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Hepatic uptake of asialoglycoprotein is different among mammalian species due to different receptor distribution

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Isolated hepatocytes of rat, rabbit and guinea pig were found to take up and degrade ^{125}I-labelled asialoorosomucoid at different rates with the rank order: rabbit > rat > guinea pig. Measurement of ^{125}I-asialoorosomucoid binding at 4°C to these hepatocytes revealed that all these cells had two classes of receptors with a major difference occurring in the number of high-affinity binding sites. The average binding affinity constants (K) and receptor concentration (N) calculated from a least-square analysis of the Scatchard plots were $K_1 = 1.15 \cdot 10^9$ M^{-1}, $K_2 = 0.93 \cdot 10^7$ M^{-1}, $N_1 = 0.049$ pmol/mg cell protein and $N_2 = 0.27$ pmol/mg cell protein for the rat; $K_2 = 3.16 \cdot 10^7$ M^{-1}, $N_1 = 0.027$ pmol/mg cell protein and $N_2 = 0.13$ pmol/mg cell protein for the guinea pig and $K_1 = 0.74 \cdot 10^9$ M^{-1}, $K_2 = 3.85 \cdot 10^7$ M^{-1}, $N_1 = 0.205$ pmol/mg cell protein and $N_2 = 0.37$ pmol/mg cell protein for the rabbit hepatocytes, respectively. Measurement of the total number of cellular receptors after solubilization with Triton X-100 also revealed the same receptor concentration rank order of rabbit (5.8 pmol/mg cell protein) > rat (0.55 pmol/mg cell protein) > guinea pig (0.18 pmol/mg cell protein). Intravenous injection of ^{125}I-asialoorosomucoid into anesthetized animals of matched body weight also indicated that the rate of plasma clearance and the rate of appearance of the degraded product of the tracer were different among these species with the same rank order as that observed with isolated hepatocytes. Thus there is a fundamental difference in the number of asialoglycoprotein receptors both on the cell surface and inside hepatocytes of these mammalian species.

Introduction

The survival time of many mammalian plasma glycoproteins depends highly on the integrity of their carbohydrate structure. Removal of terminal sialic acid resulted in a very rapid clearance from the circulation due to a specific receptor-mediated uptake and degradation by the liver hepatocytes. The cellular mechanism of this process involves binding of the desialylated glycoprotein to a galactose/*N*-acetylgalactosamine-specific receptor, endocytosis of the receptor-ligand complex, dissociation of the ligand from the receptor in an acidified endosomal compartment and transport of the ligand to the lysosomes in which the ligand is degraded [1]. The asialoglycoprotein receptors have been isolated from the livers of rabbit [2], rat [3] and human [4] and were shown to be glycoproteins consisting of either one or more polypeptide chain(s). The primary structure of the human receptor has been shown to be quite homologous to

Abbreviations: ASOR, asialoorosomucoid; DMEM, Dulbecco's modified minimum essential medium; FBS, fetal bovine serum; BSA, bovine serum albumin; DPBS; Dulbecco's phosphate-buffered saline; buffer A, 25 mM Tris-HCl/150 mM NaCl/0.6% BSA/10 mM $CaCl_2$, final pH 7.8.

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the major rat receptor protein [5]. Although the presence of similar hepatic aisaloglycoprotein receptors may suggest the existence of similar mechanisms of uptake of desialylated glycoprotein in these mammalian species, here we present evidence that among rabbit, rat and guinea pig, there is a difference in the rate of clearance of ^{125}I-asialoorosomucoid (^{125}I-ASOR) due to different densities of the cell surface receptor.

Materials and Methods

Human orosomucoid, asialoorosomucoid (ASOR) and ^{125}I-ASOR were prepared as described [6]. Collagenase type II (166 U/mg) was obtained from Cooper Biochemicals. Gentamycin sulfate was obtained from Sigma. Dulbecco's modified minimum essential medium (DMEM) and heat-inactivated fetal bovine serum were obtained from Grand Island Biological Co. Streptomycin and penicillin mixed solution (500 μg/ml and 5000 IU/ml, respectively) was obtained from Flow Laboratories. Carrier-free Na^{125}I was purchased from Amersham-Searl. Triton X-100 was purchased from Research Products International Corp.

