Specific Cell Behavior of Human Fibroblast onto Carbohydrate Surface Detected by Glycoblotting Films

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We synthesized an aminooxyl polymer that is reactive with the reduced end of carbohydrates using our sugar-displaying approach. The carbohydrates were easily immobilized on the polymer film (glycoblotting film) by simple immersion in a in sugar solution through stable oxime bond. The in vitro behaviors of human fibroblasts on the carbohydrate-coated surface were investigated. The adhesion of human fibroblasts on the cellobiose- and cellotriose-coated surfaces was much greater than on the other coated surfaces and the noncoated surface. This result indicated that simple structural differences in carbohydrates induced biological changes in human cells, especially cell adhesion. Our approach provides a high-throughput assay system for carbohydrate-related cell adhesion and proliferation.

Introduction

The cell surface landscape is richly decorated with carbohydrates anchored to proteins or lipids within the plasma membrane.[1] Cell surface carbohydrates mediate the interactions of cells with each other and with extracellular matrix components. Through these interactions, the carbohydrates play an important role in regulating various biological functions such as cell differentiation, adhesion, and proliferation.[2,3] Synthetic carbohydrates and glycoconjugates provide materials for correlating structure with function. Our previous studies showed that glycosaminoglycans, which are complicated carbohydrates, enhance the biological reaction of chondrocytes and fibroblasts[4−7] and have the potential to enhance tissue regeneration from these cells.

Some kinds of cells bind to oligosaccharide surfaces in a carbohydrate-specific and concentration-dependent manner with synthetic polyacrylamide matrices.[8−10] The development of glycoconjugate engineering has made it possible to create artificial materials from bioactive carbohydrates as a new class of biomimetic molecules. Several studies have demonstrated that carbohydrate-coated materials provide superior biological activities for various cells.[11−17] Introducing glycopolymers into the materials for tissue regeneration has several advantages. First, artificial materials, including carbohydrates, cause various biological effects without resulting in immunological reactions in living tissues or organs. In scaffold materials currently used in the field of tissue engineering, such as collagen and alginate, one of the most considerable limitations is the probability of inflammatory reactions occurring due to their antigenicity.[18−20] We think that this limitation could be overcome by the use of carbohydrate-based materials. Second, though glycosaminoglycans and proteoglycans were commonly used as the scaffold materials in tissue engineering, these materials are expensive and difficult to handle to use as biomaterials.

To use carbohydrates as a component of scaffold materials, several problems must be solved. Technically, the method for incorporating carbohydrates into materials has not established. The previous technique for creation of carbohydrate-based materials often required a time-consuming synthetic approach.[8] We have developed an effective and practical trap-and-release method based on chemoselective ligation of carbohydrates with reactive aminooxyl groups displaying polymer for high-throughput glycomics. The glycoblotting polymer captured carbohydrates in aqueous solution under mild conditions.

In the present study, we applied our glycoblotting technique to creating sugar-displaying cell adhesion substrate. We hypothesized that simple carbohydrates would have specific biological effects on fibroblasts. To test this hypothesis, we incorporated six carbohydrates into the glycoblotting film. The objectives of this study were to quantify the in vitro adhesive and proliferative behaviors of human fibroblasts in culture dishes coated with carbohydrates and to determine the specificity of each simple carbohydrate for different biological cell behaviors. These results will make it possible to utilize cell-specific carbohydrates as scaffold materials.

Experimental Section

Methacrylic anhydride (MAH), ethylenedioxybis(ethylamine) (EDBEA), methyl methacrylate (MMA), and azobis(isobutylonitrile) (AIBN) were purchased from Sigma−Aldrich Co. (St. Louis, MO). Boc-aminooxyacetic acid (Boc-AOAA) was purchased from Novabiochem, Merck Biosciences (AG, Switzerland). All other chemicals were ultrapure grade and were purchased from Wako Pure Chemistries (Osaka, Japan). Enzyme-linked immunosorbent assay (ELISA) plates (96-well) were purchased from Sumitomo Bakelite Co (Tokyo, Japan).

