Dose-Dependent Uptake of Radioactivity by Liver Parenchymal and Non-parenchymal Cells after Intravenous Administration of Fractionated 3H-Heparin to Rats

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The dose-dependent uptake of fractionated 3H-heparin in the subpopulations of liver cells, parenchymal and non-parenchymal cells, was characterized in rats in vivo. Following the intravenous administration of fractionated 3H-heparin, the radioactivity in plasma was eliminated according to the first order kinetics at each dose. However, the elimination rate constant decreased with dose over the dose range of 0.3 to 100 U/kg, suggesting nonlinear elimination. In accordance with the delay in the plasma elimination, the uptake rate constant of radioactivity by parenchymal as well as non-parenchymal cells of liver, the major distribution organ, also decreased. Although heparin has long been considered to be taken up by a reticuloendothelial system (RES) such as non-parenchymal cells in the liver, the uptake of fractionated 3H-heparin by parenchymal cells was found to be comparable with that by non-parenchymal cells at the lowest dose of 0.3 U/kg, and even larger than that by non-parenchymal cells at the highest dose of 100 U/kg. The uptake clearances of fractionated 3H-heparin at the dose of 0.3 U/kg were 86.4 and 504 ml/100g cells/d, respectively, for parenchymal and non-parenchymal cells. These values were much larger than those reported for polyvinylpyrrolidone, which has been suggested to be taken up by fluid phase endocytosis. Thus, the present study revealed the significant contribution of parenchymal cells in the hepatic uptake of fractionated 3H-heparin. The dose-dependent uptake with clearance values in both parenchymal and non-parenchymal cells provides an in vivo suggestion of the specialized transport of fractionated heparin in these two subpopulations of liver cells.

Keywords fractionated 3H-heparin; dose-dependent uptake; liver parenchymal cell; liver non-parenchymal cell

Heparin, an anticoagulative mucopolysaccharide, is polydispersable (M.W. 6000 to 20000 Da) and heterogeneous in its biological and chemical properties. Heterogeneity could be disadvantageous in establishment of pharmacokinetic and pharmacodynamic profiles of heparin in gaining more control for its use in therapy and exploiting other biological activities than anticoagulative activity. To overcome the problem of heterogeneity and obtain more homogeneous and biologically active heparin, we proposed to fractionate it by affinity chromatography on protamine-Sepharose in regard to the heterogeneity, and thereafter by gel filtration chromatography on Sephadex G-100 in regard to the polydispersity. In our previous studies, fractionated heparin was demonstrated to show different disposition characteristics than unfractonated, more rapid elimination from plasma and higher accumulation in the liver.1–5 The involvement of a specialized transport system in the uptake of fractionated heparin has also been suggested in the primary culture of liver parenchymal cells, though the transport mechanism is yet to be fully clarified.6,7 The transport of fractionated heparin was reported to be inhibited by the analogues of heparin, and by organic anions, but not by metabolic inhibitors or known inhibitors of endocytosis, suggesting a mechanism different from the established endocytosis and the involvement of negative anion charge.7

In the present study, we further characterized the in vivo disposition of fractionated heparin in rats, covering a wider dose range and examining the uptake by parenchymal and non-parenchymal cells of liver, the major distribution organ.

Materials and Methods

Materials [3H(G)]Heparin sodium salt of porcine mucosal origin (No. 2275-214; specific anticoagulant activity was 147.0 units/mg and specific radioactivity was 11.1 MBq/mg; M.W. 6000–20000 Da) was purchased from New England Nuclear Research Products (Boston, Mass., U.S.A.). Heparin sodium salt of porcine mucosal origin (No. 29F-0314; anticoagulant activity was 178 U/mg) and collagenase (type I) were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Percoll, cyanogen bromide-activated Sepharose 4B and Sephadex G-100 were purchased from Pharmacia Fine Chemicals (Upssala, Sweden).

Fractionation of 3H-Heparin

3H-Heparin was fractionated by affinity chromatography on protamine-Sepharose and the fraction which had affinity for protamine-Sepharose was then fractionated by gel filtration chromatography on Sephadex G-100 as described.1,2 Unlabeled heparin was fractionated in the same manner as 3H-heparin. Fractionated 3H- and unlabeled of 25000 Da were used throughout this study.