Preparation of rat, guinea pig and rabbit hepatocytes. Hepatocytes were prepared by the collagenase perfusion technique as described previously [6]. In all species, animals of 250–350 g were used and guinea pigs were perfused for 50 min with the same amount of collagenase used for preparation of rat and rabbit hepatocytes. The isolated cells were resuspended to $1 \cdot 10^6$ viable cells/ml in DMEM containing 10% heat-inactivated fetal bovine serum (DMEM/FBS) and 1 ml of the cell suspension ($1 \cdot 10^6$ viable cell/ml) was added into each well of the 6-well combination culture plate (Costar) which was precoated with rat collagen. After 4 h at 37 °C in a humidified incubator equilibrated with 5% CO_2/95% air, the unattached cells and medium were removed by aspiration. The cell monolayers were then washed twice with 1 ml of Kreb-Hensleit solution containing 1% BSA, replenished with 1 ml DMEM/FBS and cultured overnight before each study.

Determination of uptake and degradation of ^{125}I-asialoorosomucoid in vitro. Uptake and degradation of ^{125}I-asialoorosomucoid ($3.0 \cdot 10^{-8}$ M) by various hepatocyte monolayer cultures were determined in DMEM/1% BSA as described previously [6,7]. In all cases, the receptor-mediated uptake and degradation were corrected for the values obtained in the presence of 10 μM ASOR at each time point, which were assumed to be due to uptake and degradation via nonspecific fluid pinocytosis. All the data were presented as pmol of ^{125}I-ASOR internalized or degraded per mg of cell protein through the receptor-mediated process.

Determination of uptake and degradation of ^{125}I-asialoorosomucoid in vivo. Uptake and degradation of ^{125}I-ASOR was also determined in vivo in urethane-anesthetized animals. Rats, guinea pigs or rabbits (3 weeks old) of matched body weights (300–350 g) were anesthetized with intraperitoneal injection of 50% urethane (0.7 ml/100 g body weight). A catheter was prepared cannulating the left jugular vein with a 0.58 mm polyethylene tubing to inject ^{125}I-ASOR toward the heart. Another catheter of the same size of tubing was placed in the right jugular vein in a direction in which the blood could be drawn from the brain. After injection of 0.2 ml of ^{125}I-ASOR ($5 \cdot 10^{-7}$ M) either alone or in the presence of 0.5 mg of unlabeled ASOR, 0.6 ml blood samples were collected into an Eppendorff centrifuge tube through a heparinized syringe at 0.5, 1, 3, 5, 8, 12, 20, 30, 45 min and 1 h. After centrifugation to pellet red cells, an aliquot of 0.1 ml plasma was counted for total radioactivity and 0.1 ml was treated with phosphotungstic acid to determine the acid soluble and insoluble counts remained in the plasma as described [6,7]. These results were expressed in terms of percentage of total injected radioactivity per ml of plasma.

Binding of ^{125}I-ASOR to hepatocytes. Following an overnight incubation in DMEM/FBS at 37 °C, the hepatocyte monolayer cultures were pre-incubated at 4 °C in Dulbecco's phosphate-buffered saline (DPBS) containing 0.1% bovine serum albumin for 30 min before adding $9.5 \cdot 10^{-11}$ M of ^{125}I-ASOR and various concentration of unlabeled ASOR ($(0–5.0) \cdot 10^{-6}$ M) at 4 °C for 2 h. The medium was then removed and the cell layer was washed three times with ice-cold Dulbecco's phosphate-buffered saline. The cells were then dissolved in 1 ml 0.1 N NaOH/0.5% sodium dodecyl

sulfate. An aliquot of 0.8 ml was counted in an LKB 1274 RIA gamma counter for 2 min and an aliquot of 0.1 ml was diluted 10-fold with distilled water before measurement of protein concentration. The binding assay was run in triplicate at each unlabeled ASOR concentration in each preparation of the hepatocytes. The average bound radioactivity measured at $5 \cdot 10^{-6}$ M was regarded as nonspecific binding and was subtracted from each data point.