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Synthesis of the Oxylamine Attached Polymer. Polymerization was performed via a standard radical polymerization method (Scheme. 1). The monomer (10 g) and MMA (7.71 g) were dissolved in 200 mL of methanol. The solution was kept at 60 °C and stirred under a nitrogen atmosphere. AIBN (0.515 g, 3 wt % monomers) was added to the mixture to start the polymerization reaction, and the mixture was stirred for 16 h. The obtained polymer was purified by use of a dialysis membrane (molecular weight cutoff 3500) followed by freeze-drying. The yield was 65%. Molecular weights were determined by gel-permeation chromatography. The HPLC was performed on TSK-gel GMHHR−-N and G1000HHR columns (Tosoh Bioscience, Tokyo, Japan) at a THF flow rate of 0.5 mL/min at 40 °C. The weight-averaged molecular weight was 1730. The number-averaged molecular weight was 6700. Polydispersity was 2.60.

Polymer Coating on the Culture Plate. Polymers were dissolved in methanol at a concentration of 1 wt %. The polymer solutions were spotted to each well of the 96-well plate and the solvent was slowly removed in air. Aqueous trifluoroacetic acid (20%) was added to each well, and the plate was incubated for 2 h at room temperature and then rinsed three times with pure water to produce the aminooxyl-displaying plate.

Carbohydrates (10 mg) were dissolved in 1 mL of HCl/KCl buffer (pH 2). A 96-well plate was coated with 100 μL of the carbohydrate solution in each well and incubated at 60 °C for 16 h in a 5% CO_2 incubator. This reaction condition was determined on the basis of our previous data (data not shown). Nonspecific binding sites were blocked by incubating the plates with 100 μL of 1% bovine serum albumin for 1 h at room temperature. The wells were then washed three times with PBS.

Adsorption of Fluorescence-Labeled Lectin onto the Surface of the Carbohydrate-Displayed Culture Plate. For characterization of carbohydrate-displayed surface, fluorescein isothiocyanate- (FITC-) labeled lectins were purchased from Seikagaku Corp. (Tokyo, Japan). The lectin solutions were prepared by diluting the stock solution to a concentration of 0.1 μg/mL with PBS buffer (pH 7.4; 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 0.2% Tween20). Nonspecific binding sites were blocked by incubating the oxiamine polymer-cast polystyrene plates with 100 μL of 1% bovine serum albumin for 1 h at room temperature. The polystyrene plates were fully submerged in the lectin solution and gently shaken for 1 h. The slide was washed with PBS buffer with gentle shaking for 30 min. These dishes were imaged on a microscope (FV300, Olympus, Tokyo, Japan) in the air.

Cell Culture. Human fibroblasts (NHDF), provided by Cascade Biologics, Inc. (Portland, OR), were incubated in tissue culture flasks containing complete Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, MO) with 10% fetal bovine serum (Invitrogen Corp., Carlsbad, CA), 100 IU/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B under a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The medium was renewed three times weekly. Cells at an 80% confluent state were rinsed and detached with 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS) for 3−5 min at 37 °C. The detached cells were washed with fresh medium three times by centrifugation (1500 rpm, 5 min) and suspended in fresh medium until used for experiments.

Cell Adhesion Assay. A cell adhesion assay was performed essentially according to the method described previously.[21] Microtiter plates (96-well) were coated with 100 μL of carbohydrate solution. As controls, coated polymer plates without carbohydrates were also prepared. Nonspecific binding sites were blocked by incubating the plates with 100 μL of 1% bovine serum albumin for 1 h at room temperature. 1-Methoxy-5-methylphenazinium methosulfate (1-methoxy-PMS) and other reagents were obtained from Dojindo Laboratories (Kumamoto, Japan). Tetrazolium (1 mM) and 50 mM citrate (for pH 5−6) or 50 mM Tris (for pH 7−9) were prepared and used. Fibroblasts (2.5×10^4 cells/well) in 100 μL of serum-free medium were added to the wells and incubated for 1 h at 37 °C in a 5% CO_2 incubator. After the removal of the unbound cells by gently rinsing the wells three times with PBS, 10 μL of the working solution containing WST-8 and 1-methoxy-PMS (0.5 mM and 20 μM, respectively, as the final concentrations) was added to each well. Then, the mixtures were incubated for an additional 2 h. The absorbance of each well was measured at 450 nm (ref 630 nm) with Microplate Reader Benchmark Plus (Bio-Rad, Hercules, CA). The results were obtained from three independent experiments.

