Key words: human epithelial cells, glucose-displayed surface, glucose transporter-mediated anchoring, morphological change, cellular roundness, receptor saturation model

INTRODUCTION

In cultures of anchorage-depending mammalian cells, adhesion-promoting proteins of extracelluar matrix (ECM), such as fibronectin and vitronectin, are complex multifunctional elements which interact with other matrix molecules and also with cytoplasmic receptors. In general, the attachment of cells onto a surface is initiated by mediation of trans-membrane receptors, mainly integrins, associated with cytoskeletal formation (1-3). Integrin-mediated binding on a surface leads to the formation of focal contacts through a cascade of phosphorylation events, resulting in linking the ECM proteins on the extracellular face of the cytoplasmic membrane to cytoskeletal proteins with actin filaments on the intracellular face (4, 5). Moreover, these events give rise to the recruitment and assembling of actin-binding proteins, being attributed to the stimulation of intracellular signal transduction pathways. This coordination between the integrins and their binding sites ultimately concerns the cellular fates with respect to adhesion, spreading, migration, division and differentiation (6-11).

The mechanisms of integrin-mediated changes in cell morphology have been considered to be attributed to linkages between the integrin domains on the cytoplasmic membrane (5, 6). Various elements of ECM contribute to morphological changes accompanied with variation in cytoskeletal organization. In particular, variation in the surface quantity of integrin-mediated binding sites has been demonstrated to be an important factor for morphological changes in several cell species (12). A recent technique for the regulation of surface adhesion ability of cells is the coating of RGD (Arg-Gly-Asp), which is a functional domain of fibronectin and other ECM molecules

(13, 17). This technique was extended to preparing substrates with localized ligand display. The development of these substrates for cell adhesion can provide new insights into cell biology as well as sophisticated methodology for controlling morphogenesis of cultured cells and tissues (10, 11, 16, 18).

The carbohydrate moieties have been used to mediate attachment of hepatocyte cells in the field of tissue engineering to address the asialoglycoprotein receptor as well as transporters (19-21, 22, 23). Akaike and co-workers reported that the molecular recognition between asialoglycoprotein receptor and galactose ligand resulted in specific adhesion of hepatocytes on synthetic matrix and preserved the differentiated hepatic functions by promoting the formation of round adherent cells and aggregates (24, 25). Among natural carbohydrates, glucose has been focused as a specific cell recognition molecule because it is a common source for cellular component synthesis and biological energy yielding. The passive uptake of glucose via glucose transporters (GLUTs) is necessary for mammalian cells' metabolism (26, 27), and several different GLUTs works on the cytoplasmic membranes in various kinds of cells (28).

In the previous work (29), the morphological variation was observed in cultures of rabbit chondrocyte cells on D-glucose displayed surface where the ratio of D- and L-glucose displayed was changed. To gain access to the initial cellular events on D-glucose displayed surface, in the present study, we investigate the influence of displaying D-glucose on the attachment and morphology of human epithelial cells with changed expression of GLUTs by addition of insulin. Moreover, the fundamental mechanisms of cell and culture surface interaction are discussed concerning the formation of actin cytoskeleton and binding domain.

MATERIALS AND METHODS

Preparation of glucose-displayed surfaces Unless otherwise stated, the conventional plastic surface in an 8-well culture plate (surface area: 8.6 cm^2, Nalge Nunc, Roskilde, Denmark) was used as a starter material for displaying glucose. To create a template with hydroxyl group on the surface, an aqueous solution of 50 $\mu mol/cm^3$ potassium *tert*-butoxide was poured into each well, and then the well was incubated for 1 h under an ambient condition, followed by irrigating three times with sterilized water. An aqueous solution of 360 $\mu mol/cm^3$ glutaraldehyde was introduced into the well which was then allowed to stand for 1 h and washed thoroughly with sterilized water. The well was treated with 360 $\mu mol/cm^3$ tris(2-aminoethyl) amine solution (adjusted to pH 9.0 with 1 mol/cm^3 NaOH) for 1 h and rinsed with sterilized water.