For determination of the cell surface to the total cell receptor ratio concentration, ^{125}I-ASOR binding to the monolayers of hepatocytes in the absence and presence of Triton X-100 were measured according to the method of Pricer et al. [8]. The hepatocytes in triplicate were incubated in a buffer of 25 mM Tris HCl-150 mM NaCl/0.6% BSA/10 mM $CaCl_2$ with the pH adjusted to 7.8 (buffer A) in the absence or presence of 1% Triton X-100 (w/v) at 4°C with $1.1 \cdot 10^{-7}$ M ^{125}I-ASOR for 2 h at 4°C. The cells incubated in the absence of Triton X-100 were washed three times with 1 ml of Dulbecco's phosphate-buffered saline at 4°C and dissolved in 1 ml of 0.1 M NaOH/0.5% sodium dodecyl sulfate. An aliquot of 0.8 ml of the cell solution was counted for radioactivity while 0.1 ml was diluted 10-fold with H_2O for determination of protein. The cells incubated in the presence of Triton X-100 were resuspended with a pipet at 4°C and 0.4 ml of the suspension was transferred to a test tube and then mixed well with an equal volume of saturated ammonium sulfate solution. The precipitate formed was collected on a Whatman GF/C glass fiber filter, washed twice with the tube rinse with 45% saturated ammonium sulfate containing 10 mM $CaCl_2$ and 0.1% BSA (pH 7.8) (with Tris base) and then five times with 1 ml of the same solution at 4°C. The filter was then counted in a gamma counter. Nonspecific binding was determined as the bound radioactivity determined from cells incubated in the presence of $1.0 \cdot 10^{-5}$ M unlabeled ASOR under the same experimental conditions. These counts were subtracted from the bound counts of the samples.

Measurement of total receptor in tissue homogenates. The receptor concentration in fresh tissue homogenate was also measured after solubilization with Triton X-100. Each animal was anesthetized with intraperitoneal injection of urethane as described above. The abdominal cavity was opened with scissors and blood was drained through a catheter placed in the abdominal aorta. Immediately after draining of the blood was stopped, liver was excised and placed in a ice-chilled beaker. A piece of 1–1.5 g tissue was then taken from each lobule and homogenized in 3 ml of 10 mM Tris HCl/2 mM $CaCl_2$/0.28 M sucrose (pH 7.6 at 4°C). The final volume of the homogenate was recorded. An aliquot of 0.25 ml liver homogenate was then mixed well with 0.7 ml of buffer A containing 1% Triton X-100 either in the absence or presence of 10 nmol (400 μg) of unlabeled ASOR. An aliquot of ^{125}I-ASOR (50 μl) was then added to a final concentration of $5.56 \cdot 10^{-8}$ M and the reaction mixture was incubated at 4°C for 2 h. An aliquot of 0.2 ml of the reaction mixture was taken and mixed with an equal volume of ice-cold saturated ammonium sulfate (adjusted to pH 7.8 with Tris base). After 15 min at 4°C, 0.1 ml of the suspension was added to a Whatman GF/C glass fiber filter (2.5 cm) prewashed with 45% saturated ammonium sulfate/10 mM $CaCl_2$ (pH 7.8). The filter was washed five times with 1 ml of the above-mentioned 45% saturated ammonium sulfate solution and counted. Blank tubes using 0.25 ml of homogenizing buffer were also incubated in the absence and presence of ASOR and processed under the same conditions. These counts (2.4%of total added counts), which were the same in the absence or presence of ASOR, were substracted from counts obtained with the liver homogenates. The corrected counts obtained in the absence and presence of unlabeled ASOR were regarded as total and nonspecifically bound counts, respectively. The fractions of nonspecific binding to total binding were found to be 9, 11 and 5.5% for rat, guinea pig and rabbit liver homogenates, respectively.

Other analytical method. Protein concentration was determined by the method of Lowry et al. [8] using crystalline bovine serum albumin as standard.

