30. Studies on Hemoglobin Metabolism. V

*Kinetic Aspects of the Interaction between Hemoglobin-Haptoglobin and its Receptor in the Rat Liver Plasma Membrane**

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As has been reported previously,¹⁾ hemoglobin-haptoglobin (Hb-Hp) formed by intravascular hemolysis is incorporated into rat liver parenchymal cells through a receptor specific for the molecule.

Among the hitherto reported interaction between the cell surface receptor and ligand, Hb-Hp seems to be one of the most complicated molecules as a ligand, that is, Hb-Hp is a complex of two different proteins and has four moles of heme. It is necessary to investigate further details of events on the cell surface, since the binding of Hb-Hp to liver parenchymal cells is the initial step of the subsequent degradation of the protein and heme moiety.

The present investigation was designed to clarify the kinetics of binding of Hb-Hp to freshly isolated liver cells, liver cells in primary culture, and isolated liver plasma membrane. Available evidence indicates that two binding sites, high-affinity and low-affinity binding sites, for Hb-Hp exist on the isolated liver plasma membranes. The high-affinity binding site, however, is lost on the freshly isolated liver cells after perfusion with collagenase, and this is accompanied by the lack of the incorporation of Hb-Hp into liver cells.

Materials and methods. *Biological materials.* ¹²⁵I-Hb (2×10^8 cpm/mg) was prepared after Sonoda and Schlamowitz,²⁾ and [¹²⁵I-Hb]-Hp was prepared by the addition of the ¹²⁵I-Hb to a partially purified Hp.

Isolation and primary culture of rat liver parenchymal cells. Rat liver cells were isolated from male Wistar rats by a modification³⁾ of the method of Seglen.⁴⁾ At 5 min after [¹²⁵I-Hb]-Hp administration into lateral tail vein of the rat, liver was perfused with Ca²⁺-free Hank's solution containing 5 mM EGTA and then digested with

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0.05% collagenase solution *in situ*. Separation of the parenchymal and non-parenchymal cells was achieved according to Nilsson and Berg.⁵⁾ The cells (1×10^6) in 2 ml of Williams medium E supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μ g/ml), dexamethazone (10 mM), and insulin (1 mM) were seeded into 40 mm of plastic dishes and cultured as monolayers in a humidified incubator at 37° under 5% CO₂ and 95% O₂. The medium was changed after the first 24 h and then once every 2 days.

Binding and degradation of Hb-Hp by the liver cells in primary culture. [¹²⁵I-Hb]-Hp (30 μ g) in 100 μ l of buffered saline was added into the dish of 1×10^6 cells in 2 ml of Williams medium E. At an appropriate time, supernatant of the medium was pipetted out and 0.5 ml of the sample was mixed well with 20% TCA and centrifuged at $12,000 \times g$ for 30 min, and then the supernatant was counted as an amount of degradation. Cells attached to dishes were harvested with a rubber policeman of 3 times of chilled 1 ml of Hank's solution containing 0.5% of bovine serum albumin, and 3 ml of cell suspension was overlaid to 5 ml of 20% iso-osmotic Percoll solution and centrifuged at $450 \times g$ for 2 min. All of the precipitated cells were collected and counted in a γ well type scintillation counter.

Uptake and degradation of [¹²⁵I-Hb]-Hp by freshly isolated liver cells. Freshly isolated liver parenchymal cells (3.5×10^7 cells) were incubated with [¹²⁵I-Hb]-Hp in 10 ml of Hank's solution containing 1% bovine serum albumin and 10 mM Hepes, pH 7.3, at 37°. At an appropriate time, 1 ml of aliquot from the cell suspension was overlaid to 5 ml of chilled iso-osmotic Percoll solution and centrifuged at $450 \times g$ for 2 min. Radioactivity of the precipitated cells was counted and the amount of degradation was determined from upper layer of cell-free medium on Percoll solution as an amount of 10% TCA soluble fraction.

Subcellular fractionation by Percoll density-gradient centrifugation. Isolated liver cells or perfused liver were homogenized in 4 volumes of the buffer containing 5 mM triethanolamine-acetate, pH 7.4, 0.25 M sucrose, and 1 mM EDTA using a motor-driven Teflon-glass homogenizer. The resulting homogenate was centrifuged at $760 \times g$ for 10 min at 4°. The supernatant was diluted to a 6-volume with homogenizing buffer, and mixed with iso-osmotic Percoll density-gradient medium to give a density of 1.082 g/ml, and then centrifuged at $107,000 \times g$ for 20 min at 4°. The sample was recovered in 20 fractions. The marker enzyme activities were determined as described previously.⁶⁾

Binding of [¹²⁵I-Hb]-Hp to isolated liver plasma membrane. Liver plasma membrane was isolated from male Wistar rats by the

method of Ray.⁷⁾ The binding assay of [¹²⁵I-Hb]-Hp to liver plasma membrane was carried out as described previously.¹⁾ Amount of Hb-Hp administered, bound to liver cells or liver plasma membrane were expressed in terms of Hb.