Cell Proliferation Assay. A cell proliferation assay was also performed by WST-8 procedures as described previously.[22,23] Microtiter plates (96-well) were coated with 100 μL of carbohydrate solution. As controls, coated polymer plates without carbohydrates were also prepared. Nonspecific binding sites were blocked by incubating the plates with 100 μL of 1% bovine serum albumin for 1 h at room temperature. The fibroblast suspension (100 μL, 5.0×10^4 cells/well) was added to the wells and the plates were incubated at 37 °C for 24 h. Aliquots (10 μL) of the working solution containing WST-8 and 1-methoxy-PMS (0.5 mM and 20 μM, respectively, as the final concentrations) was added to each well. Then, the mixtures were incubated for an additional 2 h. The absorbance of each well was measured at 450 nm (ref 630 nm) with Microplate Reader Benchmark Plus (Bio-Rad, Hercules, CA). The results were obtained from three independent experiments.

Adhesion Blocking Assay. To confirm the data obtained from the cell adhesion assay, a carbohydrate adhesion blocking assay was performed. Microtiter plates (96-well) without polymer were prepared. Fibroblasts (2.5×10^4 cells/well) in 100 μL of each concentration of carbohydrate (5, 10, and 50 mg/mL) with serum-free medium were added to the wells and incubated for 1 h at 37 °C in a 5% CO_2 incubator. To evaluate the prevention of cell adhesion by carbohydrates, the cell adhesion assay was performed as described above from three independent experiments.

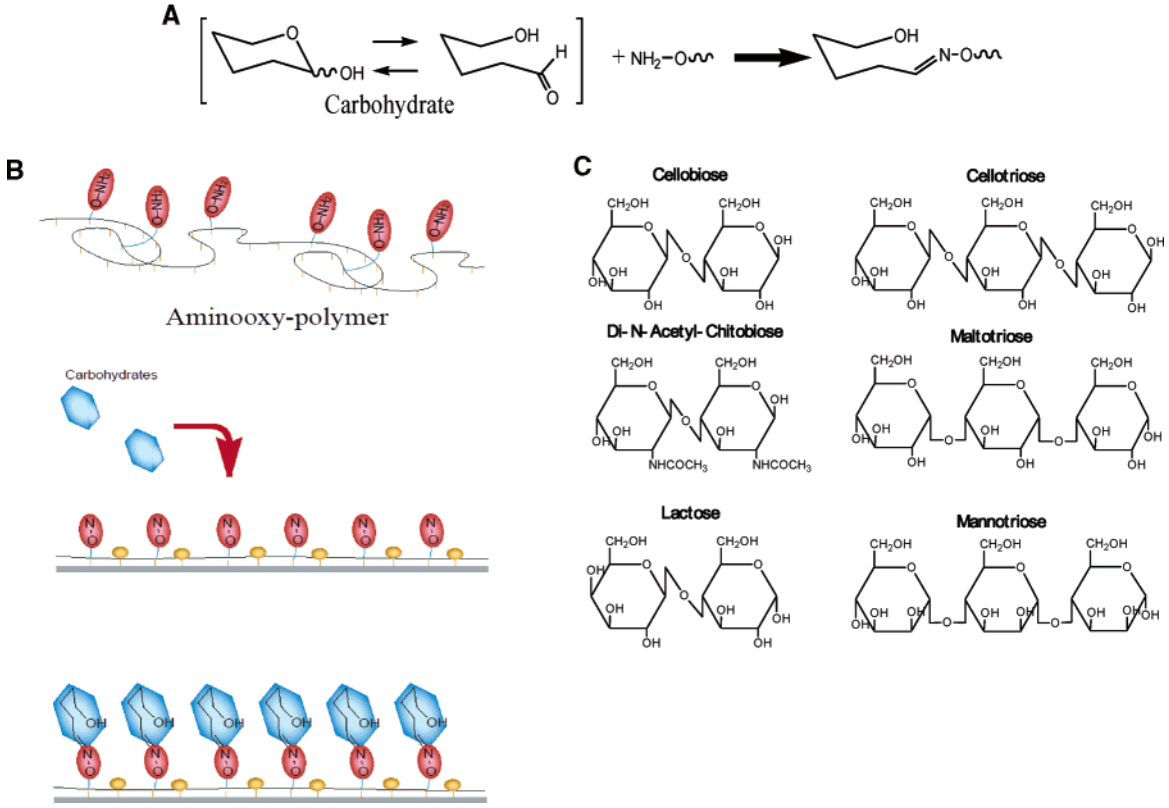
Incorporation of Carbohydrate-Displayed Quantum Dots Conjugate Stain. To confirm the data obtained from the cell adhesion assay, the affinity of the cell surface for the carbohydrates was evaluated by fluorescent beads stain. Fibroblasts were cultured on glass chamber slides (Asahi Techno Glass, Tokyo, Japan) in Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, MO) with 10% fatal bovine serum (Invitrogen Corp., Carlsbad, CA), 100 IU/mL penicillin,-100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B under a humidified atmosphere of 5% CO_2 and 95% air at 37 °C overnight. Cells were fixed with 4% formaldehyde for 10 min and were blocked for 20 min in PBS containing 1% (w/v) BSA. Sugar-displaying quantum dots were prepared by the strong interaction between Q-dot streptavidin conjugate (Quantum Dot Corp., Hayward, CA) and biotin-attached cellobiose. Fixed and BSA-blocked cells were incubated sequentially with carbohydrate-displaying quantum dots (20 nM) for 30 min. As control, quantum dots without carbohydrate were used. After staining, unbound quantum dots were removed with PBS washes. Cells were imaged on a microscope (AX80, Olympus, Tokyo, Japan). The obtained images were captured with a digital microscope camera (DP50, Olympus, Tokyo, Japan).

