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Interaction Study between Synthetic Glycoconjugate Ligands and Endocytic Receptors Using Flow Cytometry

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Flow cytometric analysis of synthetic galactosyl polymers, asialofetuin and LDL derivatives labeled with FITC (Fluorescein Isothiocyanate) was carried out to determine the phenotypes of endocytic receptors, such as asialoglycoprotein (ASPG) and the LDL receptor, on various types of cells. When FITC-labeled galactosyl polystyrene (GalCPS), being a synthetic ligand of ASPG, was applied to rat hepatocytes and human cancer cells (Hep G2 and Chang Liver), surface fluorescence intensities varied according to receptor expression on the cells. The fluorescence intensity originates from the calcium-dependent binding of the FITC-labeled GalCPS. Although unaltered by pre-treatment with glucosyl polystyrene (GluCPS), fetuin and LDL, the fluorescence intensity was suppressed by pre-treatment with (non-labeled) GalCPS and asialofetuin. Flow cytometry allowed us to demonstrate that the calcium-dependent binding of FITC-labeled LDL (prepared from rabbits) upon the addition of 17α-ethinyl estradiol enhances LDL receptor expression, and the expression is suppressed upon the addition of a monoclonal antibody to the LDL receptor. The binding efficiency based on the combination of FITC-labeled ligands suggests a possible application for the classification of cell types and conditions corresponding to endocytic receptor expression without the need for immuno-active antibodies or radiolabeled substances. Furthermore, the synthetic glycoconjugate (GalCPS) is shown to be a sensitive and useful marker for classification based on cell phenotype using flow cytometry.

Key words: asialo-glycoprotein receptor, flow cytometry, LDL receptor, synthetic glycoconjugate ligand.

Abbreviations: GalCPS, Galactose-carrying polystyrene; GluCPS, Glucose-carrying polystyrene; ASGP, asialo-glycoprotein; Hepatocytes, liver parenchymal cells; LDL, Low density lipoprotein; FITC, Fluorescein Isothiocyanate; HBSS, Hank's balanced salt solution; FCS, fetal calf serum; NaN_3, sodium azide.

Membrane proteins of mammalian hepatic cells participate in the uptake of extracellular molecules by receptor-mediated endocytosis. Asialo-glycoprotein (ASGP) receptors interact specifically with glycoproteins containing galactose or *N*-acetylgalactosamine residues at the non-reducing terminals of oligosaccharide chains to mediate their endocytosis and lysosomal degradation (*1–3*). Low density lipoprotein (LDL), the major cholesterol transport protein in plasma, enters the cell through coated pits by receptor-mediated interaction with apolipoprotein B/E receptors (*4–6*), and is subject to lysosomal degradation. Liver diseases are often associated with defects in these endocytic receptors. It has been reported that the expressions of ASGP receptors (*7–9*) is decreased significantly by malignant differentiation of hepatomas, as well as by proliferative, premature-hepatocytes (*10*, *11*). Furthermore, liver injury resulting from hepatitis frequently in the cholesterol level in the blood, and induced cholestasis leads to an increase in cholesterol by the production of a lipoprotein isoform (*12*). Thus, hepatic disease is due to a disorder in the lipoprotein-metabolic system involving disfunctioning LDL receptors.

Several studies have shown that synthetic and semi-synthetic asialo-glycoconjugates (*13–17*) interact with ASGP-receptors on mammalian hepatocytes. We also have reported that galactose-carrying polystyrene (GalCPS: poly [*N*-*p*-vinylbenzyl-*O*-β-D-galactopyranosyl-(1→4)-D-gluconamide]) interacts with rat hepatocytes (*10*, *11*, *18)*. When hepatocytes were cultured on a polymeric (polystyrene) dish coated with GalCPS, a calcium-dependent attachment (*14*) was observed that subsequently promoted the formation of three-dimensional multi-cellular aggregates (*19*) on the GalCPS-coated dishes. These findings suggest that GalCPS can be used as a specific marker for cells expressing the ASGP receptor.

