Specific Binding of Glucose-derivatized Polymers to the Asialoglycoprotein Receptor of Mouse Primary Hepatocytes*

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Sang-Heon Kim†‡, Mitsuaki Goto§, and Toshihiro Akaike†¶

From the †Department of Biomolecular Engineering, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8501, Japan and §BioQuest Research Ltd., 1-15-16 Minami-Aoyama, Minato, Tokyo 107-0062, Japan

In this study, we designed a novel amphiphilic poly-((p-N-vinylbenzyl-4-O-glucuronamide) (PV6Gna) modified at the 6-OH position of glucose for hepatocyte recognition to address the mechanism of the interaction between mouse primary hepatocytes and the PV6Gna. PV6Gna bound to lectins specific for glucose but not galactose as did other glucose-derivatized polymers. However, hepatocyte adhesion onto the PV6Gna surface was inhibited in the presence of galactose and its analogues but not in the presence of glucose and its analogues. We also showed that hepatocyte adhesion to the PV6Gna surface was inhibited dose dependently by asialofetuin (ASF). Interactions between soluble PV6Gna and hepatocytes were inhibited by GalNAc, ASF, and EGTA in flow cytometry analysis using fluorescein isothiocyanate-conjugated PV6Gna. Hepatocyte adhesion to the PV6Gna surface was inhibited more effectively by GalNAc than by methyl β-D-galactose. In flow cytometry analysis and cell adhesion assay, ASF competed for the inhibition of interaction between PV6Gna and hepatocytes 0.5–4 × 10^4-fold more effectively than did GalNAc. These results demonstrate involvement of asialoglycoprotein receptors (ASGPRs) in the interaction between PV6Gna and hepatocytes. Furthermore, to clarify the mechanism of the interaction between glycopolymers modified at the 6-OH position of glucose and the hepatocyte, we prepared a gel particle containing 6-O-methacryloyl-β-D-glucose (PMglc) synthesized by an enzymatic method. ASGPRs could be detected using Western blot analysis following precipitation with PMglc in hepatocyte cell lysate. The precipitation of ASGPRs was inhibited in the presence of galactose, ASF, PV6Gna, and EGTA. The precipitation was inhibited more effectively by GalNAc than by methyl β-D-galactose. ASGPRs were rarely precipitated by PMglc in the cell lysate that had been treated with ASF-conjugated Sepharose. Taken together, we suggest that mouse primary hepatocytes adhere to the PV6Gna surface mediated by ASGPRs, which specifically interacted with the glycopolymers modified at the C-6 position of glucose.

Carbohydrate-mediated interactions play an important role in biological processes such as receptor-mediated endocytosis, opsonization, apoptosis, and metastasis and have been applied to cell recognition studies as well as designs for biomedical materials (1–5). Multivalent glycopolymer ligands have been designed for clustering of L-selectin leading to the activation of the leukocyte cell surface and the inhibition of L-selectin-mediated leukocyte rolling (6, 7). Interaction between the carbohydrate and the carbohydrate-binding proteins (CBPs) is achieved through hydrogen bonding of the hydroxyl group of the carbohydrate to the polar amino acid side chain of the protein, including the packing of a hydrophobic sugar face against the aromatic amino acid side chain of the protein (8). CBPs interact with a particular carbohydrate by recognizing subtle differences in carbohydrate structure (9, 10). Thus, the carbohydrate specificity to the cells varies according to the kind of CBPs distributed on the cell. Asialoglycoprotein receptors (ASGPRs) are lectins for receptor-mediated endocytosis found at the hepatocyte cell surface that are bound to galactose/GalNAc-terminated ligands in a calcium-dependent manner (1, 11–14). Because ASGPRs are widely used as a model for the study of receptor-mediated endocytosis, galactose-derivatized materials have been a source applied to a cell-targeted drug delivery system for hepatocytes and as an artificial adhesion matrix for liver tissue engineering (15–18).

