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Specific Interaction between Erythrocytes and a Glucose-Carrying Polymer Mediated by the Type-1 Glucose Transporter (GLUT-1) on the Cell Membrane

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A reducing glucose-carrying polymer, called poly [3-*O*-(4′-vinylbenzyl)-D-glucose] (PVG), interacted with erythrocytes carrying the 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 a PVG polymer chain is responsible for the increase in the interaction with erythrocytes. In contrast to the PVG homopolymer, other sugar-carrying polymers showed lower interaction with erythrocytes. The affinity of erythrocytes and PVG was studied using FITC-labeled glycopolymers. The fluorescence intensity significantly changed, whereas a small change in fluorescence intensity was observed for other homopolymers. The specific interaction between GLUT-1 on erythrocytes and the PVG polymer carrying reducing glucose was suppressed by the inhibitors, phloretin, phloridzin, and cytochalasin B, and a monoclonal antibody to GLUT-1. Direct observation by confocal laser microscopy with the use of FITC-labeled PVG demonstrated that erythrocytes interacted with the soluble form of the PVG polymer *via* GLUT-1, while fluorescence labeling of the cell surface was prevented on pretreatment with the monoclonal antibody to GLUT-1.

Key words: erythrocyte, glucose-carrying polymer, monoclonal antibody, type-1 glucose transporter (GLUT-1).

Biomimetic cell-recognizable glycopolymers are highly desired for specific gene and drug delivery to target cells as well as bioartificial organs. For the design of simplified glycopolymers for specific cell recognition, one of the most important things is what kind of receptor-ligand combination should be focused on. However, it has rarely been reported the successful construction of receptor-ligand combinations except for those of asialoglycoprotein receptors (ASGP-R) and a β-galactose carrying polystyrene derivative (PVLA) (*1-8*).

Glucose moieties are facilitatedly transported through a number of integral membrane glycoproteins, called glucose transporters (GLUTs). Glucose is well-known as one of the most important sources of energy as well as a substrate for a variety of cellular components. Various different isoforms of glucose trasnporters, such as GLUT-1, 2, 3, and 4, are widely distributed and expressed in various mammalian cells and tissues, such as erythrocytes, and brain, kidney, liver, pancreas, small intestine, and other tissues (*9*). The structures, ligand specificities, molecular mechanisms, and regulation of GLUTs have been well investigated (*10*). But no attempts at designing a cell-recognizable polymer mediated by GLUT were reported. There has been great interest in the synthesis of simplified glycopolymers recognized by GLUTs to clarify mechanism of the glucose transportation through GLUT-1.

A few years ago, we reported that poly [3-*O*-(4′-vinylbenzyl)-D-glucose] (PVG) carrying a reducing glucose moiety on every repeating monomer unit could be a specific substratum for erythrocyte attachment and proposed the possibility of GLUT-1 mediated cell recognition, which could not be directly demonstrated (*11*).

Here we examined the interaction between a glucose-carrying PVG polymer in a soluble state and erythrocytes. A few kinds of synthetic glycopolymers were prepared and compared to clarify the specific recognition of the sugar-moieties of glycopolymers by erythrocytes. Specific inhibitors of GLUT, such as phloretin and phloridzin (*12, 13*), as well as a monoclonal antibody to GLUT-1 were used for flow cytometric analysis to confirm the GLUT-1-mediated interaction. Finally, we demonstrated the inhibition of the GLUT-1-mediated interaction between erythrocytes and PVG polymer by the monoclonal antibody to GLUT-1 by means of confocal laser microscopy.