Flow cytometry (*20*, *21*) with fluorescent antibodies is an established technique enabling the determination of various individual cell characteristics, by which subpopulations of peripheral blood mononuclear cells can be analyzed and classified (*22*). In addition, flow cytometry one make it possible to carry out multi-parametric assays due to the variety of antibodies using a combination of light scattering and induced fluorescence (*20*, *21*). In the present study, synthetic GalCPS and natural ligands,

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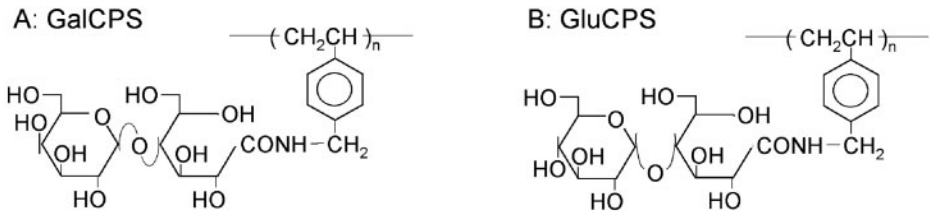

Fig. 1. **Structures of poly-*N*-*p*-vinylbenzyl-β-D-lactonamide (A: GalCPS) and poly-*N*-*p*-vinylbenzyl-α-D-malutonamide (B: GluCPS).** These polymers were labeled with FITC for use in flow cytometry.

i.e. asialofetuin and LDL for endocytic receptors (ASGP and LDL receptors) on mammalian cells, were conjugated with FITC. The FITC-labeled ligands were applied to rat hepatocytes and human cancer cells (Hep G2 cells and Chang Liver cells) expressing endocytic receptors to different extents. And to distinguish normal cells from cancer cells, the interaction of those ligands with the endocytic receptors on the cells was estimated by flow cytometry.

MATERIALS AND METHODS

Preparation of Cells—Rat liver parenchymal cells (hepatocytes) were isolated from female Sprague-Dawley (SD) rats (150–200 g; CLEA Japan Inc., Tokyo, Japan) using a two-step collagenase perfusion technique including some modifications, as reported previously (*23*). Briefly, a cannula was inserted from the portal vein, and subsequently the sub-hepatic inferior vena cava was cut. The liver was perfused through the cannula with PBS (Ca^{2+} and Mg^{2+}-free) containing 0.5 mM of EDTA (Gibco Laboratories, Grand Island, NY, USA) at 37°C until the blood in the liver was completely removed. Then the solution was exchanged with collagenase (0.05 wt%; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and trypsin inhibitor (0.005 wt%; Sigma Chemical Co., St. Louis, MO, USA) solution. After 5 min of perfusion, the liver was excised and dispersed in cold Hank's balanced salt solution (HBSS). The cells obtained from the collagenase-treated liver were then collected by filtration through a 300-gauge mesh. Parenchymal hepatocytes were separated by centrifugation (50 g, 1 min) 5 times in HBSS. The isolated hepatocytes were suspended in William's E medium supplemented with 10 mM HEPES and 1 mg/ml BSA (Wako Pure Chemical Industries, Ltd.). In order to enhance LDL receptor expression, 17α-ethinyl estradiol (Sigma Chemical Co.) was administrated to rats for 2 days (*24–26*).

Chang liver cells and Hep G2 cells (human cancer cell line; Riken Cell Bank, Saitama, Japan) were cultured in MEM (ICN Biomedicals Inc., Costa Mesa, CA, USA) containing 10 wt% of FCS (fetal calf serum). The cells were treated for 2 min for detachment with an aqueous trypsin solution (GIBCO Laboratories) containing 0.5 mM EDTA, and were incubated in the same medium for 90 min at 37°C prior to use.

Preparation of FITC-Labeled Conjugates—GalCPS was synthesized *via* homopolymerization of *N*-*p*-vinylbenzyl-*o*-β-D-galactopyranosyl-(1→4)-D-gluconamide (*18*, *23*). GluCPS was also synthesized *via* honopolymerization of *N*-*p*-vinylbenzyl-4-*O*-α-d-glucopyranosyl-(1-4)-D-gluconamide (*23*). FITC (50 mg, 1.28×10^{-4} mol; FITC-I form; Wako Pure Chemical Industries, Ltd.) and dibutyltin dilaurate (15 mg; Wako Pure Chemical Industries, Ltd.) were added to a GalCPS solution (500 mg, 1.0×10^{-3} mol monomer unit) in 5 ml of dried DMSO containing 3 drops (about 0.1 ml) of dried pyridine. The mixture was heated for 2 h at 90°C and poured into an excess amount of ethanol. The products were purified by dissolving them in water and precipitating 3 times with ethanol. FITC-labeled GalCPS was finally collected by filtration. One molecule of FITC was bound to every 40 structural units of a polymer chain as determined by fluorescence spectrometry (Spectrofluophotometer RF-500; Shimazu Corp., Kyoto, Japan). FITC-labeled GluCPS was prepared using the same procedure as FITC-labeled GalCPS. The structures of GalCPS and GluCPS are shown in Fig. 1.