To display glucose as a ligand on the template surface, D- and/or L-glucose solution (totally 0.1 $\mu g/cm^3$) was added to and left in each well for 2 h. The ratio of D- to L-glucose in the applied solution was changed so as to obtain the surface preparations with 0, 25, 50, 75 and 100% D-glucose display. A 0.5 $\mu mol/cm^3$ sodium borohydride solution was poured into the well, and after standing for 24 h, the well was washed twice with phosphate buffered saline (PBS, Sigma-Aldrich, St. Louise, MO, USA) prior to cell seeding.

When a 25-cm^2 T-flask (Nunclon Delta Flask; Nalge Nunc) was used, the procedures for displaying glucose on the surface were almost the same as describe above.

Cell cultures Human mammary epithelial cells (hTERT-HME1; Clontec Laboratories, San Diego, CA, USA) were obtained in a frozen state, and then the cells in vials were thawed according to the supplier's instruction. These cells were subcultured in a 25-cm^2 T-flask at 37 °C under a 5% CO_2 atmosphere using HuMedia-KG2 serum-free medium with 6.0 μmol/cm^3 D-glucose (Kurabo Ind., Osaka). For the experiments, the initial concentration of viable cells, determined by trypan blue exclusion, was fixed at $X_0 = 5.0\times10^3$ cells/cm^2, and the cultures were conducted on the prepared culture surfaces using the medium with or without insulin (10 μg/cm^3 when added) under the same conditions as described above.

For tracing dynamic variation in cell morphology, time-lapse observation of individual cells was conducted, and the images captured through a CCD camera system were processed according to the previous papers which described details of tools (29, 30). The value of roundness, R_c, of each cell was determined by the following equation, employing the projected area and periphery, respectively a_c and l_c of a single cell, which were obtained by extracting the cellular edge using a line-drawing tool (WinROOF, Mitani Co., Fukui).

$$R_c = \frac{2(\pi a_c)^{1/2}}{l_c} \quad ; \quad 0 < R_c \leq 1 \tag{1}$$

To evaluate cell attachment and apparent cell morphology at 24 h after seeding, the triplicate wells were picked up, and the bottom surface images were captured at five different positions in each well by using a CCD camera (CS6931; Toshiba Teli, Tokyo) attached to a microscope (area of captured image: 2.4 mm^2). The efficiency of cell attachment, X_{24}/X_0, defined as a ratio of attached cells at 24 h against seeded cells on

five images, was determined in each well. The overall roundness, R_{oc} of cells in each well was calculated by the following equation.

$$R_{oc} = \frac{2(\pi A_{oc})^{1/2}}{L_{oc}}; \quad 0 < R_{oc} \leq 1 \tag{2}$$

Here, the overall area and periphery, respectively A_{oc} and L_{oc}, of all cells projected on five images in each well were employed.

The values of X_{24}/X_0 and R_{oc} were recorded as means, represented by $\overline{X}_{24}/\overline{X}_0$ and $\overline{R}_{oc}$ respectively, from the measurements for triplicate wells.

Treatments of cells for blocking GLUTs and integrin For antibody treatments, the cells were recovered from the subculturing flasks by trypsin digestion, and were in advance incubated with 0.5% Block Ace (Dainippon Sumitomo Pharma Co., Ltd., Osaka) for 30 min at 37°C for masking nonspecific binding sites on the cells. The resultant cells were resuspended at 6.4×10^6 cells/cm^3 in HuMedia-KG2 medium containing 0.5% Block Ace. A polyclonal antibody against GLUT1 (1:100 dilution, Alpha Diagnostic International Inc., San Antonio, TX, USA), and monoclonal antibodies against GLUT4 (1:200 dilution, Biogenesis, Poole, UK) and integrin $\alpha_5\beta_1$ (1:50 dilution, Chemicon International, Inc. Temecula, USA) were adequately blended with the medium, and the cells were treated with the respective antibodies for 1 h at 4°C with gentle mixing every 20 min. The antibody-treated or non-treated cells were cultured on the indicated surfaces to examine the cell attachment and morphology.

