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Effect of galactose on binding and endocytosis of asialoglycoprotein in cultured rat hepatocytes

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Background: ^{99m}Tc-diethylenetriaminepentaacetic acid-galactosyl-human serum albumin (^{99m}Tc-GSA) has been applied clinically in scintigraphy to estimate functioning liver mass, but it is not so sensitive in differentiating mild liver injury from normal liver. ^{99m}Tc-GSA is thought to bind to the asialoglycoprotein receptor (ASGP-R) and is then internalized and degraded in the hepatocytes. The aim of this study is to know whether D-galactose inhibits GSA binding or internalization to hepatocyes because ASGP-R recognizes galactose residues of ligands. *Methods:* Isolated rat hepatocytes were obtained by collagenase perfusion, pre-cultured for 2 h after plating, and then cultured for 16 to 18 h until use. The effect of galactose on GSA binding and internalization into cells was investigated by using cultured hepatocytes. *Results:* Galactose non-competitively inhibited GSA binding to cultured hepatocytes, but its Ki value was quite high (505 ± 38 mM). Galactose significantly inhibited GSA internalization into hepatocytes at 27 mM. *Conclusion:* It was clarified that D-galactose inhibited GSA internalization rather than binding at a low concentration. Further *in vivo* studies in rats are needed to know whether an administration of galactose prior to performing ^{99m}Tc-GSA scintigraphy can brake it possible to estimate the functioning mass in mild liver injury.

Key words: asialoglycoprotein, cultured hepatocyte, galactose, inhibition

INTRODUCTION

THE ASIALOGLYCOPROTEIN RECEPTOR (ASGP-R) is localized exclusively in the parenchymal cells of the mammalian liver and functions in the rapid removal of desialylated glycoproteins from the circulation by receptor-mediated endocytosis.[1,2] These receptors recognize and bind galactose-terminated glycoproteins by a second-order chemical reaction.[3] After binding, they are transported to the lysosome where the ligands are degraded, and the receptor is recycled to the plasma membrane.[4,5] Based on this specific characteristic of ASGP-R, clinical imaging techniques to evaluate hepatic function have recently been developed.[6] ^{99m}Tc-diethylenetriaminepentaacetic acid-galactosyl-human serum albumin (^{99m}Tc-GSA) is a newly developed analog ligand, and is used clinically for scintigraphy to estimate liver function by using the receptor number as an indicator of functioning hepatic mass.[7–9] ^{99m}Tc-GSA scintigraphy is considered useful for estimating resting liver function in patients with liver cirrhosis or with partial hepatectomy,[10] but ^{99m}Tc-GSA scintigraphy is not sensitive enough to distinguish liver dysfunction in patients with chronic hepatitis 2a and 2b according to a European classification.[11] It was not thought that decreasing numbers of ASGP-R are reflected in parameters to evaluate liver function as ^{99m}Tc-GSA is used at a very low dose in clinical work. Because increasing the dose of ^{99m}Tc-GSA, a ligand, is difficult in clinical work, the use of inhibitors might be effective in detecting minor liver dysfunction.

D-galactose is a hexose that is rapidly removed from the circulation by the liver and phosphorylated within liver cells.[12] D-galactose has been used for many years to evaluate liver function in patients with chronic liver injury.[13,14] ASGP-R recognizes galactose-terminated glycoproteins,[1] but little is known about the effect of D-galactose on asialoglycoprotein.

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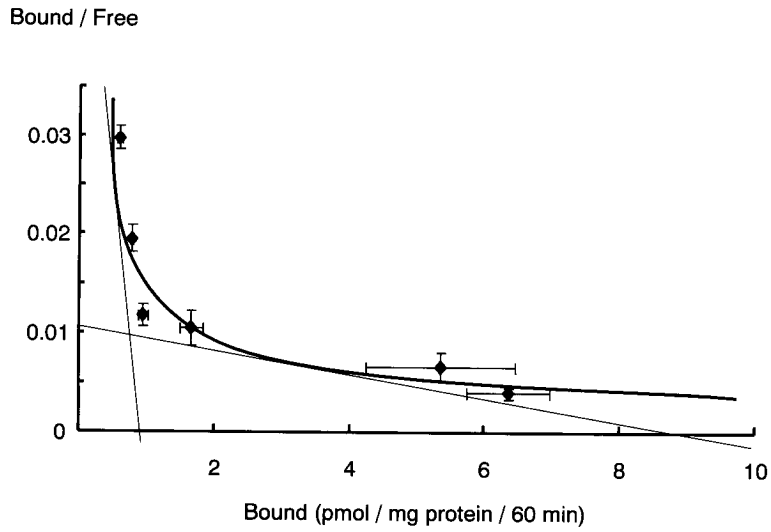

Fig. 1 Scatchard plot of the binding of diethylenetriaminepentaacetic acid-galactosyl-human serum albumin (GSA) to cultured hepatocytes. A monolayer (1.5×10^6/dish) was incubated at 4°C for 60 minutes as described in "Materials and Methods." Data are expressed as mean ± SE.