Results and Discussion

The results shown in Fig. 1 suggest that at the same initial concentration ($3 \cdot 10^{-8}$ M), ^{125}I-ASOR

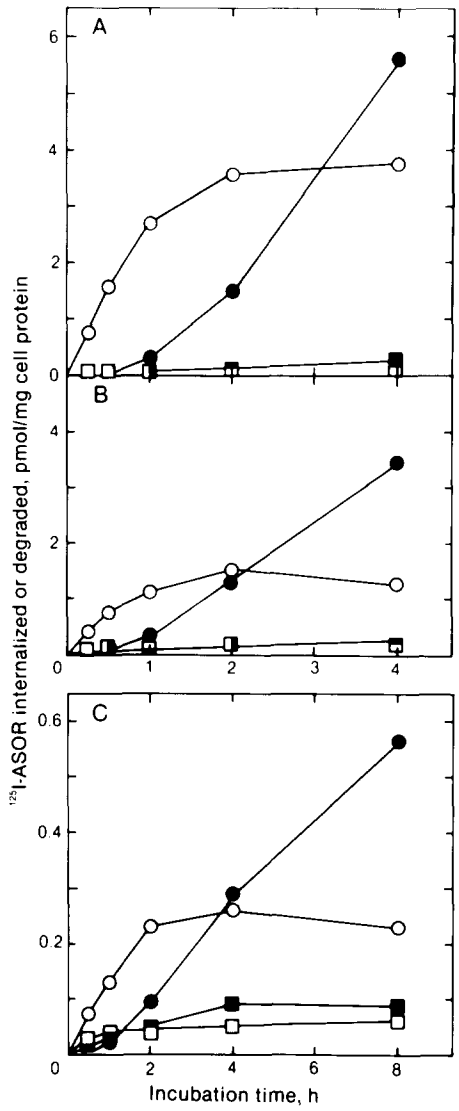

Fig. 1. The rate of internalization and degradation of ^{125}I-ASOR by isolated hepatocytes of rabbit, rat and guinea pig. Isolated hepatocytes in monolayer cultures were incubated with $3 \cdot 10^{-8}$ M ^{125}I-ASOR in the absence or presence of 10^{-5} M unlabeled ASOR at 37 °C for various time periods as indicated. The EGTA-resistant cell-associated radioactivity (open symbols) and phosphotungstic acid-soluble radioactivity released into the medium (filled symbles) were determined as tracer internalized and degraded, respectively. The squares respresent tracer internalized or degraded in the presence of unlabeled ASOR (nonspecific internalization and degradation). The circles respresent the receptor-mediated internalization or degradation of tracer obtained after substracting the non-specific values from the radioactivity determined in the absence of unlabeled ASOR at the corresponding points. The panels represent average data obtained from (A) rabbit ($n = 3$), (B) rat ($n = 5$) and (C) guinea pig ($n = 5$) hepatocytes, from n preparations of the cells.

was internalized and degraded via asialoglycoprotein receptor at different rates by the hepatocytes of rabbit, rat and guinea pig. In rabbit hepatocytes (Fig. 1A), there was a very rapid initial uptake of the ligand at 50.7 fmol/mg cell protein per min. A steady-state intracellular ligand concentration of 3.7 pmol/mg cell protein was attained after 2 h of incubation and the ligand was degraded rapidly at 28.5 fmol/mg cell protein per min. In rat hepatocytes (Fig. 1B), the initial uptake rate of ^{125}I-ASOR (28.7 fmol/mg cell protein per min) was slower than that of rabbit hepatocytes and led to a lower steady-state intracellular ligand concentration (1.4 pmol/mg cell protein) and slower rate of degradation (17 fmol/mg cell protein per min). In guinea pig hepatocytes (Fig. 1C), the rates of uptake and degradation were even slower than those observed in rat hepatocytes with an initial uptake rate of 2.3 fmol/mg cell protein per min, a steady-state ligand concentration of 0.25 pmol/mg cell protein and a rate of ligand degradation at 1.4 fmol/mg cell protein per min.

The difference in uptake and degradation of ^{125}I-ASOR by these hepatocytes appeared to be due to different concentrations of the asialoglycoprotein receptor on the cell surface. The binding data as shown in Fig. 2 exhibited non-linear Scatchard plots [10] indicating the existence of heterogeneity in the receptors [11]. Although it is not certain whether this heterogeneity represent binding of the ligand to the unclustered receptor and those concentrated in the coated pit [12,13], or to different binding proteins [2,3,14], these data do show a difference in total binding capacity among the hepatocytes of these three species. Using a model of one ligand with two classes of noninteracting receptors and the least-square curve fitting method of Feldman [15], the binding parameters were calculated and are summarized in Table I. Rat hepatocytes had a higher average affinity constant ($1.15 \cdot 10^9$ M^{-1}) than guinea pig ($0.79 \cdot 10^9$ M^{-1}) or rabbit ($0.74 \cdot 10^9$ M^{-1}) hepatocytes of the high-affinity receptors. Conversely, the average affinity constant of the low-affinity receptors in rat cells ($0.93 \cdot 10^7$ M^{-1}) was 3 to 4-times lower than those determined in guinea pig ($3.16 \cdot 10^7$ M^{-1}) and rabbit ($3.85 \cdot 10^7$ M^{-1}) cells. The most significant difference among these