Plasma containing hyper lipoproteins was collected from the peripheral blood of rabbits (Japanese White Rabbits; 2 kg, male, CLEA Japan Inc.) that had been fed with a semi-synthetic diet containing 5 wt% cholesterol for several months. LDL was purified by Hatch's method using ultra centrifugation (*27*). FITC (25 mg) was added to 60 ml of LDL solution (2.1 mg/ml, pH 8.5), and the solution was stirred for 4 h at 4°C. After filtration, non-reacted FITC was removed by ultra centrifugation twice (5.5×10^3 rpm, 3 h). Fetuin (Sigma Chemical Co.) and asialofetuin (Sigma Chemical Co.) in 0.1 M of carbonate buffer (pH 8.5), FITC (20 mg, 0.05 wt%) were added to each sample of fetal calf serum (1 wt%), and the samples were stirred for 4 h at 4°C. FITC-labeled conjugates were purified through a Sephadex G-50 (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) column.

Flow Cytometry—FITC-labeled conjugates were dissolved in PBS containing 0.1 wt% BSA and NaN_3 (Sodium azide). The cells (5.0×10^5 cells) were added to the FITC-labeled conjugate solution and were incubated in an ice bath at 4°C for 40 min with occasional shaking. The cells were washed twice with cold PBS at 4°C and diluted to $50–100 \times 10^4$ cells/ml.

Non-labeled GalCPS, GluCPS, asialofetuin, fetuin, and LDL were dissolved in PBS [containing Ca^{2+} (as 0.1 mg/ml $CaCl_2$ (anhyd.)) and Mg^{2+} (as 0.1 mg/ml $MgCl_2 \cdot 6H_2O$)] to obtain the indicated concentrations. For binding-inhibition assays, hepatocytes were pre-treated with PBS (including Ca^{2+} and Mg^{2+}) containing unlabeled ligand for 40 min at 4°C. Hep G2 and Chang Liver cells were pre-treated with 50 μl of monoclonal antibody against human LDL receptor [100 μg/ml of IgG, LDL-R (AB-1); Oncogene Science, Inc., Uniondale, NY, USA] for 40 min at 4°C. After the pre-treatments, FITC-labeled conjugates were applied to the pre-treated cells. For calcium-dependent binding assays of FITC-labeled ligands to the cells, FITC-conjugates were dissolved in PBS without Ca^{2+} and Mg^{2+} containing 0.5 mM EDTA, 0.1 wt% of BSA and NaN_3 and applied to the cells.

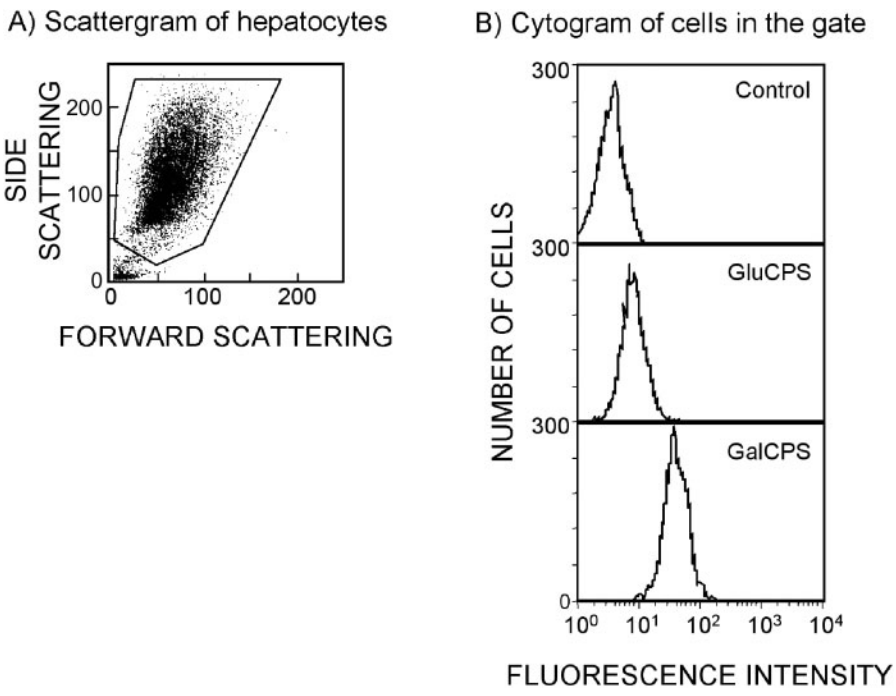

Fig. 2. **Flow cytometry with FITC-labeled GalCPS and GluCPS on rat hepatocytes.** The light scattering of cells (A) treated with FITC-labeled GalCPS and GluCPS was determined in a flow cytometer. Gated scattering shows the fraction of hepatocytes developing the cytograms (B). The control indicates a cytogram of untreated hepatocytes.