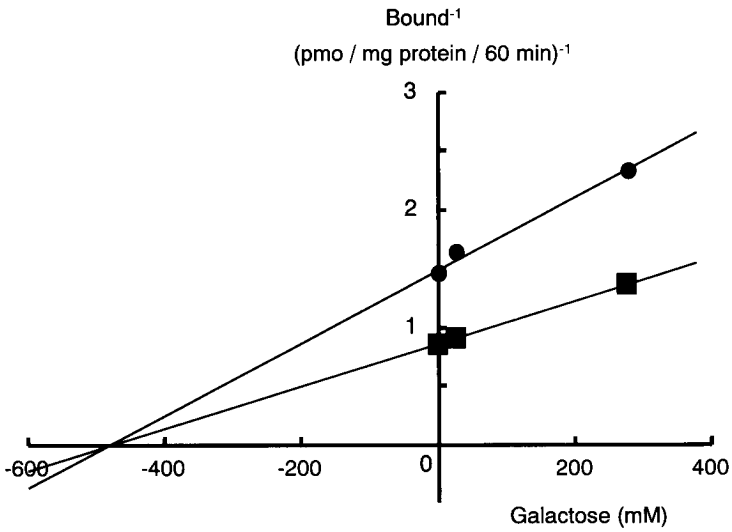

Fig. 2 A representative Dixon plot analysis of D-galactose inhibition of GSA binding at ●, 65.8 nM; ■, 132 nM. Data are expressed as the mean of duplicate experiments. Similar results were obtained in three other experiments.

In this study, to determine whether galactose can be an inhibitor, we first studied the effect of D-galactose on GSA binding to ASGP-R and internalization through ASGP-R in primary cultured rat hepatocytes.

MATERIALS AND METHODS

Materials

Adult male Sprague-Dawley rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan) weighing 200 to 250 g were housed in a room at 22 ± 2°C under normal laboratory lighting conditions. They were maintained on a commercial pelleted diet and water ad libitum. All the rats received care according to methods approved under the institutional guidelines for the care and use of laboratory animals in research.

Isolation and Short-Term Culture of Rat Hepatocytes

Hepatocytes were isolated from rat liver and cultured as previously reported.[15] In brief, the liver was perfused with collagenase type IV (Sigma Chemical, St. Louis, MO, USA), and the isolated hepatocytes were suspended in culture medium consisting of Waymouth's 752/1 (GIBCO, Grand Island, NY, USA) containing 5% heat-inactivated fetal bovine serum, 2.5 mM additional $CaCl_2$, 5 μg/m*l* bovine insulin (Sigma), 100 U/m*l* penicillin (GIBCO) and

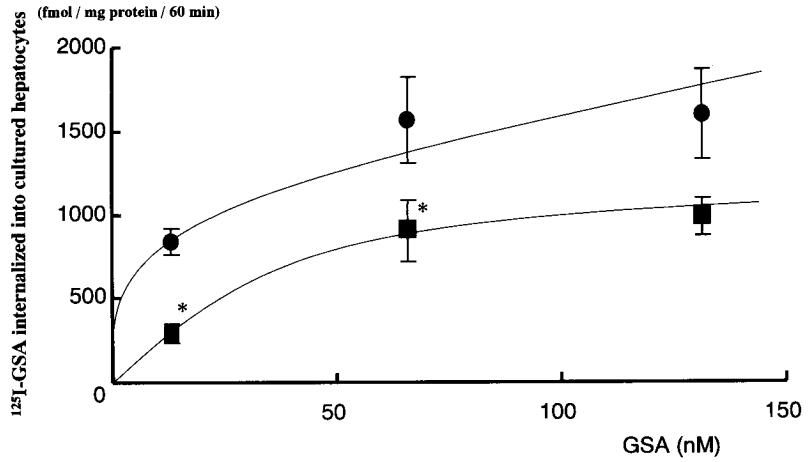

Fig. 3 Inhibition by 27 mM D-galactose of GSA endocytosis in serum-free medium (SFM) where NaCl was replaced by isosmotic D-galactose. ●, control; ■, 27 mM D-galactose. Values are expressed as mean ± SE of six determinations in three separate experiments. *: significant difference ($p < 0.05$ by Mann-Whitney test) from GSA endocytosis in SFM (control).