Results and discussion. Incorporation and degradation of [¹²⁵I-Hb]-Hp by isolated liver cells were studied. The amounts of [¹²⁵I-Hb]-Hp associated to the cells increased with time, however, it was unable to detect the radioactive degraded materials excreted into the medium up to 75 min. The same was true in the binding and degradation of [¹²⁵I-Hb]-Hp in the liver cells in primary culture. The amounts of the labeled Hb-Hp bound to the cells were saturated within 15 min and remained constant for further 45 min, however, no degradation of the Hb-Hp was observed up to 60 min. In order to explore the reason why the freshly isolated liver cells or liver cells in primary culture did not degrade the [¹²⁵I-Hb]-Hp, the cells were homogenized and the homogenate was subjected to Percoll density-gradient centrifugation, after 60 min of incubation with [¹²⁵I-Hb]-Hp. The major peak of the radioactivity was present in the fractions of low density and never in the fractions of high density. The distribution of marker enzyme activities in these cells were comparable to the patterns in liver homogenate as described previously.⁶⁾ The result together with other data suggests that [¹²⁵I-Hb]-Hp is not internalized in the liver cells though it is bound to the surface of the cells.

The subsequent experiment has been performed to solve the discrepancy between the results of the binding and degradation of [¹²⁵I-Hb]-Hp in the freshly isolated liver cells or liver cells in primary culture and the phenomena *in vivo* in which labeled Hb-Hp is transferred from low density to high density fractions and degraded in the fractions of high density.⁶⁾ Rat liver was perfused with Ca²⁺-free Hank's solution *in situ*, and then with a buffer containing [¹²⁵I-Hb]-Hp for 30 min. After perfusion, liver was excised and homogenized and the homogenate was subjected to Percoll density-gradient centrifugation. Bimodal distribution of the radioactivity was observed after perfusion with [¹²⁵I-Hb]-Hp showing a small peak in the fractions of low density and a large one in the fractions of high density. The results coincided well with the data *in vivo*,⁶⁾ and indicated that the labeled Hb-Hp was readily internalized into liver cells by perfusion of liver *in situ*.

Scatchard plots⁸⁾ of the binding of [¹²⁵I-Hb]-Hp to freshly isolated liver cells and to isolated liver plasma membrane are shown in Fig. 1. Binding of labeled Hb-Hp to isolated liver cells revealed linearly with a K_d value of 1.4×10^{-6} M ($r = -0.959$), and the number of binding sites was shown to be 6.4×10^5 per cell. While binding of

labeled Hb-Hp to the isolated plasma membranes which had been characterized in the previous paper¹⁾ revealed two-phase feature; a high-affinity binding site with K_d of 1.3×10^{-7} M ($r = -0.955$) and a low-affinity binding site with K_d 4.0×10^{-6} M ($r = -0.921$). The number of a high-affinity binding sites, however, represent only 6% of the total binding sites. The low-affinity binding site in plasma membrane presumably corresponded to that in isolated liver cells, and the high-affinity binding site in the isolated liver cells was impaired during the isolation of the cells by collagenase treatment. It is indicated from the evidences that two binding sites for Hb-Hp exist on the outer surface of the liver parenchymal cells and the high-affinity binding site represents 6% of the total binding sites and exhibited a 30-fold higher K_d value as compared with that of the low-affinity binding site. It is noteworthy that the loss of the high-affinity binding sites after collagenase treatment as observed in freshly isolated liver cells or liver cells in primary culture affects the internalization of Hb-Hp into the liver cells.

The phenomena of Hb-Hp receptor on the isolated liver cells are similar to that of the mutant receptor for low density lipoprotein

Fig. 1. Scatchard plots of the binding of [125 I-Hb]-Hp to freshly isolated liver cells and to liver plasma membranes. Isolated liver cells, 6×10^6 cells, were incubated with various amounts (4.5–994.5 μ g) of [125 I-Hb]-Hp at 37° for 60 min in air. Binding assay to liver cells ($\circ$) was as described under "Materials and methods". Liver plasma membrane, 54 μ g, was incubated with various amounts (4.8–612.8 μ g) of [125 I-Hb]-Hp at 37° for 60 min, and the reaction mixture was poured onto Whatman GF/C glass filter under reduced pressure, then the filter was counted in a γ counter ($\bullet$).

reported by Goldstein *et al.*^{9),10)} They presented a mutation that impaired the ability of the low density lipoprotein receptors to localize into the coated pits and thus impaired the internalization and degradation of lipoprotein in the cells though this mutant receptor could bind the ligand. They postulated the presence of two binding sites in the receptor molecule—a binding site and an internalization site. An internalization site would correlate the specific region of the cell surface, coated pit structure.¹¹⁾

From the evidences described above, at least three steps of mechanisms are seemed to be necessary in the internalization of the Hb-Hp molecule into the liver parenchymal cells; binding of the Hb-Hp to the receptor, clustering of receptors themselves by “site-site interaction”,¹²⁾ and localization of the Hb-Hp-receptor complexes into specific region of the cell surface such as the coated pits.

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