The cell-suspensions were added into a flow cytometer (FACScan; Becton Dickinson Immunocytometry Systems, San Jose, CA), and the fluorescence intensity on the cells was measured. In addition, the intensity with FITC-labeled GalCPS (0.01 and 0.05 wt%) and FITC-labeled GluCPS (0.01 wt%) were determined as reference values to estimate binding to the ASGP-receptor and LDL receptor, respectively.

RESULTS

Synthetic FITC-Labeled Ligands on Rat Hepatocytes Detected by Flow Cytometry—GalCPS, GluCPS, asialofetuin, fetuin and LDL were labeled with FITC. Hepatocytes were subsequently treated with these FITC-labeled conjugates in PBS (containing Ca^{2+} and Mg^{2+}) supplemented with 0.1 wt% of BSA and NaN_3, and subjected to flow cytometry. Depending on the cellular size, forward and side angle light scattering developed (Fig. 2A). The light scattering of hepatocytes is caused by fluorescence intensity on the plasma membrane (Fig. 2B). When the cells were treated with FITC-labeled GalCPS, a higher fluorescence intensity was measured. While the shift in the spectrum of FITC-labeled GluCPS-treated cells was smaller than FITC-labeled GalCPS. Similarly, the shifts in the spectra of the FITC-labeled proteins (asialofetuin, fetuin, and LDL) were smaller than FITC-labeled GalCPS (data not shown).

Calcium-Dependent Binding of FITC-Labeled Ligands to Rat Hepatocytes—FITC-labeled GalCPS and GluCPS in PBS (containing Ca^{2+} and Mg^{2+}) were applied to rat hepatocytes, and the fluorescence intensity was measured by flow cytometry, and the fluorescence intensity of the cells with 0.1wt% FITC-labeled GalCPS or asialofetuin was then calculated as relative fluorescence intensity. The FITC-labeled GalCPS yielded significant fluorescence on hepatocytes at low concentrations (10^{-3} to 10^{-4} wt%), while the application of FITC-labeled GluCPS did not result in high fluorescence intensity at these low concentrations (Fig. 3A). The strong fluorescence resulting from the interaction with FITC-labeled GalCPS at low concentrations dramatically decreased in the absence of calcium ions (Fig. 3A). The interaction between FITC-labeled asialofetuin and hepatocytes is also calcium-depended (Fig. 3B). Only a weak calcium-dependence was found in the interaction between FITC-labeled fetuin and hepatocytes (Fig. 3B).

Competitive Inhibition of the Binding of FITC-Labeled GalCPS on Hepatocytes—The effect of various competing inhibitors on the cell binding of FITC-labeled GalCPS was investigated on hepatocytes (Table 1). Although pretreatment of hepatocytes with GluCPS, fetuin and LDL had no influence on the binding to FITC-labeled GalCPS [respective relative fluorescence intensity 94.2, 95.6 and 94.3%, with non-treated hepatocytes (100%)], the relative fluorescence intensities were significantly reduced by pretreating with asialofetuin and GalCPS to 81.7% and 34.9%, respectively.

Binding of FITC-Labeled GalCPS and Asialofetuin to Hepatocytes and Cancer Cells—FITC-labeled GalCPS and asialofetuin were applied to rat hepatocytes and human cancer cell lines, *i.e.* Hep G2 and Chang Liver cells. Hep G2 cells, a human hepatoma cell line, maintain many liver-specific functions (*28*, *29*), as well as the expression of ASGP receptors (*30*, *31*). Chang Liver cells from a human hepatic tumor can be converted into the cells expressing the Hela marker (*32*, *33*); to our knowledge, there has been no report identicating that Chang Liver cells are able to express ASGP receptors.