PVG was synthesized *via* homopolymerization of the corresponding monomer (*14*). Poly [*N*-*p*-vinylbenzyl-*o*-β-D-galactopyranosyl-(1-4)-D-gluconamide] (PVLA) and poly [*N*-*p*-vinylbenzyl-*o*-α-D-glucopyranosyl-(1-4)-D-gluconamide] (PVMA) were also synthesized as reported (*7*). Figure 1 shows the chemical structures of the synthesized polymers. Each of the polymers was fluorescently labeled

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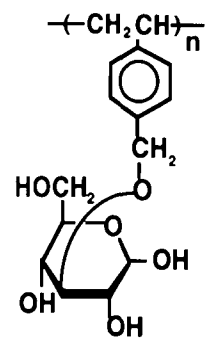
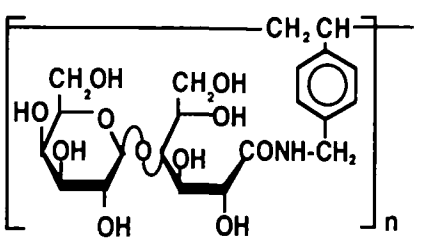
with fluorescein isothiocyanate (FITC). To a solution of a polymer (500 mg) in 5 ml of dimethylsulphoxide (DMSO), 50 mg of FITC and 15 mg of dilaurated dibutyltin were added, followed by standing for 2 h at 90°C. The mixture was then added to an excess of ethanol to precipitate on the polymerusing a DMSO-EtOH system. Finally, the polymer was dissolved in 30 ml water and dialysed against 1,000 ml of alkaline water (pH 8.0, adjusted with NaOH) for 1 day and then against distilled water for 3 days with exchange of the water three times. FITC-labeled polymers were obtained by freeze-drying. By means of this procedure, approximately one FITC molecule was introduced per hundred repeat units in each polymer, as determined by UV spectrometry at 488 nm. Blood (2 ml) was taken from 4-5 week old Sprague-Dawley rats (90-150 g), diluted with 10 ml of a 0.04% EDTA solution, and then centrifuged at 1,000 rpm for 10 min. The isolated erythrocytes were washed with a phosphate-buffered solution (10 ml, pH 7.4; 1.5 mM Ca^{2+} and Mg^{2+}) by repeated suspension and centrifugation (1,000 rpm for 10 min) three times. The cell suspension was incubated with FITC-labeled polymers for 1 h at 4°C. The cells were washed with PBS three times and then subjected to flow cytometric analysis. All measurements were performed with a Cyto ACE-150 (Jasco, Tokyo). From each sample, 10,000 cells were analyzed with logarithmic amplification of the fluorescence intensity (FI). Phloretin was dissolved in 150 μl of dimethyl sulfoxide (DMSO) and then diluted with 20 ml of a phosphate-buffered solution (pH 7.4) to obtain a 5×10^{-4} M solution. 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 the phosphate-buffered solution to obtain a 5×10^{-4} M solution. The PVG polymer solution (500 μg/ml) and glucose solutions (6 mg/ml) were made in the phosphate-buffered solution only. An erythrocyte suspension (200 ml) prepared by repeated rinsing and centrifugation three times of 1 ml of blood removed from a 4-5 week old rat was diluted with each inhibitor in the phosphate-buffered solution (pH 7.4 ~10 ml) to a density of 10^6 cells/ml, followed by preincubation at 37°C for 1 h. Erythrocytes suspended in 1.5 ml of the inhibitor solution have mixed with the FITC-labeled PVG polymer and then stored at 4°C for 1 h. After completion of the interaction between the cells and the FITC-labeled PVG polymer, the cells were washed with PBS three times and then subjected to flow cytometric analysis. Monoclonal antibodies to GLUT-1 and 2 were purchased from Biogenesis. The solution of the antibody to GLUT-1 to be added to the erythrocyte solution was prepared by dilution to 1/10,000 of the purchased concentration. Assaying of the inhibition of glycopolymer binding to erythrocytes by the antibody to GLUT-1 was also performed by the same method with the use of inhibitors of GLUT such as phloretin and phloridzin.