Recently we developed a novel amphiphilic glucose-derivatized PV6Gna as an artificial adhesion matrix for hepatocyte cultures and investigated the effects of the substituted position of the hydroxyl group of glucose on recognition of mouse hepatocytes (19). Mouse primary hepatocytes were specifically recognized by PV6Gna due to the modification of the C-6 position of glucose but not the C-1 and C-3 positions. The adhesion of hepatocytes to the PV6Gna surface was dependent on Ca^{2+} and independent of temperature unlike integrin-dependent adhesion. These results indicated that hepatocytes might be interacting with PV6Gna mediated by a CBP such as a glucose transporter as well as an ASGPR on the cell membrane surface. Mammalian hepatocytes were mainly recognized by glycopolymers that act as natural and artificial ligands having terminal galactose/GalNAc moieties, although chicken hepatocytes were recognized by terminal GlcNAc moieties (20–22). According to these reports, there are many reports that address the behavior of hepatocyte adhesion to artificial polymer surfaces having termi-

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† To whom correspondence should be addressed. Tel.: 81-45-924-5790; Fax: 81-45-924-5815; E-mail: takaike@bio.titech.ac.jp.

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nal galactose moieties (23–25). We have also developed poly-(N-p-vinylbenzyl-4-O-β-D-galactopyranosyl-0-gluconamide) (PVLA) having terminal galactose moieties as an artificial matrix for hepatocyte cultures (26). Previous studies examined hepatic functions and gene regulation of hepatocytes cultured on a PVLA surface compared with different extracellular matrices (27, 28).

To our knowledge, PV6Gna is the first polymer having glucose moieties capable of binding to mammalian hepatocytes. In this study, we evaluated the selectivity of PV6Gna against lectins and further found that the mechanism of binding of PV6Gna to hepatocytes was caused by recognition between ASGPRs and glucose moieties.

**EXPERIMENTAL PROCEDURES**

### Materials

Glycopolymers including PV6Gna were synthesized and conjugated with fluorescein isothiocyanate (FITC) according to a method described previously (19). 13C NMR spectra were recorded on a Varian UNITY plus 400 spectrometer using tetramethylsilane as external standard. Polyethylene (PS) plates or dishes were obtained from Becton Dickinson. Biotin-labeled lectins and peroxidase-conjugated streptavidin were purchased from EY Laboratories and Vector Laboratories, respectively. Monosaccharides and glycosides were purchased from Wako Pure Chemical (Osaka, Japan) except for methyl α-D-glucose, methyl β-D-glucose, and α-D-galactose acid, which were obtained from Nacalai Tesque (Kyoto, Japan). Collagenase and trypsin inhibitor were purchased from Wako Pure Chemical and Nacalai Tesque, respectively. Williams’ medium E, Dulbecco’s modified Eagle’s medium, and fetal bovine serum were purchased from Life Technologies, Inc. Asia.

### EXPERIMENTAL PROCEDURES

**Polyethylene-glycol conjugates**

PV6Gna were selected from the region of PI-unlabeled hepatocytes to nonspecific adhesion. Nonadherent hepatocytes were removed from the dish by washing with PBS three times, and protein was extracted from the adhered hepatocytes with 0.25 wt% NaOH solution. The percentage of cell adhesion was determined from the amount of protein measured by the improved Lowry method (DC Protein Assay kit). All values were normalized to the percentage of adhesion of the maximum, which was taken as 100%.

**Preparation of Erythrocytes and Cell Line Culture**

Blood (0.1 ml) was collected from the heart of the ICR mouse, diluted with 0.04% EDTA (0.5 ml) in PBS solution, and washed with PBS solution (0.5 ml) by three centrifugations (1000 rpm, 5 min). Hepa-1 and NIH 3T3 cells were incubated in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum under a humidified atmosphere of 5% CO2 and 95% air at 37°C. Human umbilical vein endothelial cells were incubated in Endothelial Cell Growth Medium BulletKit-2 (EGM-2 BulletKit, Clonetics) under the same conditions described above. The media was renewed twice weekly.

### Flow Cytometry Analysis

Freshly isolated hepatocytes (5 x 10^6 cell/ml) were incubated with 100 μg/ml FITC-conjugated PV6Gna in Williams’ medium E containing 0.1 wt% BSA and 1 μg/ml propidium iodide (PI) for 1.5 h at 4°C. 1% NaN3 was added in the lysates in advance. Pellets were washed with the fresh media three times by centrifugation (100 x g, 5 min) and suspended in the fresh media until used for experiments.