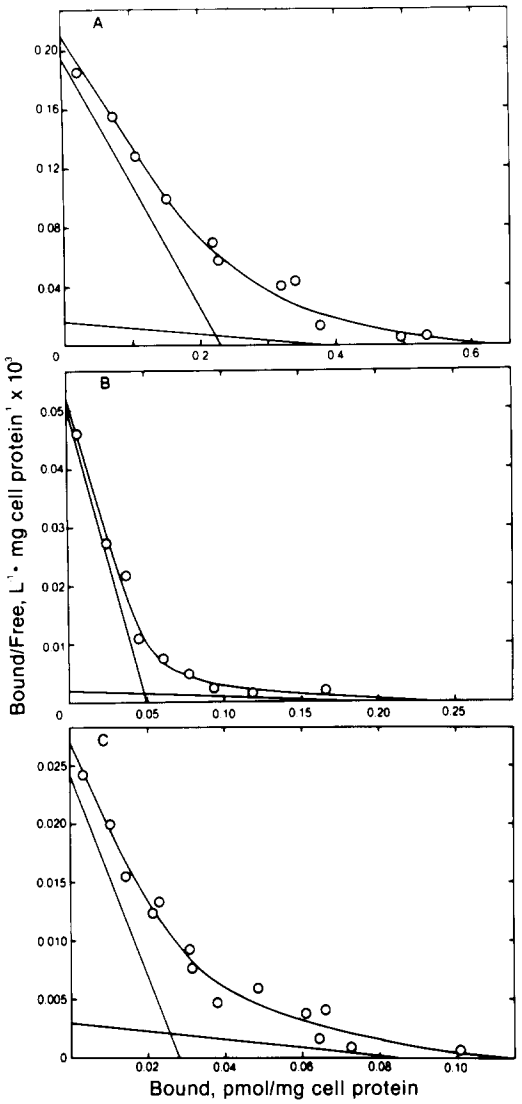

Fig. 2. Scatchard plots of binding data of ^{125}I-ASOR to rabbit, rat and guinea pig hepatocytes. In each panel, the line connecting the data points represents a least-square binding curve of two independent classes of binding sites, while the two straight lines represent the theoretical binding curves at high- and low-affinity binding sites. Each panel represents the results of a single experiment, while the average binding parameters of several experiments determined by the same method are summarized in Table I. (A) Rabbit hepatocytes, (B) rat hepatocytes, (C) guinea pig hepatocytes.

three species, however, was found in the number of high-affinity receptors with the order: rabbit (0.205 pmol per mg cell protein) > rat (0.049 pmol per mg cell protein) > guinea pig (0.027 pmol per mg cell protein). The same rank order existed for the number of low-affinity receptors, but the differences among these species were less significant. Thus, the combinding effect of both the affinity and the number of high-affinity receptors appeared to be the main source of difference among these species. It should be noted that the receptor concentration measured in the monolayers described above was 2- to 3-fold lower than that which was measured in freshly prepared hepatocytes. However, we found that the rank order of the receptor activities measured in the freshly prepared cells was the same as that observed in the monolayer cultures (data not shown). Therefore, it was unlikely that the differences among these three species were the result of variation in changes in the receptor concentration during the formation of the monolayers.