Animals

Male Wistar rats, weighing 180–230 g (6 to 7 weeks old) were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan) and used without fasting. The rats were cannulated into the right external jugular vein under light anesthesia with ether before the administration of fractionated 3H-heparin.

Administration of Fractionated 3H-Heparin Each rat received an intravenous administration of fractionated 3H-heparin at a dose of 0.3, 1.0, 10 or 100 U/kg via the cannula at the jugular vein, or 0.111 Mbq/kg for a dose of 0.3 U/kg and 0.555 Mbq/kg for the other doses. Unlabeled fractionated heparin was used with fractionated 3H-heparin to set the dose for 10 and 100 U/kg. The specific anticoagulant activity for fractionated heparin is about 170 U/mg, though determined in preparations other than the one used in this study.

Blood samples of 0.25 ml were withdrawn through the cannula at 5, 10, 15, 20, 30, 45 and 60 min after administration. The blood samples were centrifuged at 4°C and 3000 rpm for 15 min to obtain plasma. Sixty minutes after administration, the liver cell isolation procedure described in the following section was started and the rat was sacrificed. Spleen, kidney and intestine were excised and homogenized for the measurement of radioactivity.

Isolation of Liver Cells

Parenchymal cells were isolated according to the method of Berry and Friend with slight modifications. The liver was perfused with perfusion buffer (120 mM NaCl, 6 mM KCl, 0.74 mM K2HPO4, 12 mM NaHCO3 and 5 mM glucose) for 10 min and then with similar buffer containing 0.05% collagenase and 1 mM CaCl2 for 15–20 min at 37°C and at a flow rate of 25–30 ml/min. The whole liver cell suspension was centrifuged at 200 rpm for 1 min to separate parenchymal cells into pellets. The supernatant was processed to obtain non-parenchymal cells by the method of Pertoff et al. with some modifications. The supernatant was centrifuged at 500 rpm for 1 min to eliminate parenchymal cells as pellet.
This procedure was repeated two more times. The final supernatant was centrifuged at 1200 rpm for 10 min. The precipitate was suspended in 50 ml of a 1:1 mixture of buffer (131 mM NaCl, 5.2 mM KCl, 0.9 mM MgSO₄, 0.4 mM CaCl₂, 3 mM Na₂HPO₄, 1.05 mM Tris and 0.04 mM HCl) and Percoll and centrifuged at 18000 rpm for 15 min to remove erythrocytes. Non-parenchymal cells were obtained at the top layer. All centrifugation procedures were performed at 4°C. Significant radioactivity was not detected in the collagenase perfusion buffer or supernatants after centrifugation procedures, suggesting that their release of radioactivity from liver cells was negligible during the procedure isolating them.

**Determination of Radioactivity** Radioactivities were determined by liquid scintillation counting using a LSC-1000 (Aloka Co., Tokyo, Japan). The plasma, tissue homogenates and cell suspensions were solubilized with 1 ml of Protosol and 10 ml of Biofluor was added to determine radioactivity by this means.

**Data Treatment** The plasma level versus time profiles of fractionated ³H-heparin were fitted to an one-compartment model, C = C₀ e⁻ᵏₑₑᵗ, using a nonlinear regression program, MULTI²⁵ to estimate the initial concentration, C₀, and the elimination constant, kₑₑᵗ.

The uptake clearance was calculated by dividing the hepatic uptake of radioactivity by the area under the plasma level-time curves within 60 min after intravenous administration. The area under the plasma level-time curve was estimated by the trapezoidal method.

**Results and Discussion**

**Effect of Dose on Plasma Level** Figure 1 shows the time courses of radioactivity in plasma, elimination profiles normalized by the dose, after the administration of fractionated ³H-heparin. The radioactivity in plasma was eliminated according to the first order kinetics up to 20 min at the dose of 0.3 U/kg, and up to 60 min at other doses. The first-order elimination of radioactivity was considered to represent the distribution phase, which is followed by the pseudo-steady state as reported previously.¹⁹ Those plasma level versus time profiles were analyzed using the one-compartment model to compare the distribution phase among various doses. For the dose of 0.3 U/kg, the data up to 20 min were used for the analysis, since the profile appeared to have reached the pseudo-steady state earlier than those for other doses. Estimated pharmacokinetic parameters are summarized in Table I. The elimination rate constant (kₑₑᵗ) and total body clearance (Clᵣᵣᵣ) at 100 U/kg were about one sixth and one fourth of those at 0.3 U/kg, respectively. Thus, the elimination was delayed depending on dose, suggesting nonlinearity in elimination.