FITC-labeled GalCPS and asialofetuin were detected on hepatocytes, Hep G2 cells and Chang liver cells (Table 2). The relative fluorescence intensity resulting from 0.01 wt%

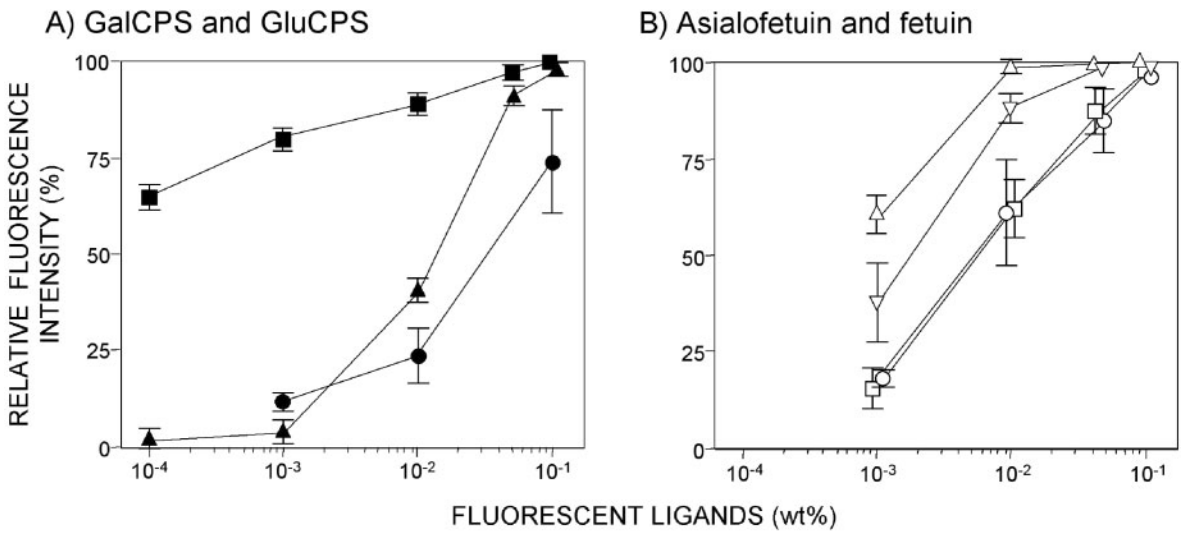

Fig. 3. **Calcium-dependent binding of FITC-labeled ligands to hepatocytes.** Hepatocytes were incubated for 40 min at 4°C in phosphate-buffered saline (PBS) containing FITC-labeled GalCPS (solid squares), GluCPS (solid circles), fetuin (inverted triangles), or asialofetuin (open triangles). Hepatocytes were also incubated in PBS without Ca^{2+} and Mg^{2+} with EDTA (0.5 mM) containing FITC-labeled GalCPS (solid triangles), fetuin (open squares), or asialofetuin (open circles).

Table 1. **Effect of pre-treatment with competing molecules on the specific binding of FITC-labeled GalCPS to hepatocytes.**

Reagents for pre-treatment[a]	Relative fluorescence intensity of GalCPS[b]
None	00 ± 1.6
GalCPS	4.9 ± 3.3
GluCPS	4.2 ± 1.1
Fetuin	5.6 ± 1.2
Asialofetuin	1.7 ± 2.9
LDL	4.3 ± 0.9

Hepatocytes were suspended in PBS containing 0.1 wt% BSA and 0.1 wt% NaN_3. GalCPS, GluCPS, fetuin, asialofetuin, or LDL as pre-treatment reagent was added to the suspension. Hepatocytes were incubated 40 min at 4°C for in PBS containing 0.01 wt% of FITC-labeled GalCPS after pre-treatment. The relative fluorescence intensities were objectively estimated by flow cytometry to measure the surface fluorescence intensities of cells with FITC-labeled GalCPS. [a]GalCPS, GluCPS, fetuin, asialofetuin and LDL were dissolved in PBS to obtain concentrations of 0.05 wt%, and 2.5 mg/ml. The binding assay of FITC-labeled GalCPS to hepatocytes without pre-treatment (None) was carried out as a control. [b]Relative fluorescence intensity was calculated as the value resulting from FITC-labeled GalCPS (0.05 wt%).

Table 2. **Receptor-mediated binding of galactose-carrying materials to cells.**

FITC-labeled ligand	Relative fluorescence intensity (%) on:		
	Hepatocytes	Hep G2	Chang liver
GalCPS	88.1 ± 0.8	34.4 ± 0.6	5.9 ± 4.5
Asialofetuin	59.2 ± 4.9	16.8 ± 4.0	13.5 ± 1.5

Fluorescence intensity of cells was measured in a flow cytometer after incubation with the fluorescent material for 40 min at 4°C. Relative fluorescence intensities with FITC-labeled GalCPS and asialofetuin were calculated from standard fluorescence level resulting from 0.05 wt% FITC-labeled GalCPS and 0.01 wt% of FITC-labeled asialofetuin, respectively.