Fluorescent microscopy for cytoskeletal observation and GLUT localization For visualization of the cytoskeletal elements and GLUTs, the cells were washed three times with PBS and fixed with 4% paraformaldehyde in PBS for 10 min at room

temperature. They were then rinsed with PBS and permeabilized by incubating for 3 min in 0.1% Triton X-100. After washing again with PBS, nonspecific binding sites on the cells were masked with Block Ace by incubating for 1 h at room temperature. The cells were then treated with anti-GLUT1, anti-GLUT4 and/or anti-vinculin primary antibodies (Sigma-Aldrich) that were adequately diluted in PBS containing 10% Block Ace. After washing with PBS and rinsing with Tris-buffed saline (DakoCytomation Carpinteria, CA, USA), the cells were subjected to immunolabeling with Alexa Fluor 568 goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA). For the immunolocalization of F-actin, the cells were washed with PBS and then stained with Alexa Fluor 488 phalloidin (Molecular Probes). The treated cells were washed three times with PBS and then mounted on a sample slide with Prolong Antifade solution (Molecular Probes). Fluorescent images were acquired using a confocal laser scanning microscope (Model FV-300, Olympus, Osaka). For stereoscopic observation of cells, a series of images was piled up longitudinally at a 0.2 μm interval.

RESULTS

Cellular behaviours on glucose-displayed surfaces To examine the interaction between epithelial cells and glucose molecules displayed on the surface, the cultures were performed with the insulin administration at 24 h of culture time. Figure 1 shows the changes in R_c values of representative cells in the courses of cultures on the 0, 50 and 100% D-glucose-displayed surfaces. After seeding in the insulin-free medium, the cells on 50% D-glucose-displayed surface started to adhere and maintained a stretched shape with R_c = 0.66–0.78 (Fig. 1B) while the R_c values on both 0 and 100%

FIG. 1

D-glucose-displayed surfaces were 0.92–0.99 and 0.90–0.99, respectively (Fig. 1A and C). After the insulin administration, on 100% D-glucose displayed surface, the cell immediately elongated and reached $R_c = 0.70$ at $t = 60$ min (Fig. 1C), while the cells on 50% D-glucose-displayed surface exhibited round shape with $R_c = 0.89$ at $t = 60$ min (Fig. 1B). These results mean that the different morphological changes occurred, accompanied with the extension and contraction of the cells after the insulin administration on 50 and 100% D-glucose-displayed surfaces. These morphological differences between the cells on the three surfaces are also obvious on typically selected snapshots presented at the upper side of Fig. 1. Thus the time-lapse observation revealed that the cells made the distinct response to the insulin administration when cultured on the surfaces with different ratios of D-glucose display.

To examine in detail the cell behaviors on the glucose-displayed surfaces, the cultures were conducted for 24 h in the presence or absence of insulin on the surfaces with various ratios of D-glucose display. As shown in Fig. 2A, there was no significant difference in the $\overline{X}_{24} / \overline{X}_0$ values on the glucose-displayed surfaces, showing the same levels in efficiency of cell attachment whether insulin existed or not.

FIG. 2

The morphological examination was also conducted at 24 h after seeding in terms of the $\overline{R}_{oc}$ value (Fig. 2B). In the presence of insulin, the $\overline{R}_{oc}$ value decreased with increasing ratio of D-glucose displayed on the surfaces, being $\overline{R}_{oc} = 0.88$ and 0.76 at 0 and 100% D-glucose display, respectively. In the insulin-free culture, on the other hand, a concave profile of $\overline{R}_{oc}$ value was obtained and the value on 100% D-glucose-displayed surface was $\overline{R}_{oc} = 0.87$, the value of which was comparable to that on 0% D-glucose-displayed surface. On 50% D-glucose-displayed surface, the value

of $\overline{R}_{oc}$ was minimized to be 0.77 which was 13% reduction, as compared to that on the surface without D-glucose display.