Rat hepatocytes also contain a large number of intracellular receptors for asialoglycoprotein [8] which have been shown to participate in the receptor-mediated endocytosis of asialoglycoprotein [16]. The fewer surface receptors found in guinea pig hepatocytes could be due to a lower surface distribution of the cellular receptors. We therefore measured the ratio of the number of the receptors on the cell surface to those of total cellular pool in the hepatocytes of all three species according to the method of Pricer et al. [8]. The results as

TABLE I

BINDING PARAMETERS FOR ASIALOOROSOMUCOID OF RAT, GUINEA PIG AND RABBIT HEPATOCYTES

The binding parameters were calculated by least-square curve fitting according to the method of Feldman [15] using a model of one ligand binding to two independent classes of binding sites. K_1 and K_2 are the binding affinity constants calculated for the high- and low-affinity binding sites, respectively. N_1 and N_2 are the concentration of high- and low-affinity binding sites calculated as pmol per mg cell protein. The data represent mean ± S.D. of the binding parameters calculated for four separate experiments using monolayers of four preparations of hepatocytes.

Species	K_1 (10^{-9} M^{-1})	K_2 (10^{-7} M^{-1})	N_1 (pmol/mg)	N_2 (pmol/mg)
Rat	1.15 ± 0.43	0.93 ± 0.42	0.049 ± 0.015	0.27 ± 0.08
Guinea pig	0.79 ± 0.11	3.16 ± 2.0	0.027 ± 0.005	0.13 ± 0.05
Rabbit	0.74 ± 0.10	3.85 ± 0.40	0.205 ± 0.025	0.37 ± 0.02

summarized in Table II indicate that guinea pig hepatocytes contained fewer receptors not only on the cell surface but also in the entire cellular pool (hence, in the intracellular pool) than do rat or rabbit hepatocytes. The rank order of the pool size of intracellular receptors was the same as that for surface receptors. Since intracellular receptors participate in the receptor-mediated uptake and degradation of asialoglycoprotein [16], and recycling of the receptors is an essential step of the process [17], it is apparent that the number of intracellular receptors affects the number of cell surface receptors as well as the capacity of the hepatocytes to take up and degrade the ligand through the receptor. The observation that the surface to total receptor ratio observed in rabbit hepatocytes (9%) was lower than that of rat (48.5%) or guinea pig (32.8%) hepatocytes indicated that rabbit cells had a lower efficiency to distribute cellular receptors to the cell surface. Whether or not this involves a slow transport rate of the receptor remains to be studied.

In order to be certain that the difference among the three species was not an artifact due to treatment with collagenase during isolation of the hepatocytes, we also determined the plasma clearance rate of ^{125}I-ASOR in these three species using anesthetized animals. Preliminary experiments in rats indicated that the plasma clearance rate in the anesthetized rat was not different from those previously measured in conscious rats [18,19]. Because uptake of ^{125}I-ASOR in both rabbit and rat were extremely rapid, the rates of uptake of ^{125}I-ASOR in the presence of 0.5 mg unlabeled ASOR were determined in all three species and compared.

As shown in Fig. 3A, the plasma clearance rate of ^{125}I-ASOR was much higher in the rabbit and rat than in the guinea pig. The corresponding plasma half-times of the tracer were 5, 6.5 and 16.5 min for the rabbit, rat and guinea pig, respectively. In addition, the rates of appearance of the degraded product in the plasma, as shown in Fig. 3B, were also more rapid in the rabbit and rat than in the guinea pig. Moreover, in agreement with the previous report [19], we found that more than 95% of the injected radioactivity was accumulated in the liver of the rabbit and rat 10–15 min after injection of the tracer. The accumulation of the tracer in the liver of guinea pig was slower, reaching 80% at 20 min after injection. At the end of the experiment (60 min after injection of the tracer), about 19% of the injected radioactivity remained in the livers of the rat and rabbit, while in the liver of guinea pig as much as 59% remained. These observations also indicated that the rates of uptake and degradation of ^{125}I-ASOR in vivo were lower in the guinea pig. The fact that the same rank order of uptake and degradation of the ligand was observed in vivo further suggested that the results observed in the isolated hepatocytes reflected intrinsic difference among these three species. Measurement of total receptor concentration in the tissue homogenates also revealed the same rank order and, therefore, provide further support to this argument. Thus, the total receptor concentrations averaged from the data of six tissue homogenates of each species were 423.4 $\pm$ 24.8, 300.2 $\pm$ 26.6 and 159.8 $\pm$ 7.1 pmol/g tissue for rabbit, rat and guinea pig, respectively.