**Effect of Dose on Tissue Levels** Tissue distribution of radioactivity was determined 60 min after administration and is shown in Table II. The radioactivity taken up by the liver decreased with dose, from 64.4% of dose at 0.3 U/kg to 12.0% of dose at 100 U/kg, suggesting the contribution of specialized transport. The dose-dependent decrease in the hepatic uptake is supposed to be responsible for the dose-dependent delay in the elimination from plasma. The uptake by spleen also decreased with dose, though the contribution to the distribution of fractionated ³H-heparin was slight, compared to the liver.

The decrease in the liver distribution with dose may be due to the possible increase in urinary excretion,²¹ the major excretion route for fractionated heparin, though the possibility of increased distribution to the tissues which were not examined in this study cannot be excluded. The metabolism and fate of fractionated heparin taken up by the liver is yet to be fully clarified. However, H₂O as the degradation product of fractionated ³H-heparin was reported to represent the minimal portion of radioactivity in plasma at 5h and in urine at 5h, suggesting that fractionated ³H-heparin is minimally modified in terms of molecular size.²²

The liver distribution of radioactivity at 1h after the intravenous administration of 0.3 U/kg was comparable with that in our previous report at 5h,²² suggesting the negligible release of radioactivity from the liver at the pseudo-steady state for at least several hours. Thus, it could be assumed that the release of radioactivity from the liver is also negligible at the initial distribution phase and, hence, the liver distribution of radioactivity at 1h represents the uptake by unidirectional influx; this is also true of the uptake clearance estimated in the following section. Although the pseudo-steady state appeared to have been achieved at about

**Table I. Pharmacokinetic Parameters for the Elimination of Radioactivity from Plasma after Intravenous Administration of Fractionated ³H-Heparin to Rats at Various Doses**

<table>
<thead>
<tr>
<th>Dose (U/kg)</th>
<th>kₑₑᵗ (h⁻¹)</th>
<th>C₀ (10⁴ dpm/ml)</th>
<th>Vᵣ (ml/kg)</th>
<th>CLᵣᵣᵣ (ml/h/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>11.6 ± 1.6</td>
<td>1.30 ± 0.18</td>
<td>51.2 ± 6.5</td>
<td>587 ± 4</td>
</tr>
<tr>
<td>1.6</td>
<td>4.7 ± 0.2</td>
<td>1.44 ± 0.01</td>
<td>464 ± 0.9</td>
<td>219 ± 36</td>
</tr>
<tr>
<td>10</td>
<td>4.2 ± 0.6</td>
<td>1.05 ± 0.03</td>
<td>54.5 ± 3.3</td>
<td>273 ± 36</td>
</tr>
<tr>
<td>100</td>
<td>2.0 ± 0.4</td>
<td>0.86 ± 0.08</td>
<td>73.2 ± 4.3</td>
<td>145 ± 23</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. (n = 3 for 0.3, 10 and 100 U/kg, and n = 4 for 1.6 U/kg). C₀ and kₑₑᵗ were estimated by fitting the one-exponential equation; C = C₀ e⁻ᵏₑₑᵗ t, to plasma level versus time profiles as described in the section of Materials and Methods. Vᵣ and CLᵣᵣᵣ were calculated as dose/C₀ and kₑₑᵗ Vᵣ, respectively. a) Significantly different (p < 0.05) from the value for 0.3 U/kg. b) 1.6 U/kg. c) 10 U/kg.