**Preparation of PMgcl and ASGPR Precipitation Assay**

PMgcl was prepared as a new gel particle containing 6-O-methyl-1,2,3-tri-O-benzyl-d-glucopyranosylamine hydrochloride synthesized by a commercially available protease that catalyzes trans-galactosylation of glucosamine. The product was isolated by silica gel column chromatography and characterized by 13C NMR and HPLC analysis. The product was gelated in the presence of ammonium peroxodisulfate as an initiator in H2O for 30 min at 55°C and sonicated to make a fine particle. PMgcl was used following removal of large particles by filtering with a 4-μm membrane filter. For ASGPR precipitation with PMgcl, fresh isolated hepatocytes were solubilized in 1% sodium cholate, 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 wt% Nonidet P-40, 20 mM n-octyl-β-D-glucopyranoside, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 2 μg/ml aprotinin) for 1 h at 4°C. Lysates were then centrifuged at 15,000 rpm for 30 min at 4°C, and the PMgcl fraction was collected as described previously (31).

**RESULTS AND DISCUSSION**

Lectins Binding to PV6Gna—A direct lectin-enzyme assay was carried out to determine the type of the sugar moiety of PV6Gna as compared with other glycopolyomers shown in Fig. 1 that have glucose or galactose moieties. Dose-dependent binding of lectins to the glycopolyomers is shown in Fig. 2. ConA and LeCa showed a greater selectivity in binding to PV6Gna, PVG, and PV6G6c substituted C-1, C-3, and C-6 of glucose, respectively, than to PVLA and PVMEA, which have galactose moieties. Allo A bound only to PVLA, whereas PNA bound to both PVLA and PVMEA, and neither lectin bound to the glycopolyomers having glucose moieties such as PV6Gna. ConA and LeCa as lectins have specificity to α-D-mannose or α-D-glucose, whereas Allo A and PNA are lectins specific to β-D-galactose. 

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is represented by a peak at 92.7 ppm in Me$_2$SO. The C-2
not PVMA or PVG as shown in Fig. 4
been coated with 0.1 mg/ml PVLA, PVMEA, and PV6Gna but
cytes exclusively adhered onto the polystyrene surface that had
hepatocytes, a cell adhesion assay was carried out on a poly-
that PV6Gna is a polymer that has glucose moieties and binds
Inhibition of Hepatocyte Adhesion to PV6Gna Surface Exclu-
sively by Galactose-type Carbohydrates—We investigated the
selectivity of the carbohydrate against the CBP used in the
adhesion. Table I presents the inhibitory effects of various
carbohydrates on the hepatocyte adhesion onto the PV6Gna
surface. Hepatocyte adhesion to the PV6Gna surface was
dramatically reduced to -4% of control adhesion, which represents
hepatocyte adhesion to the PV6Gna surface in the absence of
competitive inhibitors and in the presence of 20 mM of D-
galactose (Gal), 4-O-β-D-galactopyranosyl-D-glucose (lactose),
methyl β-D-galactoside (methyl β-gal), 6-O-α-D-galactopyranosyl-
D-glucose (melibiose), and GalNAc but not in the presence of
20 mM of glucose and its analogues. It is very interesting that the
galactose-type carbohydrates exclusively inhibited hepatocyte
adhesion to the PV6Gna that has glucose moieties. Our
previous study showed that hepatocyte adhesion onto the
PV6Gna surface occurred in a temperature-independent and Ca$^{2+}$-dependent manner (19). Taken together the results
indicate a relationship of the C-type lectin such as ASGPR with
hepatocyte adhesion to the PV6Gna surface.