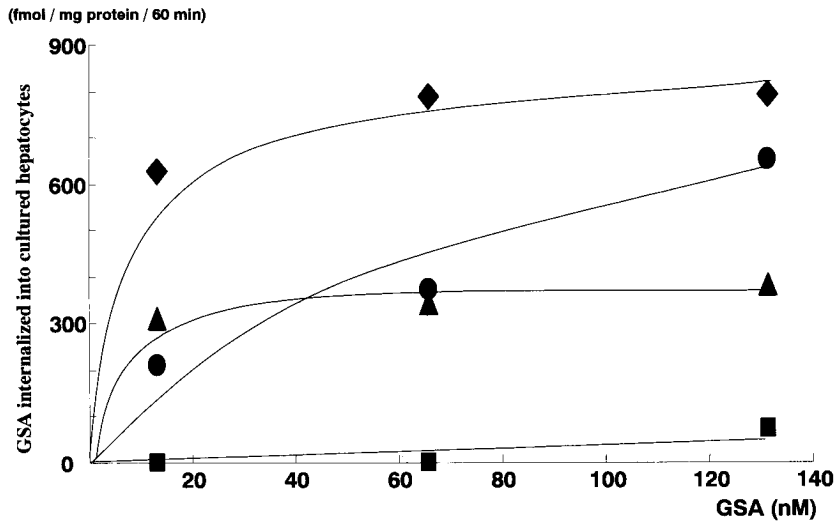

Fig. 4 GSA endocytosis into hepatocytes cultured in SFM (◆) containing 135 mM NaCl and other SFMs where NaCl was replaced with isosmotic D-galactose (■), KCl (●) or D-gluconic acid sodium salt (▲). Data are expressed as the mean of duplicate experiments.

0.1 mg/m*l* streptomycin (GIBCO). Approximately 1.5×10^6 cells in 3 m*l* were plated in individual 60-mm diameter Lux culture dishes (Corning, NY, USA), and cultured in a 5% CO_2 atmosphere at 37°C. The culture medium was changed –2 h after plating, and hepatocytes were cultured for an additional 16 to 18 h until use.

Viability of the isolated hepatocytes was greater than 90% as judged by trypan blue exclusion.

Studies of Binding and Endocytosis of GSA

Binding study

GSA (Nihon Medi-Physics Chemical Co., Ltd., Chiba, Japan), which is a synthetic neoglycoalbumin used in clinical hepatic imaging, was radiolabeled with $Na^{125}I$ (Daiichi-Kagakuyakuhin Co., Ltd., Tokyo, Japan) by the Chloramine T method.[16] To quantify binding of ligand to cell-surface receptors, monolayers were chilled to 4°C by two washes with 1.5 m*l* of serum-free medium (SFM) consisting of (in mM) 135 NaCl, 1.2 $MgCl_2$, 0.81 $MgSO_4$, 27.8 glucose, 2.5 $CaCl_2$ and 25 HEPES, pH 7.2, and incubated for 60 min at 4°C. SFM was replaced with 1 m*l* of SFM containing various concentrations of D-galactose (Sigma) and GSA with the radiolabeled GSA (^{125}I-GSA) under the tracer dose ($1–2 \times 10^6$ cpm/0.45 pmol/m*l*) and steady-state binding was reached in 60 min at 4°C. All media were adjusted to a same osmolarity (270 mOsm) by replacing the NaCl in SFM with 27 or 270 mM D-galactose. Unbound ligand was removed by four 1.5 m*l*

washes with SFM; the third wash contained 0.5 mM N-acetylgalactosamine (Sigma).[4] The cells were removed with a rubber policeman and collected with 1 m*l* of SFM. Then RI was measured in an automatic gamma counter (Model 1480 WIZARD™ 3″; Wallac Co., Ltd., Turku, Finland).

After three washes with SFM at 4°C, the cells were removed with a rubber policeman and collected with 1 m*l* of phosphate buffer to determine the cellular protein content according to the method of Lowry et al.[17]

Endocytosis study

Monolayers were washed two times with 1.5 m*l* of SFM warmed to 37°C and incubated for 60 min at 37°C with 1 m*l* of SFM containing various concentrations (27, 270 mM) of D-galactose and GSA (13.2, 65.8, and 132 nM) with a tracer dose of ^{125}I-GSA (1–2 × 10^6 cpm). Internalization was stopped by chilling hepatocytes with ice-cold SFM and unbound ligand was removed by four 1.5 m*l* washes with ice-cold SFM; the third wash contained 0.5 mM N-acetylgalactosamine.[4] The cells were removed with a rubber policeman and collected with 1 m*l* of SFM. RI was measured in an automatic gamma counter. To estimate the amount of internalized ligand, the level of radioactivity of the cell surface bound was subtracted from the radioactivity obtained in the internalization study. We also studied the effect of sodium and chloride ions on ASGP-R uptake into cultured hepatocytes. NaCl was replaced in SFM with isosmotic KCl and D-gluconic acid sodium salt, and monolayers were washed twice with these altered SFMs. The binding solutions were also made in altered SFMs.

