin antiserum showed localization of renin in the proximal tubules of the human kidney. Thus, some of the circulating human renin may be filtered through glomerular capillaries and reabsorbed by the proximal tubule.

In conclusion, human renin was cleared mainly by the liver, and glycosylation probably contributed to the hepatic clearance.

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