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A Probing of Inhibition Effect on Specific Interaction Between Glucose Ligand Carrying Polymer and HepG2 Cells

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Abstract A reducing glucose-carrying polymer, called poly [3-*O*-(4'-vinylbenzyl)-D-glucose] (PVG), was interacted with HepG2 cells including a type-1 glucose transporter (GLUT-1) on the cell membrane. The cooperative interaction between a number of GLUT-1s and a number of reducing 3-*O*-methyl-D-glucose moieties on the PVG polymer chain was found to be responsible for the increase in the interaction with HepG2 cells. The affinity between the cells and the PVG was studied using RITC-labeled glycopolymers. The specific interaction between the GLUT-1 on HepG2 cells and the PVG polymer carrying reducing glucose moieties was suppressed by the inhibitors, phloretin, phloridzin, and cytochalasin B. Direct observation by confocal laser microscopy with the use of RITC-labeled PVG and pretreatment of HepG2 cells with the inhibitors demonstrated that the cells interacted with the soluble form of the PVG polymer via GLUT-1, while fluorescence labeling of the cell surface was prevented after pretreatment with the inhibitors of GLUT-1.

Key words: Fluorescence intensity, glucose transporter, glycopolymer, HepG2 cell, specific interaction

Recently, cell-recognizable polymers have become highly attractive for a specific gene or drug delivery to target cells [13], as well as for bioartificial organs [7]. As such, understanding the characteristics of the receptor-ligand interaction is one of the most important considerations when designing simplified glycopolymers for specific cell recognition [8, 15]. However, successful construction of receptor-ligand interactions has rarely been reported despite considerable efforts to synthesize simplified glycopolymers for specific cell recognition. Consequently, significant progress has been made in the use of synthetic glycopolymers that mimic the role of carbohydrates in the biological system for cell recognition actions and medical applications [10]. Among the many kinds of natural sugar, glucose has been focused on as a specific cell recognition material, since it is one of the most important sources of energy and a substrate for a variety of cellular components. Cells accumulate glucose via a process of facilitated uptake that is mediated by a family of glucose transporters (GLUTs) [4]. In different kinds of mammalian cell and tissue, several distinct families of glucose transporters are widely distributed and expressed on the cell membrane [3]. However, glucose itself is not a suitable matrix for elucidating receptor-ligand interaction, as glucose moieties interact with GLUT before they can be transported into the cells. As such, designing a cell-recognizable polymer mediated by GLUT is of interest in the synthesis of simplified glycopolymers in order to clarify the mechanism of glucose transportation via GLUT into the cells. Previous work has reported that polyvinylbenzyl glucose (PVG), bearing a reducing glucose moiety, is a promising cell recognizable polymer, which may be a useful tool in elucidating the cellular mechanism mediated by GLUT on the cell membrane [5, 11].

Accordingly, this study attempted to determine the specific interaction between the reducing glucose moiety carrying PVG HepG2 cells using a competition test, while also confirming the specific interaction between PVG and HepG2 cells with different inhibitors such as phloretin, phloridzin, and cytochalasin B and synthetic glycopolymers. For a more detailed examination of the specific interaction between the glycopolymer and the cells, a fluorescence-labeled polymer was also used and the interaction monitored using fluorescence intensity measurements and a confocal microscopic analysis.

MATERIALS AND METHODS

Preparation of Sugar-Carrying Homopolymer

The 3-*O*-(4'-vinylbenzyl)-D-glucose (PVG), poly[N-p-vinylbenzyl-*O*-α-D-glucopyranosyl-(1-4)-D-gluconamide]