The Relationship of ASGPR to the Interaction of Hepatocytes
and PV6Gna—We examined the inhibitory effect that is
dependent on the concentration of those galactose-type carbohy-
drates on the hepatocyte adhesion onto the PV6Gna surface.
Hepatocyte adhesion to the PV6Gna surface was almost completely
inhibited by 1 mM GalNAc, 5 mM methyl β-gal, 10 mM
lactose, and 20 mM melibiose, respectively, as shown in Fig. 5.
Numerous studies have determined the difference of the affinity
for carbohydrates of C-type lectins by using an inhibition
binding assay and crystallographic analysis (36–40). For
example, mannos-binding proteins have specificity for mannose,
glucose, fucose, or GlcNAc, all of which have the equatorial
hydroxyl group at the C-4 position, whereas mammalian hep-
atic lectins have specificity for galactose/GalNAc, which has
the axial hydroxyl group of C-4. Despite 85% identity in the
sequences of both the hepatic and macrophage receptors, they
have distinct sugar binding properties. The hepatic receptor
binds GalNAc with a greater affinity than Gal, whereas the
macrophage receptor binds Gal and GalNAc with almost equal
affinity (38). Specifically, the rabbit and rat ASGPRs bound
to GalNAc with a higher affinity than Gal. A terminal β-D-galac-
topyranosyl residue bound to the ASGPRs with a higher affinity
than a α-D-galactopyranosyl residue and galactose when
assaying the ability of the carbohydrates to inhibit 125I-labeled
asialoorosomucoid binding to the liver plasma membrane or
the isolated lectin (36). In competition for binding to the carbo-
hydrate recognition domain of rat hepatic lectin-1, GalNAc
showed a greater binding affinity due to the effects of the acyl
group of GalNAc binding to the carbohydrate recognition do-
main than did Gal (39). We show in Fig. 5 that GalNAc inhib-
ited hepatocyte adhesion to PV6Gna with between -5- and
20-fold higher affinity than the other carbohydrates, indicating
that GalNAc has the most inhibitory effect on hepatocyte ad-
hesion to the PV6Gna surface, and β-D-galactopyranosyl
showed more of an increase of the inhibitory effect on the
adhesion than did α-D-galactopyranosyl. Interestingly this
means that hepatocytes may adhere to the PV6Gna surface
through ASGPR on the cell surface membrane. To verify the
possibility that ASGPR is mediating the interactions between
hepatocytes and PV6Gna, an inhibition cell adhesion assay was
carried out on the PV6Gna surface using soluble ASF, which
had been used as a natural ligand for ASGPR, as competitive
inhibitors against the ASGPR-PV6Gna interaction. Hepatocyte
adhesion to the PV6Gna surface was drastically reduced in the
presence of soluble PVLA, PV6Gna, and ASF but not fetuin
and PVMA (data not shown). Hepatocyte adhesion to PV6Gna was
almost completely inhibited by 0.02 µM ASF (normalized with

Inhibition of Hepatocyte Adhesion to PV6Gna Surface Exclu-

Glycopolymer Selectivity of Hepatocytes—To investigate spe-
cific interactions between the synthetic glycopolymers and
hepatocytes, a cell adhesion assay was carried out on a poly-
styrene dish coated with a particular glycopolymer. Hepatocy-
etically adhered onto the polystyrene surface that had
been coated with 0.1 mg/ml PVLA, PVMEA, and PV6Gna but
not PVMA or PVG as shown in Fig. 4a. Mammalian primary
hepatocytes are specifically recognized by the glycoproteins
that act as natural and artificial ligands having terminal ga-
lactose/GalNAc moieties. As expected, hepatocytes interacted
with PVLA and PVMEA having terminal galactose moieties.
On the other hand, hepatocytes interacted only with PV6Gna
among the glycopolymers that have glucose moieties. A
photonicrograph of hepatocytes adhered to the PV6Gna surface
is shown in Fig. 4b indicating that it did not result from contam-
ination by nonparenchymal cells, such as Kupffer cells and
sinusoidal endothelial cells, of our hepatocyte preparation.
The results indicate that hepatocytes specifically recognize PV6Gna
due to the substitution of the C-6 position of glucose but not at
the C-1 and C-3 positions.