In a previous study, a reducing moiety glucose-carrying polymer (PVG) exhibited strong interaction with erythrocytes (*11*). To observe the interaction between PVG and erythrocytes, we attempted to perform the flow cytometric assay using a FITC-labeled homopolymer in this study. The affinity of the FITC-labeled PVG to erythrocytes was much more clearly shown by histograms of erythrocytes *vs.* their fluorescence intensity. The fluorescence intensity of erythrocytes binding with the FITC-labeled PVG drastically shifted to a higher value than those for PVMA carrying non-reducing glucose and PVLA carrying non-reducing galactose (Fig. 2). It is very interesting that PVMA carrying a non-reducing glucose moiety did not show as high affinity as the PVG carrying a reducing glucose moiety. It was considered that the FITC-labeled PVG undergoes specific interaction with erythrocytes mediated by something like a receptor-ligand combination. The above results suggested that one of the candidate strong binding sites that interact with the reducing glucose-carrying polymer might be a glucose transporter (GLUT). Erythrocytes are known to have the type-1 glucose transporter (GLUT-1) on the cell membrane. It was reported that no glucose transporter exhibits as strong ability as other sugar recognizable receptors such as ASGP-R and so on. GLUT-1 exhibits a dissociation constant (K_m) of 17.0 mM toward D-glucose which is almost the same as the value for human GLUT-1 toward 3-*O*-methyl-D-glucose (K_m = 17.6-16.9 mM) (*15*). Based on our experimental results, it was considered that



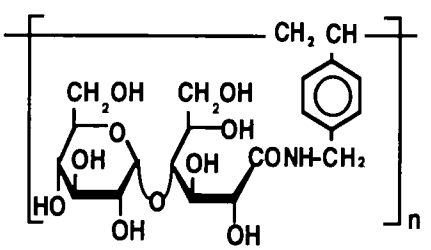
poly [3-O-(4'-vinylbenzyl)-D-glucose]

PVG



poly [N-p-vinylbenzyl-O-β-D-galactopyranosyl-(1-4)-D-gluconamide]

PVLA



poly [N-p-vinylbenzyl-O-α-D-glucopyranosyl-(1-4)-D-gluconamide]

PVMA

Fig. 1. **Chemical structures of the sugar-carrying polymers.**

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. In other words, multivalent interactions between ligands and transporters have a significantly enhanced effect on the binding of PVG and erythrocytes. As compared with other sugar-carrying polymers, the binding of PVG carrying reducing glucose to erythrocytes was found to be stronger and more selective.

In order to determine whether or not the higher affinity of PVG to erythrocytes was mediated by GLUT-1, the interaction between PVG and erythrocytes was analyzed with various inhibitors such as phloretin and phloridzin. Figure 3 shows that the fluorescence intensity of erythrocytes with FITC-labeled PVG drastically decreased on pretreatment with inhibitors of GLUT. It was reported that inhibitors of GLUT such as phloretin and phloridzin inhibited the transportation of D-glucose *via* GLUT-1 in erythrocytes (*12, 13*). The interaction between PVG and erythrocytes was also inhibited by cytocalasin B which is known to bind with GLUT-1 (*16, 17*) from the inside of the cell membrane. From the results obtained with inhibitors of the GLUT, it was strongly suggested that a GLUT plays an important role in the GLUT-mediated binding between erythrocytes and the reducing glucose-carrying polymer, PVG. Pretreatment of erythrocytes with the soluble form of the PVG polymer, which showed the highest affinity to erythrocytes, also strongly blocked the adsorption of PVG labeled with FITC. However, PVMA carrying non-reducing glucose did not affect the interaction between FITC-labeled PVG and GLUT-1 of erythrocytes (Fig. 3). This means that the soluble form of the PVG polymer interacted in advance with GLUT-1s expressed on the cell suface. Therefore, FITC-labeled PVG polymers are considered to lose their specific binding sites for erythrocytes because of their occupation by pretreatment PVG polymers.

Furthermore, we tried to use a monoclonal antibody to GLUT-1 to inhibit the GLUT-1-mediated interaction between PVG and erythrocytes. On pretreatment with the monoclonal antibody to GLUT-1 on the erythrocyte cell surface, dose-dependent suppression of the fluorescence intensity was observed in the flow cytomeric assay shown in Fig. 4. Erythrocytes preincubated with the monoclonal antibody to GLUT-1 showed significant inhibition of the binding of the PVG polymer to erythrocytes, while no changes were observed on the pretreatment of erythrocytes with a monoclonal antibody to GLUT-2 (data not shown). This result is quite strong evidence that the interaction