Blocking effects of GLUTs and integrin For further understanding the mechanism of cell attachment, the cells were in advance treated with antibodies for GLUTs1 and/or 4, and then were subjected to seeding on the glucose-displayed surfaces. As shown in Fig. 3, no significant difference in the efficiency of cell attachment was recognized on the D-glucose-displayed surfaces, giving $\overline{X}_{24}/\overline{X}_0$ value of nearly unity whether insulin existed or not. In a similar way, the cell attachment test was conducted using cells treated with the antibody for integrin $\alpha_5\beta_1$ on the D-glucose-displayed surfaces. The blocking of integrin $\alpha_5\beta_1$ caused significant reduction of cell attachment, resulting in $\overline{X}_{24}/\overline{X}_0 = 0.61$ on 0% D-glucose-displayed surface in the presence of insulin. In addition, the $\overline{X}_{24}/\overline{X}_0$ values on the D-glucose-displayed surfaces in the presence of insulin slightly increased on 100% D-glucose ($\overline{X}_{24}/\overline{X}_0$ = 0.78), compared to that on 0% D-glucose-displayed surface (Fig. 3). These results can support the view that cell attachment mainly depends on the integrin mediation on the glucose-displayed surfaces examined in this work.

FIG. 3

The test of morphological change was also conducted by culturing the cells with the GLUT blockings for 24 h. As shown in Fig. 4, in the insulin-free cultures on the D-glucose-displayed surfaces, the values of $\overline{R}_{oc}$ for the single blocking of GLUT1 and double blocking of GLUTs1 and 4 were kept to be levels over $\overline{R}_{oc}$ = 0.87, meaning no notable morphological variation due to inhibition of cell stretching. However, in the culture of the cells with single blocking of GLUT4, the $\overline{R}_{oc}$ profile on various

FIG. 4

D-glucose-displayed surfaces was overlapped with that in the culture of the cells with non-blocking of GLUTs. In the insulin-containing culture, the single blocking of GLUTs1 or 4 caused the concave profile of $\overline{R}_{oc}$ value against the ratios of D-glucose displayed on the surfaces, and the minimum $\overline{R}_{oc}$ values of $\overline{R}_{oc}$ = 0.77 and 0.78 were obtained on 50 and 75% D-glucose displayed surfaces, respectively. When the cells experienced the double blocking of GLUTs1 and 4, the stretching of cells was not appreciable in the presence of insulin, being no significant variation in $\overline{R}_{oc}$ value, in analogy to that in the insulin-free culture. These results indicated that the mediation between D-glucose and GLUTs participates not only in the cell attachment to surface but also in the morphological change. The concave profile of $\overline{R}_{oc}$ value was considered to attribute to the quantative balance between D-glucose displayed on the surface and GLUTs expressed on the cellular membrane.

Cytoskeletal formation and GLUT localization To confirm the cytoskeletal formation associated with integrin- and GLUT-mediated bindings, the fluorescent microscopy was applied for the cells cultured for 24 h on the D-glucose-displayed surfaces in the presence and absence of insulin. As shown in Fig. 5, distinct fibers of F-actin with vinculin spots were recognized under all the examined conditions. Especially, the vinculin spots at an inner part of cell on 100% D-glucose-displayed surface clearly appeared with transversal stress fibers of F-actin, meaning the promoted formation of focal contacts not only at a periphery but also at a whole body of cell in the presence of insulin.

FIG. 5

As shown in Fig. 6, GLUT1 was expressed under all the conditions, especially at inner parts of a cell body where filopodia and lamelipodia of F-actin did not exist, while

FIG. 6

the cells on 100% D-glucose-displayed surface in the presence of insulin exhibited nebulous distribution of GLUT1 expression at a whole cell body including the top of filopodia. In contrast with GLUT1 localization in the cells, a notable difference was seen in the distribution of GLUT4 in association with the stimulation of the cells by insulin. As demonstrated in Fig 6, GLUT4 spots were observed around the nucleus under the insulin-free condition, and the spatial distribution of GLUT4 was seldom observed on the basal side of cell. In the presence of insulin, the exhibition of GLUT4 on the cytoplasmic membrane was observed. On 100% D-glucose-displayed surface, punctuate GLUT4 spots were widely scattered on the basal and apical sides of cell, whereas the cell on 0% D-glucose-displayed surface showed intensive GLUT4 spots only on the apical side. These findings suggest that the insulin stimulation facilitates, together with D-glucose display, the localization of GLUTs1 and 4 to cellular parts near the cell edges.