Statistical Analysis. All data were represented as mean ± standard deviation. Statistical comparisons were performed by using analysis of variance (ANOVA) and Fisher's protected least significant difference post hoc test. Differences were considered significant for $p < 0.05$.

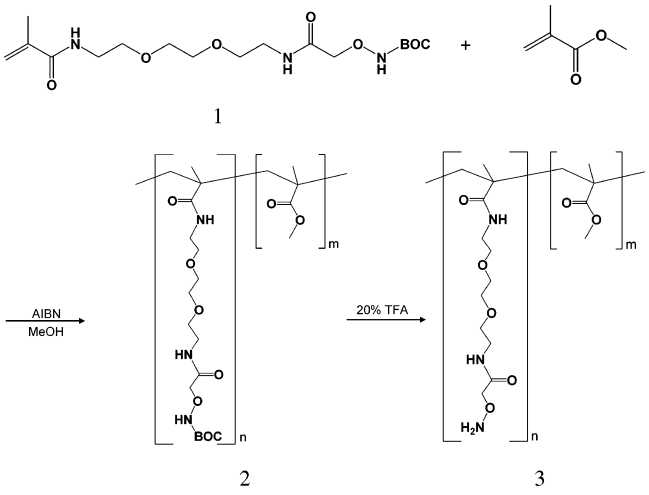
Results and Discussion

We have developed an effective and practical trap method based on chemoselective ligation of carbohydrates with reactive aminooxyl groups on displayed polymers (glycoblotting polymers) via stable oxime bond formation.[24,25] In this study, glycoblotting polymers were cast on plastic plates to provide the thin film (glycoblotting film) (Scheme 1). The chemical bonding of carbohydrates occurs on the reducing terminus of carbohydrate molecules. Considering the natural state of the

Scheme 1. (A) Ligation between Carbohydrates and the Aminooxyl Group. (B) Cartoon of the Process. (C) Chemical Formula of Six Carbohydrates



Scheme 2. Synthesis of Oxylamine-Containing Polymer



extracellular matrix (ECM) and the cell surface, homogeneous direction of carbohydrates on the polymer surface might simulate a natural state of these environments. Some sugar-based materials have already been developed by use of synthetic polymers such as polyacrylamide matrices[8−10] or polystyrene.[26] However, to prepare these materials, a time-consuming process containing multiple-step organic syntheses is required. The advantage of using the aminooxyl polymer is the simple immobilization of carbohydrates without complicated carbohydrate syntheses.