**Table II. Tissue Distribution of Radioactivity 60 min after Intravenous Administration of Fractionated ³H-Heparin to Rats at Various Doses**

<table>
<thead>
<tr>
<th>Dose (U/kg)</th>
<th>Liver</th>
<th>Spleen</th>
<th>Kidney</th>
<th>Small and large intestines</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>64.4 ± 4.4</td>
<td>1.6 ± 0.3</td>
<td>3.5 ± 0.4</td>
<td>-○</td>
</tr>
<tr>
<td>1.6</td>
<td>51.7 ± 5.3</td>
<td>1.5 ± 0.1</td>
<td>5.9 ± 1.4</td>
<td>-○</td>
</tr>
<tr>
<td>10</td>
<td>47.4 ± 8.7</td>
<td>0.9 ± 0.1</td>
<td>5.3 ± 0.8</td>
<td>9.0 ± 2.0</td>
</tr>
<tr>
<td>100</td>
<td>12.0 ± 1.9</td>
<td>0.4 ± 0.2</td>
<td>3.7 ± 0.4</td>
<td>8.9 ± 1.9</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. (n = 3 for 0.3, 10 and 100 U/kg, and n = 4 for 1.6 U/kg). a) Significantly different (p < 0.05) from the value for 0.3 U/kg. b) 1.6 U/kg. c) 10 U/kg. d) Not determined.
TABLE III. Uptake of Radioactivity by Liver Parenchymal and Non-parenchymal Cells 60 min after Intravenous Administration of Fractionated $^3$H-Heparin to Rats

<table>
<thead>
<tr>
<th>Dose (U/kg)</th>
<th>Uptake (% of dose/10^6 cells)</th>
<th>Uptake ratio (PC/NPC)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parenchymal cells (PC)</td>
<td>Non-parenchymal cells (NPC)</td>
</tr>
<tr>
<td>0.3</td>
<td>3.7 ± 0.8 $^{a}$</td>
<td>21.2 ± 2.1</td>
</tr>
<tr>
<td>1.6</td>
<td>2.3 ± 0.2 $^{a,b}$</td>
<td>15.5 ± 3.0 $^{b}$</td>
</tr>
<tr>
<td>10</td>
<td>3.5 ± 0.03 $^{a,b}$</td>
<td>5.5 ± 1.0 $^{a,b}$</td>
</tr>
<tr>
<td>100</td>
<td>1.4 ± 0.3 $^{a,b}$</td>
<td>2.3 ± 0.5 $^{a,b}$</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. (n = 3 for 0.3, 1.6 and 100 U/kg, and n = 4 for 1.6 U/kg). a) Significantly different (p < 0.05) from the value for non-parenchymal cells. b) Significantly different (p < 0.05) from the value for 0.3 U/kg, c) 1.6 U/kg, d) 10 U/kg.

TABLE IV. Contribution of Liver Parenchymal and Non-parenchymal Cells to the Hepatic Uptake of Fractionated $^3$H-Heparin to Rats 60 min after Intravenous Administration

<table>
<thead>
<tr>
<th>Dose (U/kg)</th>
<th>Uptake (% of dose/g liver)</th>
<th>Uptake ratio (PC/NPC)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parenchymal cells (PC)</td>
<td>Non-parenchymal cells (NPC)</td>
</tr>
<tr>
<td>0.3</td>
<td>4.57 ± 0.93</td>
<td>4.02 ± 0.41</td>
</tr>
<tr>
<td>1.6</td>
<td>2.91 ± 0.24 $^{a,b}$</td>
<td>2.95 ± 0.70 $^{b}$</td>
</tr>
<tr>
<td>10</td>
<td>4.32 ± 0.03 $^{a,b}$</td>
<td>1.17 ± 0.06 $^{a,b}$</td>
</tr>
<tr>
<td>100</td>
<td>1.68 ± 0.36 $^{a,b}$</td>
<td>0.42 ± 0.09 $^{a,b}$</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. (n = 3 for 0.3, 1.6 and 100 U/kg, and n = 4 for 1.6 U/kg). The values of uptake were calculated from those in Table III, assuming isolated non-parenchymal cells consisted of Kupffer cells alone and cellular composition of the rat liver to be 1.25 x 10^8 parenchymal cells and 1.9 x 10^7 Kupffer cells for one gram of liver. a) Significantly different (p < 0.05) from the value for non-parenchymal cells. b) Significantly different (p < 0.05) from the value for 0.3 U/kg, c) 1.6 U/kg, d) 10 U/kg.