FITC-labeled GalCPS was determined to be 88.1, 34.4 and 5.9 percent on hepatocytes, Hep G2 cells and Chang Liver cells, respectively. And the relative intensity resulting from 0.001 wt% FITC-labeled asialofetuin was 59.2, 16.8 and 13.5 percent on hepatocytes, Hep G2 cells and Chang Liver cells, respectively. For both FITC-labeled ligands, the expression of the ASGP receptor on the cell surface followed the same order, *i.e.* hepatocytes > Hep G2 cells >> Chang Liver cells. Indicating that normal hepatocytes express ASGP receptors at a high level and that the concentration of ASGP receptors is significantly decreased in malignant cells (Hep G2 cells and Chang liver cells). Since applying FITC-labeled GalCPS (rather than FITC-labeled asialofetuin) resulted in a wider range of intensity variation (5.9 to 88.1%) for each cell type, FITC-labeled GalCPS as a synthetic ASGP analogue may be used as a functional and susceptible marker in flow cytometric analysis.

Specific Interaction of FITC-Labeled LDL with LDL Receptor—Several reports have described that the binding and up-take of human LDL in Hep G2 cells depends on the presence of calcium ions (*28*, *34*). This calcium dependence suggests a receptor-mediated interaction between LDL and the LDL receptor on the cell surface. However, when the binding of FITC-labeled rabbit LDL to rat hepatocytes was examined by flow cytometry, the fluorescence intensities resulting from FITC-labeled LDL on the cell surface (using PBS without Ca^{2+} and Mg^{2+} containing 0.5 mM EDTA) were reduced to only about 70% (data not shown).

A hormone to enhance LDL receptor expression on hepatocytes, 17α-ethinyl estradiol, was administered to rats, as described previously (*24–26*). Fluorescent LDL (2 mg/ml) was added to the hepatocytes obtained from the estrogen-administered rats, as well as control rats (no administration), and the fluorescence intensity on the cells was measured in a flow cytometer. The ratio of the relative fluorescence intensities of the estrogen-treated cells to control cells was calculated to determine the effect of estrogen. Furthermore, human Hep G2 cells and Chang Liver cells were treated with a monoclonal antibody against the

Table 3. **Effect of estrogen and anti-human LDL receptor antibody administration on the binding of FITC-labeled LDL.**

Cell types	Cell pre-treatment (operated cells/intact cells) in FITC-labeled LDL	Ratio of fluorescence intensity
Rat liver	Subcutaneous administration of hepatocytes 17α-ethinyl estradiol prior to cells	2.80 ± 1.05
Hep G2	Pre-treatment with antibody to human LDL receptor on isolated cells	0.84 ± 0.04
Chang liver	Pre-treatment with antibody to human LDL receptor on isolated cells	0.57 ± 0.21

Cells were subjected to either administration of 17α-ethinyl estradiol to enhance LDL receptor expression or a pre-treatment with monoclonal antibody to human LDL receptor. FITC-labeled LDL (2.0 mg/ml) dissolved in PBS containing BSA and NaN_3 (0.1 wt%) was added to 5×10^5 of hepatocytes and human cancer cells after cell pre-treatment, and the cells were successively incubated for 40 min at 4°C. Relative fluorescence intensity with FITC-labeled LDL was calculated from the intensity resulting from FITC-labeled GluCPS. The values, expressed as the ratio of fluorescence intensity on pre-treated cells to the intensity on intact (control) cells, are indicative of the receptor-mediated binding of FITC-labeled rabbit LDL. Data points are the averages of 3 independent experiments.

human LDL receptor. The change in LDL binding activity after antibody treatment was also determined from calculation of the fluorescence intensity ratios, as given in Table 3. The administration of estrogen resulted in a 2.8-fold higher intensity ratio, while the treatment with anti-LDL receptor antibody resulted in a decrease in the intensity ratio to 0.84 on Hep G2 cells and 0.57 on Chang Liver cells.