The synthesis of polymer **3** is shown in Scheme 2. Monomer **1**, which has a Boc-protected aminooxyl group, and MMA were copolymerized at a ratio of 1:3 (mol/mol) in methanol. Polymer **2** was cast on the 96-well plate and deprotection was performed by addition of aqueous TFA (20%) in wells. Carbohydrates were immobilized on the film in HCl/KCl buffer (pH 2). Although the proper pH for oxime formation in an aqueous solution is around 4, the proper pH for the glycoblotting film was around 2. This is due to the decrease in pK_a of the aminooxyl group on a solid surface. The sugar-trapping efficiencies on the polymer were around 80% for aminooxyl groups. The immobilization of carbohydrates on the glycoblotting film was confirmed by specific binding of FITC-labeled lectins. FITC-labeled RCA120 (galactose-specific lectin) was bound to the lactose- [Gal-β(1−4)Glc-]immobilized glycoblotting polymer (Figure 1A). As a control experiment, when ConA (mannose-specific lectin) was added to the lactose-displaying film, no bindings were observed (Figure 1B). RCA120 showed no binding to the glycoblotting film before immobilization of carbohydrates (Figure 1C). We tested whether the immobilized carbohydrates were retained in culture medium during cell adhesion and proliferation experiments. After incubation of lactose-displaying film in culture medium for 1 and 24 h, the solution of FITC−RCA120 was bound to the film. The fluorescent image shows that RCA120 were bound on the film almost the same as initial binding, indicating that the carbohydrates strongly immobilize on the film in culture medium for 24 h (Figure 1D,E). The carbohydrate immobilization on the polymer was also confirmed by orcinol/sulfuric acid method, which is a quantitative optical sugar assay. The sugar-trapping efficiencies on the polymer were around 80% for aminooxyl groups (data not shown).

Figure 2 shows the result of the cell adhesion assay. Human fibroblast adhesion on the cellobiose- and cellotriose-coated surfaces significantly increased, compared with that on the noncoated surface (292% ± 84.3% vs 100% ± 27.6%, cellobiose vs control, $p < 0.001$; and 249% ± 57.0%, cellotriose vs control, $p < 0.001$). Results of the adhesion blocking assay are shown in Figure 3. We repeated cell-adhesion and adhesion blocking experiments three times using different sets of surfaces and cells. In three experiments, we obtained similar cellular