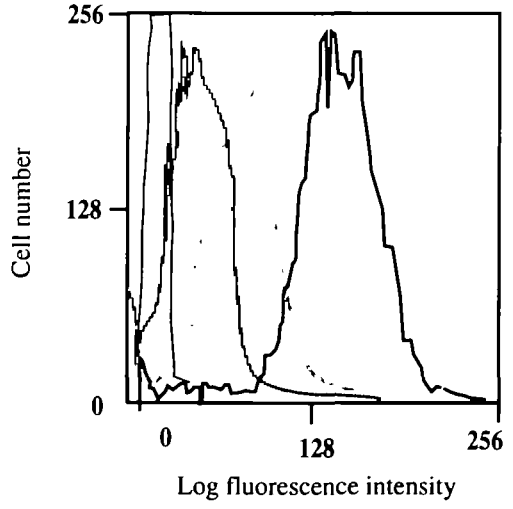


Fig. 2. **Fluorescence intensity of erythrocytes interacting with FITC-labeled polymers with different sugar moieties.** Isolated erythrocytes (1.5×10^6 cells/ml) were washed three times with PBS, mixed with a FITC-labeled polymer, and then incubated at 4°C for 1 h. After the incubation, they were washed three times with PBS to remove the unreacted FITC-labeled polymer. Fluorescence intensity was measured by flow cytometry. ——: PVG; ——: PVLA; ▬: PVMA; ——: control.

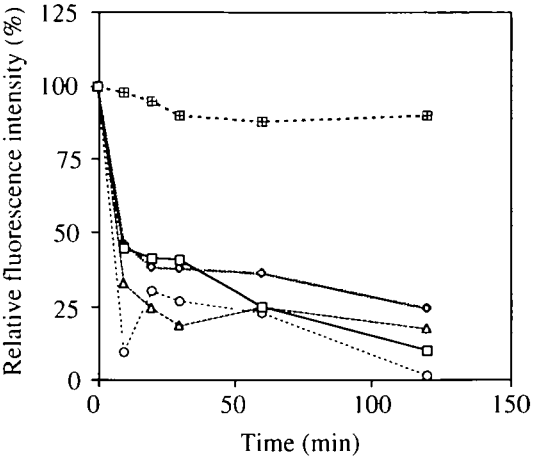


Fig. 3. **Effects of GLUT-1 inhibitors and PVG (soluble form) on the interaction between erythrocytes and FITC-PVG.** The following inhibitors, 5×10^{-4} M phloretin, 10^{-3} M phloridzin, and 5×10^{-4} M cytochalasin B, and the polymer at the indicated concentrations were added to erythrocytes, followed by incubation for 1 h at 4°C. FITC-labeled PVG was then added and the incubation was continued at 4°C. Fluorescence intensity was measured as described in Fig. 1 (○: cytochalasin B; □: phloretin; △: PVG; ◇: phloridzin; and ⊞: PVMA).

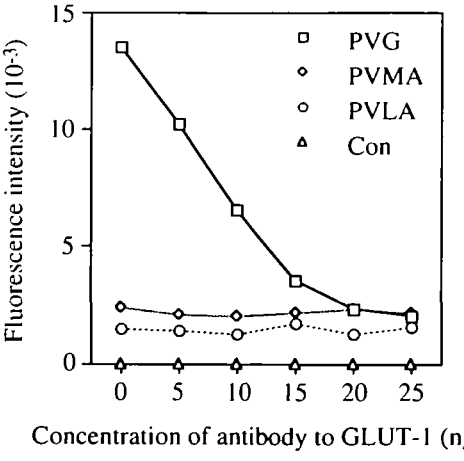


Fig. 4. **Effect of a monoclonal antibody to GLUT-1 on the fluorescence intensity of FITC-labeled erythrocytes.** Assaying of the attachment of various polymers, with FITC-labeling, was performed by flow cytometry (□: PVG; ◇: PVMA; ○: PVLA; and △: control). Before performing the flow cytometry, pretreatment with the monoclonal antibody to GLUT-1 at the indicated concentrations was performed.