FIG. 1. Molecular structure of glycopolymers.
molecular weight). The ASF that was used is a desialylated glycoprotein from calf serum fetuin (molecular mass, 48 kDa) that has an average 13.6 sialic acid residues. Hepatocyte adhesion to the PV6Gna surface was inhibited by 1 mM GalNAc as shown in Fig. 5. The relative inhibitory effect of the carbohydrates was determined from the concentration of carbohydrate required for 50% inhibition of PV6Gna binding ([I]_{50}) to hepatocyte. The results indicate that hepatocyte adhesion to PV6Gna surface was inhibited at least $4 \times 10^{10}$ fold more efficiently by ASF ([I]_{50} = 1.4 \times 10^{-6} \text{ mM}) than by GalNAc ([I]_{50} = 0.6 \text{ mM}) as shown in Fig. 5. We also showed that the soluble PVLA inhibited hepatocyte adhesion to the PV6Gna surface with a higher affinity than galactose-type monosaccharides or disaccharides (data not shown). It has already been reported that rat hepatocytes bound to PVLA with a higher affinity than lactose. In addition, we examined recognition of PV6Gna to other cell types in which ASGPRs are not expressed. Hepa 1-6 cells, erythrocytes, NIH 3T3 fibroblasts, and human umbilical vein endothelial cells rarely adhered to the PV6Gna surface compared with the collagen surface or BSA surface as shown in Fig. 6. Hepa 1-6 is a mice hepatoma cell line in which ASGPRs are not detected by reverse transcription-polymerase chain reaction and Western blot analysis (data not shown). Erythrocytes probably attached to the PS surface through a nonspecific hydrophobic interaction. The cell lines could adhere to the BSA surface coated with collagen by an integrin-mediated interaction. The results also show that the cell lines were apt to adhere to the BSA surface through a nonspecific hydrophobic interaction with adherence to PVLA and PV6Gna surfaces. We observed that the hydrophobicity of the BSA surface was higher than the hydrophobicities of the PVLA and PV6Gna surfaces (data not shown). Taken together these results indicate that hepatocyte adhesion to the PV6Gna surface is mediated by ASGPRs on the cell membrane but not by nonspecific interaction.

Flow cytometry analysis as a different assay was carried out using FITC-conjugated PV6Gna to investigate ASGPR-mediated interactions between the hepatocyte cell surface and PV6Gna (Fig. 7). As shown in Fig. 7, a, b, and c, the surface-labeled hepatocyte fraction (Region 1) was determined from the PI-unlabeled fraction (Region 1 + Region 3), which was estimated as undamaged cells. Hepatocytes were labeled to 25% of the PI-unlabeled fraction in the presence of 0.1 mg/ml FITC-PV6Gna (Fig. 7b). FITC-PV6Gna binding to hepatocytes was increased with an increase of the concentration of FITC-PV6Gna that was added as represented by a hyperbolic plot in Fig. 7d. The FITC-PV6Gna binding to hepatocytes was notably decreased by the addition of EGTA, GalNAc, and ASF but not fetuin (Fig. 7, c and e). We observed that 15–20% of hepatocytes were damaged (PI-labeled fraction, Region 2 + Region 4) during incubation. The FITC-labeled hepatocyte fraction (Region 2) in PI-labeled hepatocytes was also increased with an increase of the concentration of FITC-PV6Gna and inhibited by EGTA (Fig. 7c), ASF, or GalNAc (data not shown). Increasing doses of ASF or GalNAc as a competitive inhibitor produced a

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Kobayashi, A., Goto, M., Kobayashi, K., and Akaike, T. (1994) J. Biomater. Sci. Polym. Ed. 6, 325–342.
dose-dependent decrease of the FITC-PV6Gna binding to hepatocytes as shown in Fig. 7. Although PV6Gna binding to hepatocytes is almost completely inhibited in the presence of 10 μM ASF, it is inhibited no more than 80% in the presence of 100 mM GalNAc. The soluble FITC-PV6Gna binding to hepatocytes was inhibited 50-fold more effectively by ASF ([I]_{50} = 4 \times 10^{-4} \text{ mM}) than by GalNAc ([I]_{50} = 20 \text{ mM}). These results are compatible with the multivalent effect on the inhibition of the interaction between soluble PV6Gna and hepatocytes. We also observed that FITC-PV6Gna or FITC-PVLA binding to hepatocytes was increased with an increase of Ca^{2+} in the reaction mixture (data not shown). The optimum concentration of Ca^{2+} for the binding of FITC-conjugated PV6Gna and PVLA to hepatocytes was -0.3 and 0.1 mM, respectively. This result is in agreement with the previous observation of the Ca^{2+} requirement of hepatocytes to adhere to PV6Gna and PVLA surfaces (19). Our data did not result from nonparenchymal cell contamination because the hepatocyte region was created by adjusting forward scatter and side scatter. These results indicate that soluble PV6Gna also binds to the hepatocyte cell surface mediated by ASGPR on the cell surface.