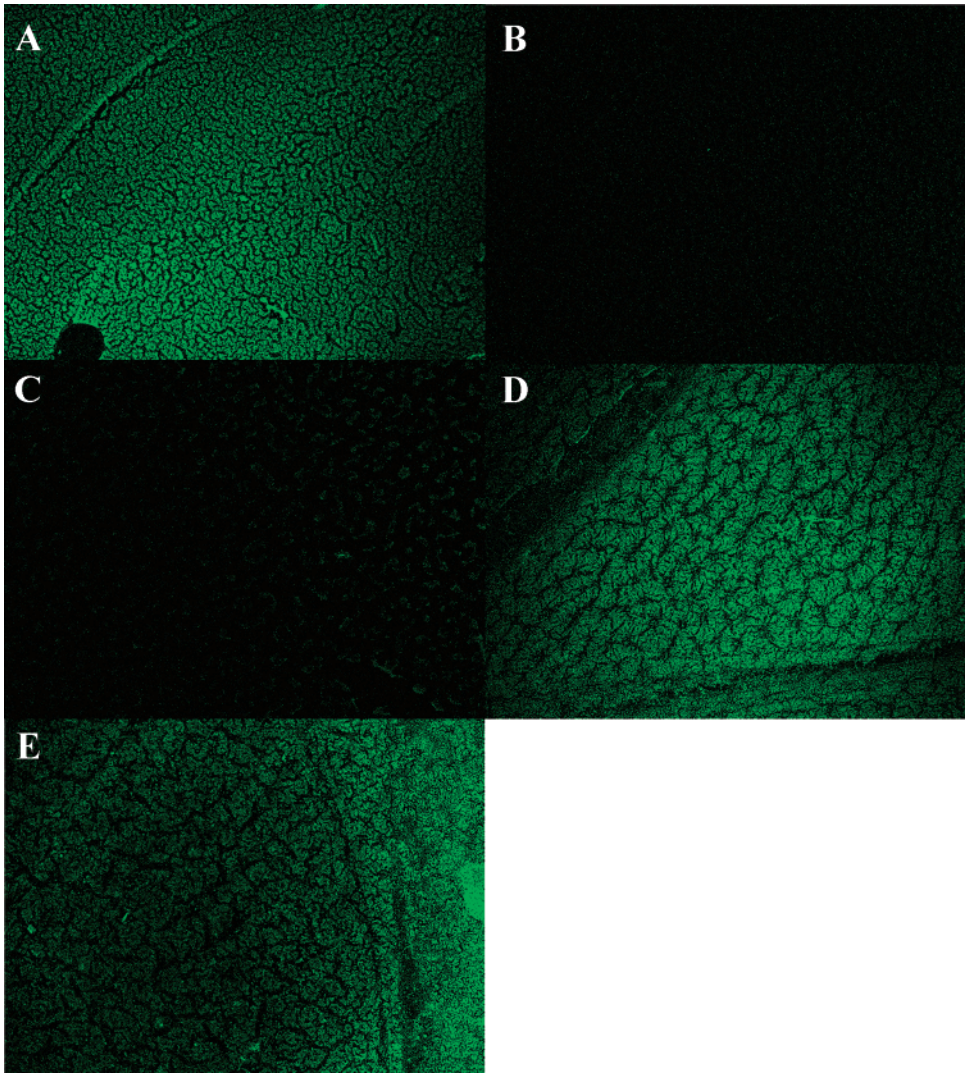


Figure 1. Lectin interactions with oxiamine-polymer before and after carbohydrate attachment. (A) FITC−RCA120 binding to lactose-displaying surface. (B) FITC−ConA binding to lactose-displaying surface (negative control). (C) FITC−RCA120 binding to the polymer without carbohydrate attachment. (D) FITC−RCA120 binding to the lactose-displaying surface after incubation in culture medium for 1 h. (E) FITC−RCA120 binding to the lactose-displaying surface after incubation in culture medium for 24 h.

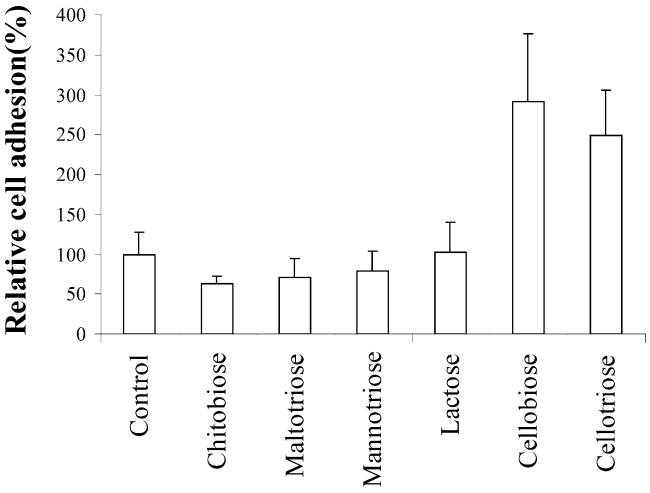


Figure 2. Cell adhesion on carbohydrate-displaying polymers. The cellobiose- and cellotriose-coated surfaces resulted in a significant increase, compared with the noncoated surface (292% ± 84.3% vs 100% ± 27.6%, cellobiose vs control, $p < 0.001$; and 249% ± 57.0% cellotriose vs control, $p < 0.001$).

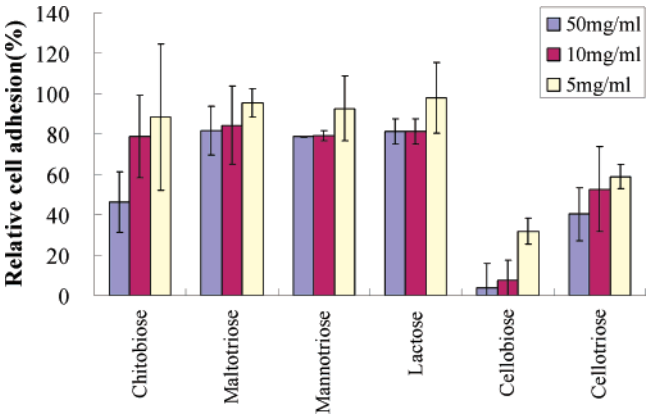


Figure 3. Cell adhesion blocking activity: Fibroblast adhesion on the polystyrene plate in the presence of free carbohydrate (10 mg/mL). Cellobiose and cellotriose significantly blocked fibroblast adhesion in each concentration (cellobiose vs all other carbohydrates, $p < 0.001$; cellotriose vs all other carbohydrates, $p < 0.001$, except vs 50 mg/mL chitobiose, $p = 0.5192$).

specificity for cellobiose and cellotriose with good reproducibility. We found that each carbohydrate inhibited cell adhesion in a concentration-dependent way. The obtained data showed that cellobiose and cellotriose significantly blocked fibroblast adhesion in each concentration of carbohydrate, compared with