Classification of Cell Types and Conditions—Relative fluorescence intensity on rat hepatocytes, Hep G2 cells and Chang Liver cells was measured using flow cytometry with FITC-labeled GalCPS and GluCPS (0.01 wt%), and FITC-labeled asialofetuin (0.001 wt%) and fetuin (0.001 wt%). In order to distinguish between normal cells and cancer cells, the ratios of the fluorescence intensities of FITC-labeled GalCPS and FITC-labeled asialofetuin versus the intensities of their controls (FITC-labeled GluCPS and FITC-labeled fetuin, respectively) were calculated. A ratio >1.0 indicates enhanced binding of FITC-labeled GalCPS and FITC-labeled asialofetuin to ASGP receptors on the cells (Table 4). Preferential coupling of GalCPS and asialofetuin was observed on rat hepatocytes and Hep G2 cells. Especially, Hep G2 cells showed an extreme high ratio (21.1 ± 7.1), partly ascribed to a reduced intensity of FITC-labeled GluCPS (control). However, Chang Liver cells did not show an enhanced fluorescence intensity by FITC-labeled GalCPS and FITC-labeled asialofetuin (only 1.1 ± 0.1 and 1.1 ± 0.3, respectively). In addition, the relative fluorescence intensity on these cells was evaluated using FITC-labeled LDL in flow cytometry. The relative fluorescence intensity of FITC-labeled LDL (5.0 mg/ml) was calculated from the standard fluorescence induced by FITC-labeled GluCPS (Table 5). The intensity of normal hepatocytes was found to be higher than that of cancer cells, and it is possible to distinguish normal hepatocytes (70.8%) from hepatomas (Hep G2 cells: 59.2%) and cancer cells (Chang Liver cells: 54.4%).

Table 4. **The specificity of GalCPS and asialofetuin for cells.**

FITC-labeled	The ratio of fluorescence intensity on conjugates		
	Hepatocytes	Hep G2	Chang liver
GalCPS/GluCPS	2.2 ± 0.1	21.1 ± 7.1	1.1 ± 0.1
Asialofetuin/fetuin	2.2 ± 0.1	2.2 ± 0.4	1.1 ± 0.3

FITC-labeled GalCPS and GluCPS (0.01 wt%), and FITC-labeled asialofetuin and fetuin (0.001 wt%) were applied to rat hepatocytes and human cancer cells. Values represent the ratio of the fluorescense intensity with FITC-labeled GalCPS with GluCPS (control) and FITC-labeled asialofetuin with fetuin (control).

Table 5. **Binding of FITC-labeled LDL to cells expressing LDL receptor.**

Applied ligand	Relative fluorescence intensity (%) on		
	Rat hepatocytes	Hep G2	Chang liver
FITC-labeled LDL	70.8 ± 6.2	59.2 ± 2.4	54.4 ± 14.7

FITC-labeled LDL dissolved in PBS containing 0.1 wt% of BSA and 0.1 wt% NaN_3. The solution with fluorescent LDL (100 ml) was added to 5×10^5 of rat heptocytes and cancer cells, and then cells were then incubated for 40 min at 4°C for with occasional shaking. Relative fluorescence intensity with FITC-labeled LDL was calculated from the intensity resulting from FITC-labeled GluCPS (0.01 wt%).

DISCUSSION

The galactose/*N*-acethyl-galactosamine–specific receptor on rat hepatocytes (*1–3*), as well as the apolipoprotein B/E receptor (LDL receptor) on fibroblasts and cells arising from the liver (*4–6*), has been reported to be an endocytic receptor. The specific interaction between an endocytic receptor and its ligand could be applied in the biomedical field. Akaike *et al.* (*14*) synthesized GalCPS as a model ligand to the ASGP receptor. Hepatocytes were found to adhere to GalCPS-coated polystyrene dishes in a calcium-dependent way (*14*). The immobilization of GalCPS onto a solid support has served as a substrate for the primary culture of hepatocytes (liver parenchymal cells) (*14*, *23*). GalCPS, after being modified to contain drug-binding domains, can also be used as a drug carrier possessing an endocytic receptor–targeting property (*35*).

On the other hand, flow cytometry with fluorescence-labeled monoclonal antibodies has been used for phenotype determination of lymphocyte subpopulations and the classification of the subpopulations resulting from these phenotypes (*36, 37*). In general, flow cytometry may serve as a phenotype analysis technology by using various fluorescence-labeled antibodies, each with a different excitation wavelength, *i.e.* a multi-parameter assay. However, the use of a fluorescent monoclonal antibody has several restrictions in general and for easy handling. In addition to the high cost of obtaining the antibody, an antibody recognizes only some part of the specific ASGP receptors, and it does not recognize ASGP receptor in various other animals and tissues and other subtypes of ASGP receptors. Therefore we have to prepare multiple monoclonal antibodies or polyclonal antibodies for the total ASGP receptors. If a phenotypic assay is carried out using a fluorescent artificial ligand rather than a monoclonal antibody, applications in clinical diagnosis may be feasible. Therefore,

FITC-labeled ligands to endocytic receptors were prepared and used as specific markers for cells arising from liver. Flow cytometric analysis using these FITC-labeled ligands has been carried out.