Statistical Analysis

All experiments were performed in duplicate. Results are expressed as means ± SE. Data were analyzed by the Mann-Whitney test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Binding study

The ASGP-R contains two binding sites, a high affinity site and a low affinity site as determined by Scatchard analysis (Fig. 1). The Kd value and number of receptors were calculated by manual graph analysis as follows (n = 6): $Kd_1 = 20.2 \pm 1.3$ (× 10^{-9} M), $Rt_1 = 4.7 \pm 0.2$ (× 10^5 sites/cell), $Kd_2 = 941.0 \pm 156.4$ (× 10^{-9} M), $Rt_2 = 38.5 \pm 4.61$ (× 10^5 sites/cell).

At all concentrations tested D-galactose showed non-competitive inhibition of GSA binding to cultured hepatocytes (Fig. 2). The Ki value for D-galactose was 505 ± 38 mM (n = 4).

Endocytosis study

The galactose inhibited GSA internalization into hepatocytes. In internalized ligand there were significant differences between treatments with no galactose (control) and 27 mM galactose at 13.2 and 65.8 nM of GSA (Fig. 3). Internalized GSA was reduced in altered SFM as compared to SFM, but this reduction was less than that caused by 270 mM galactose (Fig. 4).

DISCUSSION

In this study, two different binding sites of GSA on the cultured rat hepatocytes were suggested. High affinity dissociation constant (Kd_1) of GSA for ASGP-R was about 3 times higher than the reported Kd of asialo-orosomucoid for ASGP-R.[18] The high affinity receptor number (Rt_1) of GSA for ASGP-R in the cultured hepatocytes was quite similar to that reported previously.[18] Our studies indicated that D-galactose inhibits GSA binding to rat cultured hepatocytes non-competitively. Nevertheless, the Ki value for D-galactose was considerably higher, but this concentration cannot be achieved *in vivo* if the galactose is administered intravenously prior to scintigraphy at the levels used (–10 mM) in the galactose elimination capacity test.[13,14] Galactose inhibited endocytosis of GSA at lower concentrations, which can be achieved *in vivo* if the galactose is administered intravenously prior to scintigraphy at the levels used in the galactose elimination capacity test.[13,14]

Rat ASGP-R is known to be an integral transmembrane glycoprotein composed of three polypeptide subunits known as rat hepatic lectins (RHL).[19] Recently the RHL-1 subunit was found to be the same as lactoferrins (Lf) binding receptor, and binding occurs in a galactose-independent manner. Furthermore, at large molar excesses, β-lactose progressively blocked hepatocyte interaction of ^{125}I-Lf and of ^{125}I-asialo-orosomucoid.[20]

Galactose, a constituent of milk sugar, is a major source of calories for children, as well as an important component of glycolipids and glycoproteins. The conversion of galactose to glucose is mediated by a series of four enzymes located within the soluble fraction of hepatocytes, erythrocytes and renal cells. Two clinical syndromes, galactokinase deficiency galactosemia and uridyl transferase deficiency galactosemia, in which phosphorylation by the galactokinase takes place, are in a more serious clinical state. Galactose is usually ingested as the disaccharide lactose, which is hydrolyzed by lactase to glucose and galactose. Galactokinase catalyzes a phosphotransferase reaction involving MgATP and galactose that results in the production of galactose-1-phosphate. These processes have been studied in rat, pig liver, human red cells and placenta. Enzymatic regulation involves kinetic factors, substrate and cofactor availability, competing reactions for the same cofactors, and compartmentalization within the cell. Activities of these enzymes are modified by phosphorylation and dephosphorylation, controlled by hormones and cyclic AMP.[12] It

was determined that ASGP-R is a phosphoprotein, and rapid regulation of receptor activity occurs through the phosphorylation and dephosphorylation cycle. Therefore, phosphorylation and dephosphorylation of D-galactose might affect the rapid regulation of ASGP-R activity.

In conclusion, it was elucidated that D-galactose inhibited GSA internalization rather than binding at a low concentration, and this can be achieved *in vivo* if the galactose is administered intravenously at the levels used in the galactose elimination capacity test.[13,14] Further experimental *in vivo* studies in rats are needed to investigate whether galactose makes ^{99m}Tc-GSA scintigraph more sensitive in detecting minor liver dysfunction in clinical work.

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