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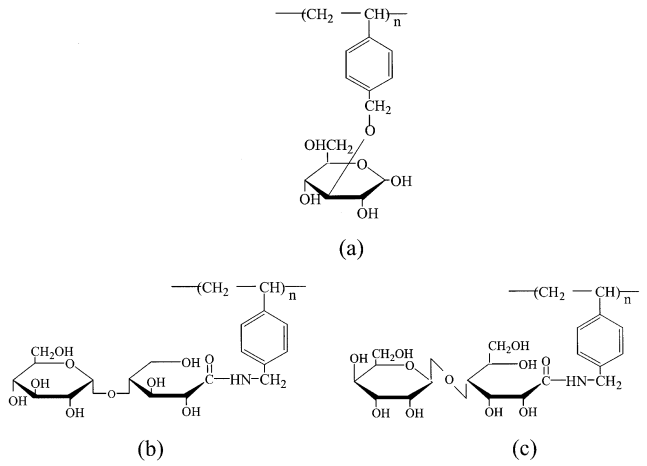

Fig. 1. Chemical structures of glycopolymers used in the current study.
(a) PVG, (b) PVMA, and (c) PVLA.

(PVMA), and poly[N-p-vinylbenzyl-*O*-β-D-galactopyranosyl-(1-4)-D-gluconamide] (PVLA) were all synthesized by previously reported methods [12]. The chemical structures of the glycopolymers used in this study are shown in Fig. 1.

Cell Culture

HepG2 cells were cultured in Dulbecco's modified eagle's medium (DMEM) equilibrated with 5% CO_2 at 37°C. The medium was also supplemented with 10% fetal calf serum, 50 mg/l streptomycin, and 75 mg/l penicillin sulfate.

Fluorescent Labeling of Polymers

Each polymer was fluorescent labeled with rhodamine-B isothiocyanate (RITC). Based on a solution of the polymer (500 mg) in 5 ml of dimethylsulfoxide (DMSO), 50 mg of RITC and 15 mg of dilaurated dibutyltin were added and allowed to stand for 2 h at 80°C. The mixture was then precipitated with excess ethanol using a DMSO-EtOH system. Thereafter, the polymer was dissolved in 30 ml of water, then dialyzed against 1-l of mild alkaline water (pH 8.0 with NaOH) for 1 day and dialyzed against distilled water for 3 days with three exchanges of distilled water. The RITC-labeled polymers were obtained by freeze-drying, and the final products were obtained after freeze-drying the solution. Using this procedure, one RITC molecule was found to be introduced per approximately one hundred repeating units of each polymer when determined using a UV spectrophotometer at 488 nm.

Assay of Fluorescence Intensity (FI)

The cells were preincubated in KRBB (pH 7.4, 3.3 mM of glucose) equilibrated with 5% CO_2 at 37°C for 20 min, followed by incubation in KRBB containing the RITC-labeled polymers at 4°C for 1 h. The HepG2 cells were washed with PBS three times, placed onto a cover glass, and placed on a slide glass. The HepG2 cells were then observed using a confocal laser microscope (Leika, Heidelberg, Germany).

Suppression of Specific Interaction by Inhibitor of GLUT

Phloretin was dissolved in 150 μl of dimethylsulfoxide (DMSO) and diluted with 20 ml of PBS to make a solution of 5×10^{-4} M. Phloridzin was dissolved in 20 ml of a phosphate buffer to obtain a 10^{-3} M solution. Cytochalasin B was dissolved in 60 μl of DMSO and then diluted with a phosphate-buffered solution to obtain 5×10^{-4} M. The cells suspended in 1.5 ml of the inhibitor solutions were added to RITC-labeled PVG and stored at 4°C for 1 h. After finishing the interaction between the cells and the RITC-labeled PVG polymer, the cells were washed with PBS three times and subjected to a microplate reader and confocal laser microscope.

RESULTS AND DISCUSSION

It has been reported that GLUT-1 exhibits a dissociation constant (K_m) of 17.0 mM for D-glucose, which is similar to the value of human GLUT-1 for 3-*O*-methyl-D-glucose (K_m=17.6– 16.9 mM) [2]. Also, our previous study indicated that the integrated and cooperative interaction between a large number of GLUT-1s and a number of reducing 3-*O*-methyl-D-glucose moieties on a PVG polymer chain was responsible for the high affinity of this polymer to erythrocytes.