Detection of ASGPR with Gel Particles Containing 6-O-Methacryloyl-D-glucose—Mammalian hepatocytes are rich in S-type and C-type lectins, which recognize galactose, glucose, or mannose. Therefore, we could not rule out that the interactions of the hepatocytes and the PV6Gna might be mediated by unknown C-type lectins capable of recognizing terminal galactose residues. To confirm the ability of ASGPRs and the polymers modified at the hydroxyl group of C-6 of glucose to associate and to further demonstrate that ASGPRs are specifically recognized by PV6Gna due to the modification of the hydroxyl group position of C-6 of glucose (19). Briefly, hepatocytes (5 × 10^4 cells/cm²) were allowed to adhere for 30 min at 37°C onto the polystyrene dish (Falcon 1008) that had been coated with a 0.1 mg/ml solution of PVLA, PVMEA, PVMA, PVG, or PV6Gna followed by blocking with 0.5 wt% BSA. The percentage of cell adhesion was determined as described under “Experimental Procedures.”

Table I: Inhibition of hepatocyte adhesion to the PV6Gna surface by carbohydrates

| Inhibitory carbohydrate (20 mM) | Adhered cells (%) |
|---------------------------------|-------------------|
| None                            | 100 ± 7           |
| D-Glucose                       | 93 ± 8            |
| Methyl α-D-glucoside            | 100 ± 4           |
| Methyl β-D-glucoside            | 97 ± 10           |
| 3-Methyl-D-glucose              | 99 ± 7            |
| D-Mannose                       | 96 ± 10           |
| D-Fructose                      | 95 ± 5            |
| Sodium D-glucuronate            | 93 ± 2            |
| D-Glucosamine                   | 91 ± 4            |
| Maltose                         | 96 ± 2            |
| Sucrose                         | 99 ± 1            |
| Cellulose                       | 91 ± 8            |
| Isomaltose                      | 94 ± 6            |
| D-Galactose                     | 4 ± 2             |
| Methyl β-D-galactoside          | 3 ± 2             |
| Lactose                         | 4 ± 4             |
| Melibiose                       | 2 ± 2             |
| N-Acetyl-D-galactosamine        | 4 ± 1             |

FIG. 3. ^13C NMR spectra of the carbohydrate moiety in PV6Gna. Carbohydrates (7 wt%) were dissolved in MeSO-4 and transferred to a thin-walled 5-mm NMR tube. Spectra were obtained on a Varian UNITY plus 400 spectrometer with 25,000 scans at 25°C. a, D-galactose; b, D-glucuronic acid; c, carbohydrate moiety in PV6Gna.