30 min for 0.3 U/kg (Fig. 1), the area under the plasma level-time curve, $AUC$, between 30 and 60 min is negligible compared with the $AUC$ up to 60 min and, so, probably, is the uptake.

Effect of Dose on the Uptake by Parenchymal and Non-parenchymal Liver Cells The radioactivity in parenchymal and non-parenchymal cells was determined 60 min after the administration of fractionated $^3$H-heparin and is shown in Table III in terms of 10^6 cells, and in Table IV, in terms of grams of liver. The uptake of radioactivity decreased with dose in both parenchymal and non-parenchymal cells, suggesting that some specialized mechanism other than diffusion may be involved in the uptake of $^3$H-heparin as reported for human and bovine endothelial cells in culture$^{11-13}$ and for rat parenchymal liver cells in culture$^{10}$.

The data in Table IV were calculated from those in Table III, assuming the cellular composition of rat liver as 1.25 x 10^8 parenchymal cells and 1.9 x 10^7 non-parenchymal (Kupffer) cells for 1 g of rat liver. The isolated non-parenchymal cells were assumed to consist solely of Kupffer cells alone. Assuming that liver weight is about 8.5 g or 4.25% of body weight, the data in Table IV provide comparable values of uptake with those estimated from the radioactivity in the whole liver cell suspension in Table II.

Non-parenchymal cells showed higher uptake than parenchymal cells in terms of 10^6 cells throughout the dose range. However, when scaled up to a gram of liver, the uptake by parenchymal cells was comparable with that by non-parenchymal cells at the lowest dose of 0.3 U/kg, and 4 times larger than that by non-parenchymal cells at the highest dose of 100 U/kg, because the liver contains an order of magnitude more parenchymal cells than non-parenchymal cells and the latter showed sharper decline in uptake by dosages. The uptake clearances of fractionated $^3$H-heparin in parenchymal and non-parenchymal cells were 86.4 and 504 ml/10^6 cells/d, respectively, at 0.3 U/kg, and 12 and 19.2 ml/10^6 cells/d, respectively, at 100 U/kg (Table V). These values were much larger than those for polyvinylpyrrolidone, which was suggested to be taken up by fluid-phase endocytosis$^{15}$ and were comparable to those for colloidal albumin and antimony sulphur colloid, which are known to be taken up by receptor-mediated endocytosis$^{16}$ (Table V), supporting the suggestion of specialized transport of fractionated $^3$H-heparin in both parenchymal and non-parenchymal cells.

We recently reported the saturable uptake of fractionated heparin and the involvement of $x$-globulin as the major binding protein in the primary culture of rat parenchymal hepatocytes. In the presence of physiological concentration of $x$-globulin, the uptake clearance of fractionated $^3$H-heparin increased with concentration in the medium concentration range, suggesting the increase in the unbound fraction due to the saturation of the binding of fractionated heparin to $x$-globulin, and then decreased because of the saturation of the uptake. Although no similar increase in hepatic uptake clearance was confirmed in this study, the decrease in the uptake clearance with dose is in agreement with the observation in the cultured parenchymal hepatocytes. The involvement of $x$-globulin in the uptake of fractionated heparin in vivo should be evaluated in the future.

The involvement of scavenger receptors has recently been suggested in the uptake of low molecular weight heparin in non-parenchymal cells$^{17,18}$. However, the transport mechanisms of fractionated heparin in parenchymal and non-parenchymal cells are yet to be fully clarified and are currently under investigation.

In conclusion, this study demonstrated the dose-dependent delay in the elimination of fractionated $^3$H-heparin from plasma owing to the dose-dependent decrease in uptake by the liver, the major distribution organ, and the significant contribution of parenchymal cells in the liver uptake. The dose-dependent uptake of fractionated $^3$H-heparin was calculated as uptake/AUC. AUC, area under the plasma level versus time curve, was estimated for 0 to 60 min by the trapezoidal method. a) Data from D. P. Fraanjeing-van Delden et al.$^{19}$.
$^3$H-heparin with high clearance values suggested the involvement of specialized transport in both parenchymal and non-parenchymal cells.

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References