Thus, to determine whether or not the higher affinity of the PVG to HepG2 cells was mediated by GLUT-1, the interaction between the PVG and HepG2 cells was analyzed

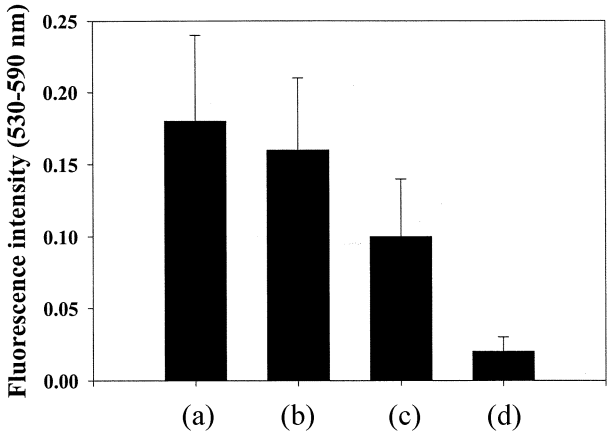

Fig. 2. Effect of glucose transporter type-1 inhibitors on fluorescence intensity against HepG2 cells.
(a) No treatment; (b) cytochalasin B; (c) phloridzin; and (d) phloretin. Data represent experimental data (mean±SD) (n=5).

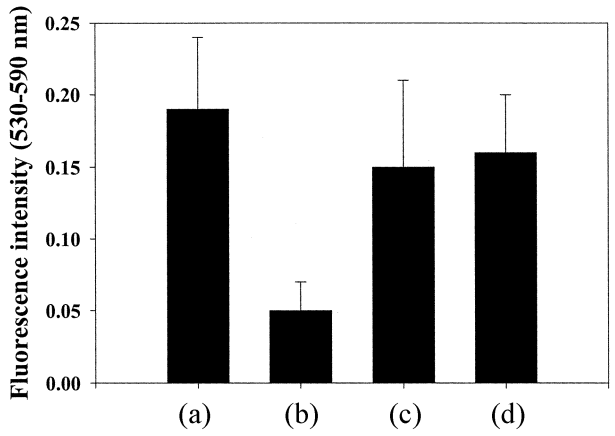

Fig. 3. Inhibition effect of cytochalasin B with different types of homopolymer on fluorescence intensity against HepG2 cells. Each sample was incubated in HEPES-balanced KRBB (without BSA) with a low glucose concentration and the labeled polymer for 1 h.
(a) No treatment; (b) PVG; (c) PVMA; and (d) PVLA.

with various inhibitors such as phloretin, phloridzin, and cytochalasin B. Figure 2 shows that the fluorescence intensity of HepG2 cells with the RITC-labeled PVG drastically decreased after pretreatment with the inhibitors of GLUT, approximately 80% less with cytochalasin B, 65% less with phloretin, and 60% less with phloridzin compared to that of the nontreated cells. It has also been reported that inhibitors of GLUT, such as phloretin and phloridzin, inhibit the transportation of D-glucose via GLUT-1 in erythrocytes.

To confirm the inhibitory effect against the PVG homopolymer, cytochalasin B was also used with other types of homopolymer (Fig. 3). Cytochalasin B has been found to include natural photo-affinity ligands that can be photo-incorporated at a low concentration into specific proteins in the human erythrocyte plasma membrane associated with the high affinity binding sites of this molecule [9, 14]. As such, in the presence of L-glucose, the polypeptide region referred to as zone 4.5 on sodium dodecyl sulfate-polyacrylamide gel electrophoretograms was photolabeled with [^{3}H] cytochalasin B. At least two major peaks were resolved in this region, one at approximately 50,000 daltons and the other at approximately 47,000 daltons. In addition, the incorporation was partially blocked when D-glucose was substituted for L-glucose in these experiments. The incorporation was time-dependent upon photolysis, did not occur in the absence of light, and was irreversible under the harsh conditions of gel electrophoresis, suggesting a covalent incorporation of this ligand into polypeptides previously identified as being associated with monosaccharide transport in these cells. Furthermore, soluble G-actin from muscle was also labeled, but at a level 100-fold lower than the transporter polypeptides. Therefore, these experiments suggest that this technique may prove useful in identifying monosaccharide transporter proteins in other cell systems as well as other types of cellular protein associated with high affinity cytochalasin binding. In the case of the PVG homopolymer, cytochalasin B strongly blocked the adsorption of PVG labeled with RITC (Fig. 3). However, PVMA, a nonreducing glucose-carrying polymer, and PVLA, a β-galactose-carrying polymer, did not affect the interaction between the GLUT-1 of HepG2 cells and the RITC-labeled PVG, indicating that the glucose interacted in advance with GLUT-1s expressed on the cell surface. As such, the RITC-labeled PVG polymers were considered to have lost their specific binding sites for HepG2 cells due to occupation by the pretreated PVG polymer.