FIG. 4. Hepatocyte adhesion to the polystyrene dish coated with glycopolymers. a, hepatocyte adhesion to polymer surfaces. Hepatocyte adhesion assay was carried out as described previously (19). Briefly, hepatocytes (5 × 10^4 cells/cm²) were allowed to adhere for 30 min at 37°C onto the polystyrene dish (Falcon 1008) that had been coated with a 0.1 mg/ml solution of PVLA, PVMEA, PVMA, PVG, or PV6Gna followed by blocking with 0.5 wt% BSA. The percentage of cell adhesion was determined as described under “Experimental Procedures.” b, photomicrograph of hepatocytes adhered to the PV6Gna surface. Scale bar = 100.2 μm.
clonal anti-human ASGPR as shown in Fig. 8c. It has been previously shown that the monoclonal anti-human ASGPR was produced by immunizing BALB/c mice with ASGPR purified from human liver and was reactive with mouse hepatic lectin-1 (40). Mouse hepatic lectin-1 was expressed as a band at 42 kDa consistent with previous reports (41, 42). We observed that the antibody was not reactive with the lysates of nonparenchymal cells as well as Hepa 1-6 cells in which ASGPR is not expressed as described above (data not shown). Dose dependence experiments showed that the increase of the precipitated ASGPR was dependent on the amounts of the PMglc added for the precipitation (data not shown). In contrast, ASGPR was rarely precipitated with the PMglc in the presence of 1 mg/ml ASF, 2 mM EGTA, or 0.1 mg/ml PV6Gna, but the precipitation was unaffected by 2 mg/ml fetuin. In addition, the precipitation was inhibited by 20 mM galactose but not glucose and mannose as monosaccharide inhibitors (data not shown). In addition, increasing doses of ASF in the reaction mixture provided a dose-dependent inhibition in the precipitation of ASGPR with PMglc (data not shown). We carried out ASGPR precipitation with PMglc in the extract that had been treated with ASF-conjugated Sepharose to remove active ASGPR from the hepatocyte lysate. As shown in Fig. 9c, increasing amounts of ASF-Sepharose caused a dose-dependent decrease of ASGPR in the extract. The precipitated ASGPR was drastically reduced to less than 10% of control when the ASF-Sepharose-treated extract was precipitated with PMglc. These results that the precipitation of ASGPRs with PMglc was inhibited by ASF are enough to provide the evidence for the involvement of ASGPR in the interaction between hepatocytes and the glucose-derivatized polymers. Furthermore, we examined the inhibitory effect of GalNAc and methyl β-gal on the precipitation of ASGPR with
PMgле. GalNAc inhibited ASGPR precipitation by PMgłe more effectively than did methyl β-gal as shown in Fig. 9b. Because hepatic ASGPR had a greater affinity for GalNAc than for β-galactoside, this result demonstrates that the precipitated ASGPRs are hepatic ASGPRs. Using the technique of $^{13}$C NMR analysis of carbohydrates (48), PMgłe showed a downfield shift of the peak at C-6 from the results of NMR analysis (data not shown). These shifts resulted from the transesterification of glucose with vinyl methacrylate at the C-6 position of glucose. However, the yield of the glucose monomer from HPLC analysis was 36% of the total product containing glucose di-(52%) and triester (12%), which might act as cross-linkers for gelation of the glucose monomer shown in Fig. 8a. We thought that the reduction of regioselectivity may have been caused by a scale-up of the enzyme reaction as compared with a previous report. Despite the low content of the glucose monomer in the PMgłe, hepatocytes recognized PMgłe in the same manner as PV6Gna (Fig. 8b). Specifically, ASGPRs bound to PMgłe in the hepatocyte cell lysate. The results show direct binding of ASGPRs to the PMgłe by a Ca$^{2+}$-dependent and galactose-specific manner, indicating that the ASGPRs on hepatocytes are specifically recognized by glycopolymers modified at the C-6 position of glucose as well as by PV6Gna. Numerous studies have been reported about the regioselective formation of polymerizable sugar esters at 6-OH with various alkylation agents using commercially available protease and lipase (31, 44–46). Our data provide new information for the design of biomimetic glycopolymers for medical applications such as liver tissue engineering and cell-targeted drug delivery.