It should be mentioned that the above results involved comparison among the three species of

TABLE II

DISTRIBUTION OF ASIALOGLYCOPROTEIN RECEPTORS IN ISOLATED RAT, GUINEA PIG AND RABBIT HEPATOCYTES IN MONOLAYER CULTURES

The experimental details are given in the text. The data respresent the mean $\pm$ SD of results obtained from the number of hepatocytes preparations given in the parenthesis.

Species	Cell surface receptor (pmol/mg cell protein)	Total cellular receptor (pmol/mg cell protein)	Surface/total ratio (%)
Rat ($n = 5$)	0.242 $\pm$ 0.101	0.545 $\pm$ 0.202	48.5 $\pm$ 10.8
Guinea pig ($n = 5$)	0.071 $\pm$ 0.019	0.183 $\pm$ 0.042	32.8 $\pm$ 15.5
Rabbit ($n = 3$)	0.466 $\pm$ 0.130	5.800 $\pm$ 2.410	9.0 $\pm$ 2.4

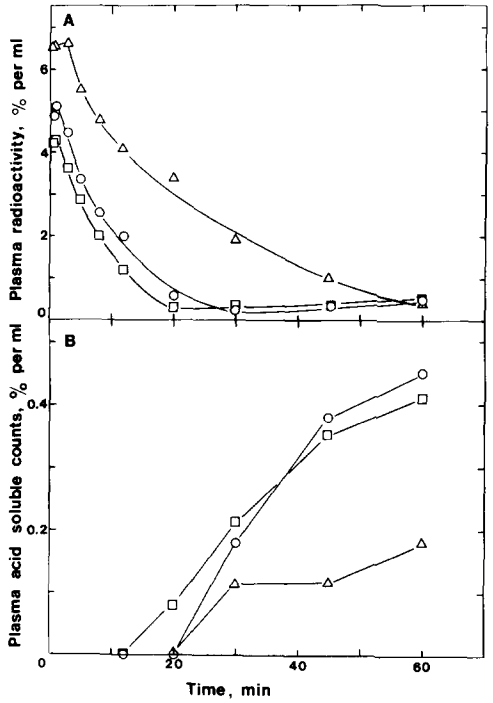

Fig. 3. Plasma clearance rates of ^{125}I-ASOR and appearrance of its degradative product in rabbit, rat and guinea pig in vivo. (A) Plasma clearance rate in the presence of 0.5 mg unlabeled ASOR. (B) rate of appearance of degradative product. □, rabbit; ○, rat; △, guinea pig. The data represent the average of results from three animals.

animals with matched body weight rather than age. It may be conjectured that the results obtained with the young rabbits (3–4 weeks old) may not represent well the receptor distribution in this species. However, Collins et al. [20] have shown in the mouse that the activity of hepatic asialoglycoprotein receptors increased rapidly after birth reaching the adult level 5 days after birth. Judging from the times before puberty in the mouse (6–8 weeks) and the rabbit (6–8 months), it appears that the rabbits used in the present study were at least as matured as a 5-day old mouse, relative to their puberty ages. Thus, if the asialoglycoprotein receptor activities are expressed at the same rate post partum in both species, then the binding parameters obtained from the rabbit hepatocytes in the present study could be very close to those of the cells of adult animals. This notion was supported by the similar binding parameters obtained from the hepatocytes of two rabbits of 7-weeks old (1.1 kg body weight, data not shown).

The results of the present study indicate that the asialoglycoprotein receptor activities are different among rabbit, rat and guinea pig hepatocytes both in vivo and in vitro. The activities in both rat and rabbit hepatocytes appeared to relate quite well with their receptor concentration. On the other hand, the rates of ligand uptake and degradation in guinea pig hepatocytes, when compared with rat or rabbit cells were below those expected from their relative receptor concentrations. Since asialoglycoprotein is internalized via coated pit containing clustered receptors resulting in quantal uptake of the ligand, it is likely that isolated guinea pig hepatocytes with low receptor concentrations might contain both fewer coated pits with receptors (due to a slower rate of receptor clustering) and fewer receptors per coated pit than rat or rabbit cells resulting in a much slower rate of receptor-mediated transport. In addition, the lower intracellular receptor concentration in guinea pig hepatocytes might also affect the rate of receptor recycling and hence, uptake of the ligand. The answer to these questions, obtained from a future study, undoubtedly will throw new light on the mechanism of asialoglycoprotein receptor-mediated transport.

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