Confocal Laser Microscopic Imaging of HepG2 Cells Inhibited by Inhibitors of GLUT

The specific interactions via the transporters, phloretin, phloridzin, and cytochalasin B, were monitored by a confocal microscopic analysis. Previous research has demonstrated a highly significant inhibition of glucose uptake in cells mediated by GLUT-1 with phloretin and cytochalasin B, and partial inhibition with phlorizin [1, 6]. However, when the inhibitors were added to the cells 20 min after the start of incubation, by which time the GLUT on the cell membrane was expected to have been suppressed, the RITC-labeled PVG binding was reduced by the same extent in the presence of the inhibitors from the start of incubation. Figure 4 shows the suppressed luminance of the RITC-labeled PVG with GLUT-1 when pretreated with the inhibitors cytochalasin B (b), phloridzin (c), and phloretin (d). When pretreated as a function of time, the luminance completely disappeared after 60 min (data not shown), indicating that the specific interaction between HepG2 cells and the PVG polymer was mediated by GLUT-1 and the ligand of the reducing glucose moiety. Therefore, these results strongly suggest that the GLUTs on the cell membrane played an important role in the GLUT-1-mediated binding between HepG2 cells and the PVG carrying reducing glucose.

Competition Test for Binding Affinity to HepG2 Cells

To determine the binding affinity, a fluorescence intensity test was conducted based on different ratios of the RITC-labeled PVG homopolymer to glucose at 10:1, 1:1, and 1:10 corresponding to Figs. 5 (b), (c), and (d), respectively. As the concentration of glucose increased, the fluorescence intensity of the RITC-labeled polymer significantly decreased. The fluorescence intensity of HepG2 cells with a 10:1 ratio of the RITC-labeled PVG homopolymer to glucose was stronger than that with any other ratio, as shown in Fig. 5(b). This intensity increased with a decrease in the ratio of the PVG homopolymer and reached more than 5-fold of the intensity of a 1:10 ratio of the RITC-labeled PVG