These experiments provide evidence of a specific interaction between the galactose group of the ligand and the ASGP receptor. When FITC-labeled asialofetuin and GalCPS were applied to isolated hepatocytes, calcium-dependent binding occurred at various concentrations of FITC-labeled ligand. The binding specificities of FITC-labeled asialofetuin and GalCPS were presented in comparison with FITC-labeled fetuin and GluCPS. Especially, synthetic FITC-labeled GalCPS was found to be a specific functional ligand for the ASGP receptor, rather than FITC-labeled asialofetuin (Fig. 3). The formation of GalCPS micelles has been confirmed by Goto and associates (*35*). From their report, it was concluded that the hydrophobic styrene in GalCPS is hidden in the core surrounded by hydrophilic sugars chains forming a shell in contact with the aqueous solution. Consequently, micelle formation by GalCPS results in a high density of functional galactose groups towards ASGP receptors on cells, and thus increasing the specific interactions. A blocking assay with unlabeled GalCPS, asialofetuin and their controls also supports the high specificity of GalCPS (Table 1).

Hep G2 cells (hepatoma from human liver) are frequently used to analyze the expression dynamics of ASGP receptors (*30*, *31*). No reports exist describing Chang Liver cells expressing ASGP receptors. Flow cytometry with rat hepatocytes, Hep G2 cells and Chang Liver cells produced evidence of an ASGP receptor–mediated interaction with FITC-labeled GalCPS and asialofetuin (Table 2). FITC-labeled GalCPS and asialofetuin quantified the interaction with the ASGP receptor in the following order: hepatocytes > Hep G2 cells >> Chang Liver cells. This finding is in agreement with another report that hepatic cancer cells as well as proliferative, premature-hepatocytes show decreased ASGP receptor expression (*10*, *11*, *38*).

LDL is incorporated and endocytosed into hepatocytes *via* the LDL receptor, a process with the physiological function of controlling the concentration of cholesterol in the blood (*5*, *6*). The receptor-mediated interaction of rabbit LDL with hepatocytes was induced by the administration of estrogen, which is known to enhance LDL receptor expression (*24–26*). The fluorescence intensity ratio on hepatocytes induced by estrogen was significantly increased (as compared with a control), and showed a much lower fluorescence intensity after pre-treatment of the LDL receptor on Hep G2 and Chang liver cells with an immuno-active antibody (Table 3), a finding identical to our previous report (*39*). These results show that flow cytometric analysis can demonstrate the receptor-mediated binding of rabbit LDL to rat hepatocytes and human cancer cells, including the LDL-metabolic system via the receptor. In addition, the use of fluorescent LDL and FITC-labeled GluCPS, which do not bind specifically to endocytic receptors, provide those cells (hepatocytes and cancer cells) with a stable and low-level fluorescence intensity background.

The expression of the ASGP receptor by various cells was evaluated using the fluorescence intensity ratio of FITC-labeled GalCPS binding over FITC-labeled GluCPS binding, as well as the fluorescence intensity ratio of FITC-labeled asialofetuin binding over FITC-labeled fetuin binding. The results show characteristic values according to cell type (Table 4). Especially, the fluorescence intensity ratio of FITC-labeled GalCPS over FITC-labeled GluCPS on Hep G2 cells resulted in a remarkable figure due to the high fluorescence intensity with FITC-labeled GluCPS. The difference in the fluorescence intensity ratio between Hep G2 cells and hepatocytes may reflect differences in the plasma membrane of each cell type, as well as the expression level of ASGP. On the other hand, the fluorescence intensity of hepatocytes resulting from the binding of FITC-labeled LDL was higher than the intensity of human cancer cells (Table 5). Thus, flow cytometric analysis using FITC-labeled ligands for receptors that mediate endocytosis is found to be useful in phenotyping, and does not require the use of a monoclonal antibody. In particular, FITC-labeled synthetic glycoconjugates, such as GalCPS, have been a successful applied to flow cytometry.

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