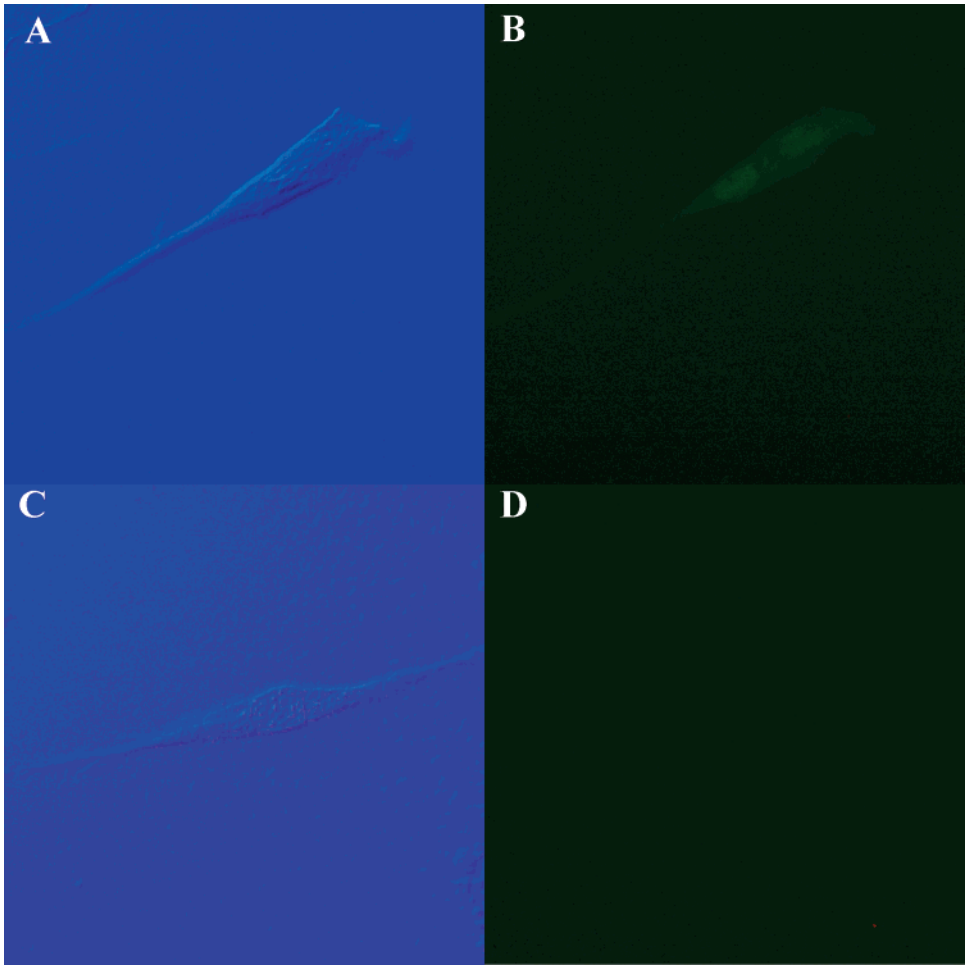


Figure 4. Incorporation of cellobiose-displaying quantum dots into fibroblasts: (A) bright field; (B) fluorescence image. As control, quantum dots without carbohydrate were incubated: (C) bright field; (D) fluorescent image.

the control (polymer without carbohydrate). These results indicated that cellobiose and cellotriose have the potential for specific adhesion to the human fibroblasts in a concentration-dependent way. Figure 4 shows the results of the carbohydrate-displayed quantum dots conjugate stain. Cellobiose-displayed quantum dots were well stained compared to quantum dots without carbohydrate. Cellobiose and cellotriose are considered to rarely exist in the human body. Those results indicate that the cell membrane has an affinity for cellobiose. Contrary to our expectation, carbohydrates that are not major components of the human body have a high capability for cell adhesion.

In the cell proliferation assay, fibroblast proliferation on all the surfaces coated with carbohydrates was significantly higher than on the surface without carbohydrate (Figure 5, $p < 0.05$, each carbohydrate vs control). Although cell proliferation on the cellobiose-coated surface tended to be higher than that on other carbohydrate-coated surfaces, there were no significant differences in the values among all the coated surfaces. Carbohydrates themselves had the potential for cell proliferation but there was no drastic specificity for cell proliferation. The proliferation of cells was obtained even if the cells adhered to the carbohydrate-coated dishes. One reason for this result was that carbohydrates are influential in loose initial cellular binding. In long-term culture such as cell proliferation assay, all cells secondarily attached onto carbohydrate-displaying surfaces after all for a short time, and significant difference was not detected for the long incubation.[9,27] Another reason is that carbohydrates promote production of fibronectin and other ECM proteins.[26] Therefore, there was a significant difference between the