DISCUSSION

Integrin and GLUTs mediate cell attachment and spreading on D-glucose-displayed surface A series of cellular behaviors including adhesion, migration, division and differentiation occurs during *in vitro* culture of anchorage-dependent cells. Especially, the cell attachment onto a surface and subsequent morphological change are important events until growth initiation after seeding. In this study, we examined the mechanism concerning the morphological response of epithelial cells to glucose molecules displayed on the surface under the culture conditions with and without insulin. Integrins are crucial transmembrane

proteins for cell-ECM bindings and initial cell attachment generally relies on the integrin-mediation (1, 5, 7). The blocking experiments for integrin $\alpha_5\beta_1$ binding indicated that the integrin mechanism mainly contributed to cell attachment rather than GLUT-mediated bindings (Fig. 3), which supported the consideration that the latter bindings made primary contribution to the morphological change of epithelial cells.

In mammalian cells, the facilitative uptake of D-glucose is mediated by a family of specialized GLUTs. According to current reports (26, 27), there are at least 13 isoforms of facultative GLUTs that are differentially expressed in various cell species. Cellular uptake of D-glucose can be performed by expressing GLUTs on cell surface through a series of events such as synthesis of GLUTs in cytoplasm, translocation of vesicle-associated GLUTs to cytoplasmic membrane and fusion of GLUT vesicles with cytoplasmic membrane. In addition, GLUTs show the sharp specificity of binding affinity against glucose isomers, that is, the affinity of GLUTs is high against D-glucose, while being extremely low against L-glucose. D-Glucose itself is continent to be an inert component to induce cell signaling. But it is most likely that such the high affinity can work as the cell anchoring mechanism via GLUT mediation for D-glucose molecules displayed on the surface. In the case of insulin-sensitive cells such as adipose cells, about 50% of intracellular GLUT4 is translocated onto the cytoplasmic membrane when activated by insulin, providing a 10-fold increase in the amount of the transporter on cell surface (28). The overall insulin-dependent shifts in the cellular dynamics of GLUT vesicle trafficking result in an increase of GLUT4 on the cell surface, while GLUT1 is localized both to the cytoplasmic membrane and intracellular storage sites (26-28, 31). These natures of GLUTs1 and 4 seem to be concordant with our results obtained in the

culture of epithelial cells on the D-glucose-displayed surfaces in the absence and presence of insulin. Insulin stimulation caused rapid change in cell morphology within 40 min on 100% D-glucose-displayed surface, possibly owing to display of GLUT4 on the cytoplasmic membrane (Fig. 1), and the immunofluoresence staining clarified the translocation of GLUT4 (Fig. 6). Furthermore, stainings of GLUTs and F-actin showed the co-localization of GLUTs on the leading edges of cells with filopodia and lamellipodia when cultured on 100% D-glucose-displayed surface with insulin, though GLUTs were observed exclusively in the cell body on 0% D-glucose-displayed surface. Especially, GLUT4 was observed in the cytoplasmic membrane on the apical side of cells when cultured on 0% D-glucose-displayed surface in the presence of insulin.

Morphological variation depends on quantative balance of GLUTs and D-glucose displayed In the present work, the drastic variation in cell morphology was also induced by changing the ratio of D-glucose displayed on the surface (Fig. 2). The morphological variation was recognized in culture of rabbit chondrocytes by changing the density of D-glucose displayed on surface (29). This morphological variation can be explained according to the "receptor saturation" model proposed by Gaudet *et al.* (32). They reported morphological variation of fibroblast cells cultured on a fibronectin-coated surface where cellular integrin and fibronectin function as receptor and binding sites, respectively. The model states that the morphological variation occurs owing to the quantitative balance between binding sites on surface and receptor sites on the cytoplasmic membrane as follows. Much lower density of binding sites, as compared with receptor sites, leads to a round shape of cells, associated with weak cell attachment on the surface. With an increase in density of binding sites, cell morphology

transfers to a stretched shape. Further increment in density of binding sites yields round shaped cells with strong cell attachment on the surface. Ultimately an appropriate balance between binding and receptor sites makes the cells stretched.