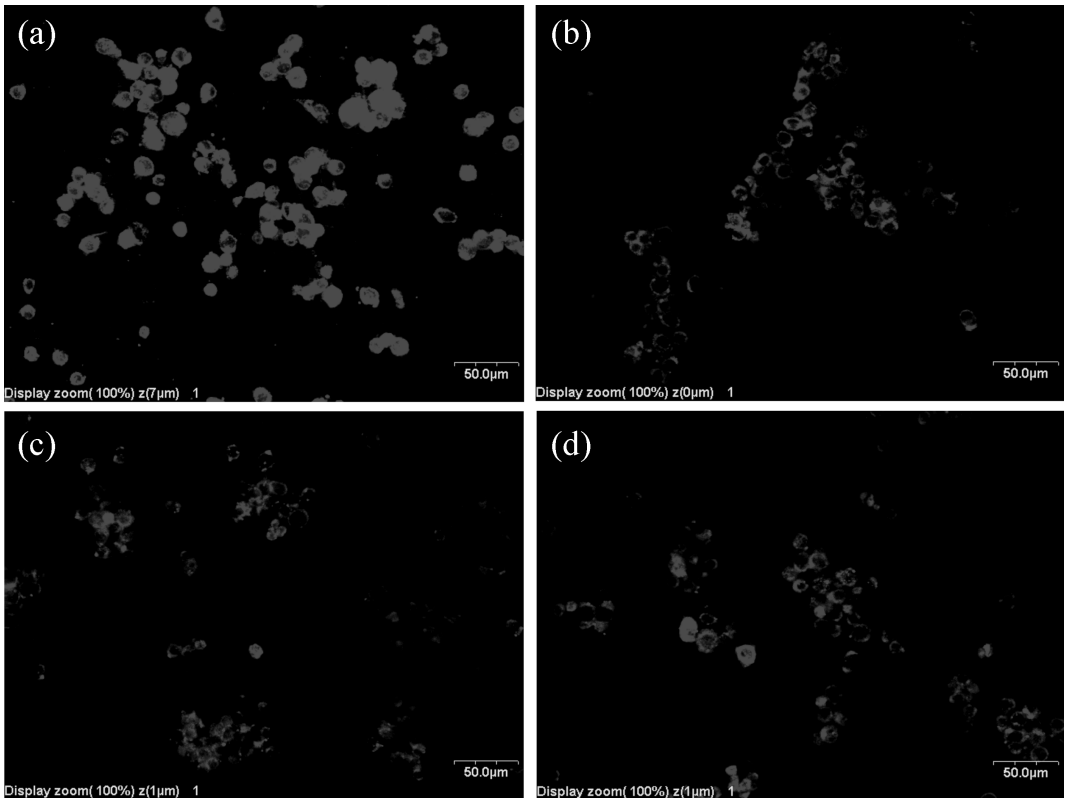

Fig. 4. Confocal laser microscopic view of cells interacted with RITC-labeled PVG homopolymer at 4°C (×100, rhodamine-B isothiocyanate as probe). Each sample was incubated in HEPES-balanced KRBB (without BSA) with a low glucose concentration and the labeled polymer for 1 h.
(a) No treatment (PVG only); (b) cytochalasin B; (c) phloridzin; and (d) phloretin.

homopolymer and glucose, suggesting that the binding process of the RITC-labeled PVG homopolymer to HepG2 cells was inhibited by the addition of glucose. However, other homopolymers, such as PVMA and PVLA, had no inhibitory effect on the specific interaction between the RITC-labeled PVG homopolymer and HepG2 cells (data not shown).

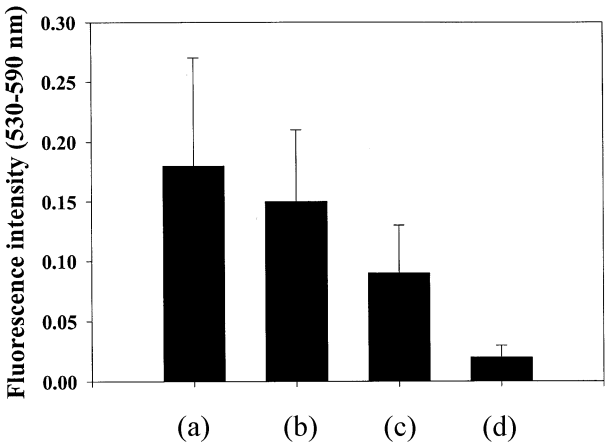

Fig. 5. Fluorescence intensity of RITC-labeled PVG homopolymer with HepG2 cells.
The feed ratios were as follows: (a) RITC-labeled PVG polymer only; (b) RITC-labeled PVG homopolymer 10: glucose 1; (c) 1:1; and (d) 1:10. The error bars represent mean±SD.

Confocal Laser Microscopic Imaging of Competition Between PVG Homopolymer and RITC-labeled PVG Homopolymer

Fluorescence microscopic observation was used to probe the specific interaction between the RITC-labeled PVG homopolymer and HepG2 cells. In Fig. 6, the RITC-labeled PVG homopolymer and glucose competed with each other based on ratios of 10:1, 1:1, and 1:10, corresponding to Figs. 6(a), (b), and (c), respectively. As the concentrations of glucose increased, the luminance of HepG2 cells gradually faded away. In Fig. 6(a), HepG2 cells were strongly luminated by the interaction with the RITC-labeled PVG homopolymer, indicating that the glucose interacted with HepG2 cells in advance based on the receptors expressed on the cell membrane. Therefore, the RITC-labeled PVG polymer apparently lost its specific binding sites with HepG2 cells due to competitive occupation by glucose.