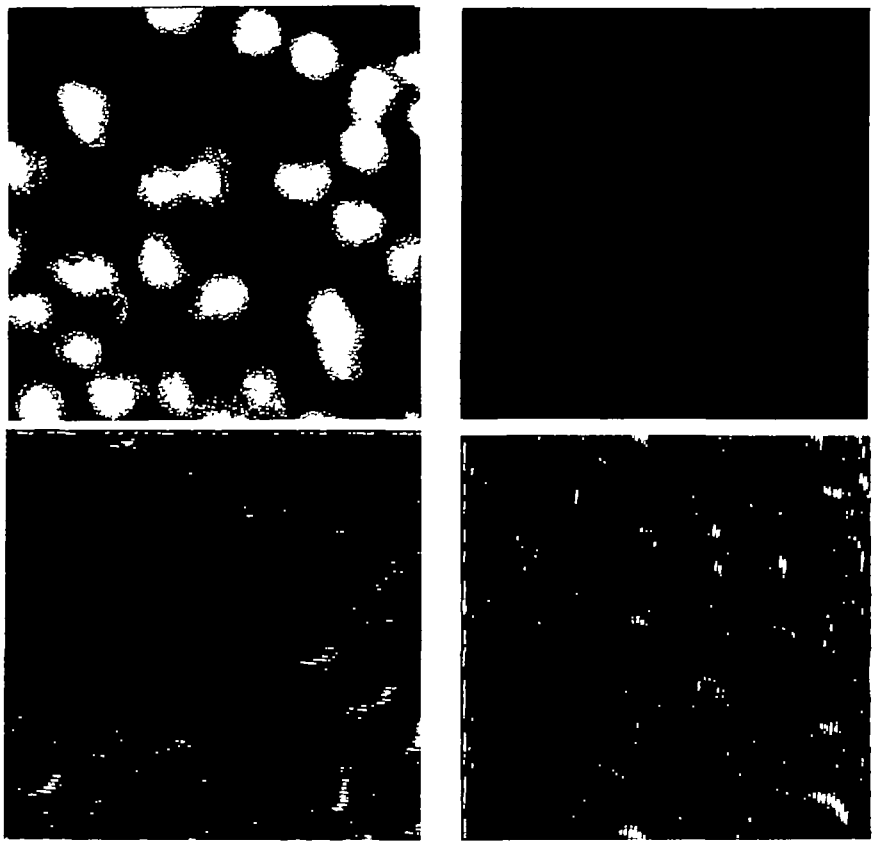


Fig. 5. **Photographs on confocal laser microscopy of erythrocytes interacted with the soluble form of the FITC-labeled PVG polymer.** Erythrocytes were incubated with the FITC-labeled PVG polymer at 4°C for 1 h. Also, erythrocytes were pretreated with the monoclonal antibody to GLUT-1 at 4°C for 1 h and then incubated with FITC-labeled PVG polymer at 4°C for 1 h. After the incubation, the cells were washed three times with PBS to remove the unreacted FITC-labeled polymer. (A) Specific interaction between the PVG polymer and erythrocytes. (B) Effect of the monoclonal antibody to GLUT-1 on the interaction between the PVG polymer and erythrocytes.

between erythrocytes and the reducing glucose-carrying PVG polymer was mediated by the interaction of GLUT-1s on the cell membrane and reducing glucose ligands on the polymer chain.

To clarify the GLUT-1 mediated specific interaction more directly, we performed fluorescence microscopical observation of erythrocytes which had interacted with the soluble form of the PVG polymer labeled with FITC by confocal laser microscopy. In Fig. 5a, erythrocytes show strong luminescence on interaction with the FITC-labeled PVG polymer. However, the luminescence almost completely disappeared on pretreatment with the monoclonal antibody to GLUT-1 (Fig. 5b). The results also supported that the specific interaction was mediated by GLUT-1. In this study, we found that a PVG polymer carrying reducing glucose interacted with erythrocytes which was mediated by GLUT-1. This study is the first to report that a glucose transporter can be labeled with a synthetic polymer.

From the above results, it is also proposed that sugar-carrying homopolymers are promising cell recognizable polymers for artificial organs, cell and tissue engineering, and drug targeting, and also useful tools for elucidating the cellular mechanisms involved in polymer-mediated cellular attachment *via* the specific receptors and transporters on cells.

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