Based on the data obtained in our experiments, D-glucose molecules and GLUTs are considered to work as binding and receptor sites, respectively, as illustrated in Fig. 7. FIG. 7 In the absence of insulin, the cells on 0% D-glucose-displayed surface became relatively round shape, likewise in the case of cells cultured on the plain surface in a conventional culture well (33, 34), owing to no binding sites for GLUT mediation. With an increase in the ratio of D-glucose on the surface (see Arrow A in Fig. 7), the cells showed excessive stretching, while cell stretching on 100% D-glucose-displayed surface was inhibited owing to shortage of GLUTs, resulting in the concave profile of $\overline{R}_{oc}$ value (Fig. 2). In the absence of insulin, however, blocking of GLUT1 caused the hindrance of stretching at any ratio of D-glucose displayed on the surface, while the profile of $\overline{R}_{oc}$ value with blocking of GLUT4 resembled the profile without blocking (Fig. 4). These facts suggest that the morphological variation in the absence of insulin was caused by fluctuation in GLUT1 expression. In the presence of insulin, with an increase in the ratio of D-glucose displayed on the surface, the cell stretching was promoted, and on 100% D-glucose-displayed surface, the stretching was the most remarkable. These facts mean that the insulin-encouraging expression of GLUT4 on the cytoplasmic membrane as receptor sites could maintain the stretch shape (see Arrow B in Fig. 7), even through the hindrance of stretching in the absence of insulin occurred owing to reduction of GLUT4 expression as receptor sites. A reduction in receptor sites was also achieved by the GLUT blocking experiment in the present work (see Arrow C in Fig. 7). The single

blocking of GLUTs1 or 4 caused the hindrance of stretching on 100% D-glucose displayed surface, again resulting in the concave profile of $\overline{R}_{oc}$ value (Fig. 4). The double blockings of GLUTs1 and 4 led to fewer receptor sites, being attributed to the hindrance of stretching at any ratio of D-glucose displayed on the surface. These results support the view that the receptor saturation model can be valid for the GLUT mediation mechanism.

According to Cox *et al.*, a biphasic relationship existed between density of coated fibronectin and activation of Rho family of GTPases in terms of regulating the spreading and motility of fibroblasts (35). This trend was concordant with our results obtained in the culture of epithelial cells on 100% D-glucose-displayed surface in the presence of insulin. On 100% D-glucose-displayed surface the cells exhibited increased formation of focal contacts, with the developments of distinct vinculin spots, suggesting that the grasping GLUTs on the cytoplasmic membrane with D-glucose on the surface caused the active formation of the contacts (Fig. 6). It is considered that D-glucose display on the surface permits the cells to be in close contact with the surface through grasping of GLUTs on the cytoplasmic membrane, which is attributable to the promotion of focal contact formation.

The present work proposed the possible mechanism of cell anchoring and morphological change in the culture of human epithelial cells on the D-glucose displayed surface. The variation in the ratio of D-glucose displayed caused the cellular morphological change which depended on the culture condition with or without insulin. The cultures of cells with blockings for GLUTs1 and 4 as well as integrin $\alpha_5\beta_1$ revealed that cell attachment and morphology were dominantly governed by the mediations via

integrin and GLUTs, respectively. In addition, the fluorescence microscopy for F-actin, vinculin, and GLUTs clarified the localization of integrin-mediated as well as GLUT-mediated anchoring, inducing the consideration that the morphological change of cells is responsible for the variation in the quantitative balance between D-glucose density on culture surface and GLUTs on the cytoplasm membrane, associated with the promoted formation of focal contacts by GLUT mediation. It is expected that the GLUT-mediated anchoring will offer a synergistic impact on the cell signaling by means of co-immobilization of D-glucose and stimulator molecules targeting receptors on the cell membrane.

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