In conclusion, the current study on the inhibition of the PVG polymer (RITC-labeled) with HepG2 cells using a

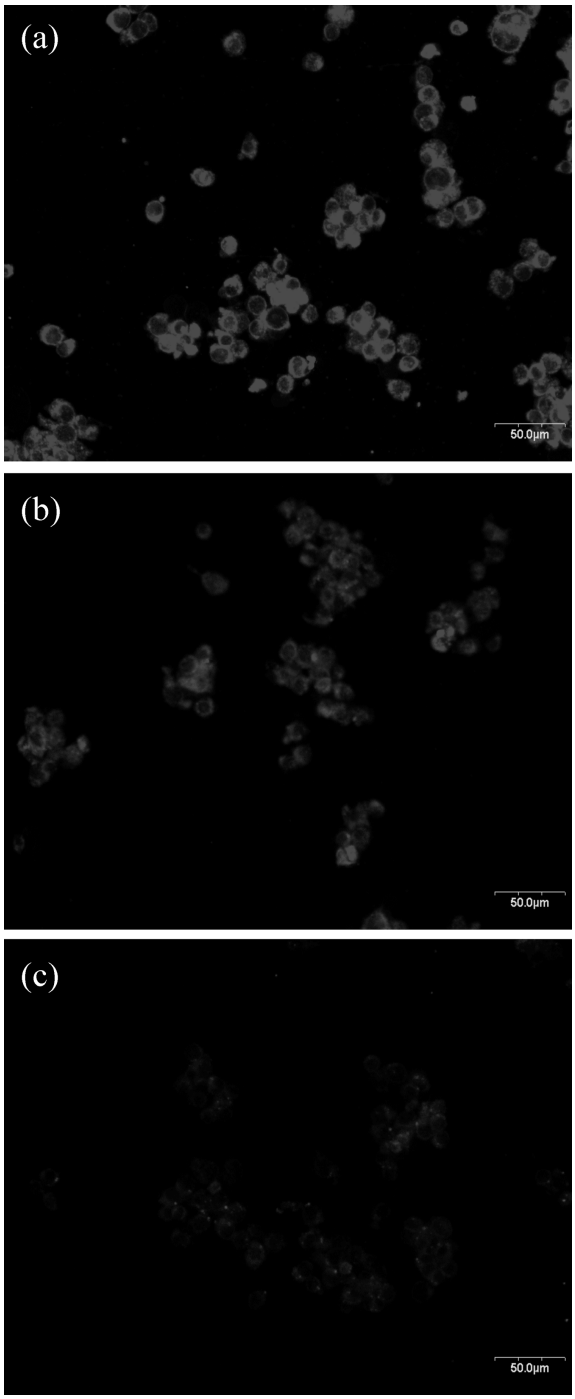

Fig. 6. Confocal microscopic images showing that RITC-labeled PVG homopolymer competed with PVG homopolymer and specifically interacted with HepG2 cells. Each sample was incubated in HEPES-balanced KRBB (without BSA) with a low glucose concentration, and the labeled and unlabeled homopolymer for 1 h.
The feed ratios were as follows: (a) RITC-labeled PVG homopolymer 10: glucose 1; (b) 1:1; (c) 1:10.

microplate reader and direct visualization with a confocal laser microscopy provided strong evidence of a receptor-mediated interaction between HepG2 cells and polymers bearing glucose moieties. Furthermore, the competitive study using the RITC-labeled PVG homopolymer and glucose revealed that the specific interaction between the PVG homopolymer and HepG2 cells was mediated by GLUT-1 on the cell membrane, thereby providing clear evidence of a glucose transporter based on the glucose moieties in the PVG polymer and offering a new tool for determining the biological event on GLUT. In addition, this PVG polymer could be also used for the imaging and diagnosis of GLUT-1s-carrying cells.

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