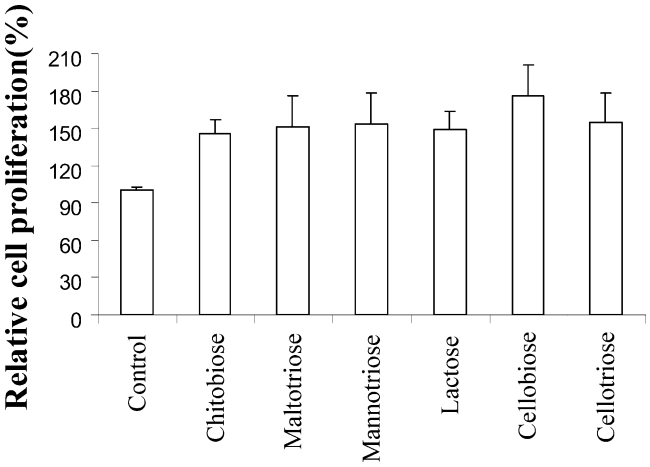


Figure 5. Cell proliferation activity on carbohydrate-displaying polymers. The proliferation on all the surfaces coated with carbohydrates were significantly higher than on the surface without carbohydrate ($p < 0.05$, each carbohydrate vs control).

carbohydrate-coated and noncoated surfaces. However, the fibronectin and other ECM proteins secreted from fibroblasts may mask the characteristics of each carbohydrate-coated surface. After all, all kinds of carbohydrate in this study increased cell proliferation. We have to elucidate this point in the future.

The relationships between carbohydrates and certain cells have been clarified.[28] For example, the relationship between galactose and hepatocytes has been investigated in detail.

Asialoglycoprotein receptors are lectins for receptor-mediated endocytosis found at the hepatocyte cell surface, and there are many reports that address the behavior of hepatocyte adhesion to artificial polymer surfaces having terminal galactose moieties.[29,30] Kim et al.[13] stated that mannose, maltose, and cellobiose are not inhibitors of hepatocyte-carbohydrate adhesion but lactose is an inhibitor.[13] In the present study, we confirmed specific adhesion of human fibroblasts on the cellobiose-coated surface and on the cellotriose-coated surface. The result of our study is different from that of Kim because asialoglycoprotein receptors do not exist on the surface of fibroblasts.

The most significant point of this study is that carbohydrates of phytogenic origin, such as cellobiose and cellotriose, have significant cell adhesion properties compared with other carbohydrates. There are some reports in which phytogenic-origin carbohydrates were used for cellular adhesions. However, they used different cells, such as hepatocytes, and showed no significant adhesivity to the phytogenic-origin carbohydrates. Our findings of the strong adhesion to phytogenic-origin carbohydrates seem to be of specific to fibroblast cells.[31,32] Most biomaterials in the medical field have been developed based on the major components of ECMs in animal body. These biomaterials contain the problems of zoonotic infection and immunological reaction. In a report, cellocarbohydrate produced by bacteria has a drastic effect on wound healing.[33] On the basis of these findings, when we choose the materials suitable for a scaffold, we must consider not only the materials of human origin but also materials of other origin. In the future, an appropriate bioactive material for a scaffold may be identified from an unexpected place.

The most considerable limitation of this study is that the adhesion mechanism between cells and cellobiose remains unclear. We did not investigate why human fibroblasts selectively adhere to cellobiose and cellotriose. Gutsche et al.[26] stated that murine fibroblast spreading was greater on polymer surfaces derivatized with *N*-acetylglucosamine than those with adenosine and chloromethyl styrene. They did not mention the mechanisms of adhesion. Inhibition of cellular adhesion by free cellobiose leads the hypothesis that there possibly is a carbohydrate-specific receptor such as asialoglycoprotein on the surface of the human fibroblasts. Further studies are necessary to clarify the mechanisms of cell adhesion for cellobiose and for cellotriose.

Conclusion

We have developed a novel technology to stabilize carbohydrates with a polystyrene polymer. This method is quite simple and the carbohydrate bonds occur on the reducing-terminal side. The differences in the potential for cell adhesion and proliferation can be detected in the six carbohydrates by using the novel carbohydrate-trapping polymer. On the basis of the results obtained here, we will make a useful scaffold material without using antigenic complex macromolecules such as collagen. This polymer will be applied to construction materials for culture applications and to scaffolds for tissue engineering.

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