CBPs interact with a particular carbohydrate by recognizing subtle differences in the carbohydrate structure. Interaction of galactose/GalNAc and ASGPR occurs through hydrogen bonds and nonpolar interactions between the carbohydrate recognition domain, Ca$^{2+}$, and the carbohydrate. It has been reported that ASGPRs essentially require the equatorial hydroxyl group of C-3 and the axial hydroxyl group of C-4 of galactose that bind to the receptors (47, 48). Nevertheless, why do ASGPRs on the mouse primary hepatocyte bind to the polymer modified at the 6-OH position of glucose? We hypothesize two combined effects to answer this question. The first combined effect is the effect of the selectivity of the substituted position of the hydroxyl group of glucose. There are many reports that demonstrate affinity for the individual hydroxyl group of galactose bound to ASGPRs. For example, substitution of another monosaccharide in the hydroxyl group of C-6 of galactose rarely affected the affinity for ASGPRs (36). Our previous study showed that hepatocytes could not interact with a glycopolymer substituted at the hydroxyl group of C-1 or C-3 of glucose. Thus, the hydroxyl group of C-6 of the carbohydrates may contribute less to recognition for ASGPRs than the hydroxyl groups of C-1, C-3, and C-4 of the carbohydrates. Alternatively, PV6Gna can bind to ASGPRs probably because the combination of 1-OH in the α-anomeric form and 2-OH mimics the equatorial-axial combination of galactose. In the PVMA, there is no such combination available because OH at C-1 of glucose was modified with a vinylbenzyl group. PVG may not be a good ligand because of the linkage of the benzyl group, which is too close to this pair of OH groups. The second combined effect is the polymeric effect of the PV6Gna engaging ASGPR in multivalent binding on the hepatocyte cell surface. Multivalent synthetic glycoprotein inhibits L-selectin-mediated leukocyte rolling more effectively than the corresponding monomer (7). Several reports have demonstrated a multivalent effect of ligands for an optimum recognition to the structure of ASGPRs (49, 50). Hepatocyte adhesion onto the PV6Gna surface was inhibited by 2 and 16 μM of soluble PVLA and PV6Gna, respectively (data not shown), although the concentrations of PV6Gna and PVLA were normalized (calculated by the molecular weight of the corresponding monomer of the polymers). The results show that on the hepatocyte adhesion, soluble PVLA (500-fold) and PV6Gna (60-fold) have a greater binding affinity than GalNAc. In addition, the hepatocyte adhesion was not affected in the presence of sodium glucuronate and isomaltose as monosaccharide or disaccharide modified at C-6 of glucose, respectively, as well as glucose. In addition, we excluded the effect of the modifying groups on the interactions of ASGPRs and the polymers modified at C-6 of glucose using a different modifying group with an amide bond or ester bond. We suggest the reason that PV6Gna can bind to ASGPR despite the loss of hydrogen bonding at C-4 of glucose might be due to the contribution differences of hydroxyl groups of the sugar and a polymeric effect of sugar ligands during the formation of hydrogen bonds.

It is well known that mammalian primary hepatocytes are
specifically recognized through ASGPRs by glycopolymers that act as natural and artificial ligands having terminal galactose/GalNAc moieties. However, our data showed that the mouse primary hepatocytes specifically interacted with the PV6Gna due to the modification at the C-6 position of glucose but not at the C-1 and C-3 positions. Furthermore, we first found that ASGPRs could bind to the glucose-derivatized polymer substituted at the hydroxyl group at the C-6 position of glucose although it has been reported that the binding of ASGPR to galactose was not affected by the modification of the hydroxyl group at the C-6 position of galactose. ASGPRs have been identified in peritoneal macrophages as well as in mammalian liver. Subsequent studies demonstrated characteristic differences between the macrophage galactose receptor and the hepatic lectin in carbohydrate specificity as well as in the number and complexity of polypeptide subunits. However, Kupffer cell lectin had a greater affinity to GalNAc than to galactoside with a multivalent effect, indicating that the carbohydrate specific- lity of Kupffer cell lectin was similar to that of hepatic ASGPR (51, 52). Kupffer cell lectin could be recognized by 6-O-phospho- D-glucose and glucosyl-BSA. In this study, we report that hepatic ASGPR recognizes glucose-derivatized polymers depending on the position of the substituted hydroxyl group. Future studies should examine whether glucose-derivatized polymers can recognize the galactose receptor of macrophages including Kupffer cells.

Carbohydrate selectivity for C-type lectins such as ASGPRs and mannos-binding proteins have been demonstrated by binding competition assay and crystallographic analysis for the carbohydrate recognition domain using monosaccharides or disaccharides as ligands. The results showed that ASGPR could not recognize glucose and its derivatives. However, most C-type lectins including ASGPR were recognized by multicar- bohydrate ligands in a living body. Although PV6Gna is an artificial glycopolymer that cannot be found in a living body, our study provides new insights into carbohydrate recognition of CBPs especially for the design of biomimetic materials based on interactions between carbohydrates and CBPs.

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