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Glucose Transporter Mediation Responsible for Morphological Changes of Human Epithelial Cells on Glucose-Displayed Surfaces

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Cellular morphology is one of the important factors for coordinating cell signaling. In this study, the morphological variation via glucose transporter (GLUT)-mediated anchoring was investigated in the cultures of human mammary epithelial cells in the presence or absence of insulin on culture surfaces with the changed ratios of D- and L-glucose displayed. With increasing ratio of D-glucose displayed on the surfaces, the cells showed a stretched shape in the culture with 10 μg/cm^3 insulin, reaching the highest extent of cell stretching at 100% D-glucose display, whereas round cells were predominant at 0% D-glucose display. In the absence of insulin, on the other hand, the extent of cell stretching showed a concave profile in terms of the ratio of D-glucose displayed, the extent being highest at 50% D-glucose display. Blocking of integrin $\alpha_5\beta_1$ or GLUTs1 and 4 on the cells using corresponding antibodies revealed that the primary mechanism for cell attachment was based on integrin-mediated binding, and that GLUTs1 and 4 contributed largely to morphological changes of cells. Confocal microscopy further revealed that GLUT4 localization occurred in response to D-glucose display as well as insulin addition. In the absence of insulin, GLUT4 spots were extensively observed in the cell body regardless of whether D-glucose was displayed or not. However, in the presence of insulin, the broad distribution of GLUT4 appeared on the basal and apical sides of cells at 100% D-glucose display, in contrast with its localization only on the apical side of cells at 0% D-glucose display. These results suggest that the quantitative balance between GLUTs on the cytoplasmic membrane and D-glucose displayed on a culture surface determines the cell morphology, as explained by the receptor saturation model.

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

In cultures of anchorage-depending mammalian cells, adhesion-promoting proteins of an 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 culture surface is initiated by mediation of transmembrane receptors, mainly integrins, associated with cytoskeletal formation (1–3). Integrin-mediated binding on a culture 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 lead to the recruitment and assembling of actin-binding proteins, which are attributed to the stimulation of intracellular signal transduction pathways. This coordination between integrins and their binding sites ultimately determines the cellular fates with respect to adhesion, spreading, migration, division, and differentiation (6–11).

The mechanisms underlying 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 by 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

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tripeptide (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 methodologies for controlling the morphogenesis of cultured cells and tissues (10, 11, 16, 18).

The carbohydrate moieties have been used to mediate the attachment of hepatocytes in the field of tissue engineering to study the asialoglycoprotein receptor as well as transporters (19–23). Cho *et al.* (24) and Park *et al.* (25) reported that the molecular recognition between the asialoglycoprotein receptor and galactose ligand results in the specific adhesion of hepatocytes onto a synthetic matrix and preserves the differentiated hepatic functions by promoting the formation of round adherent cells and aggregates. 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 the metabolism of mammalian cells (26, 27), and various GLUTs work on the cytoplasmic membrane in various types of cell (28).

In our previous work (29), morphological variation was observed in rabbit chondrocytes cultured on a D-glucose-displayed surface on which the ratio of D- and L-glucose was changed. To clarify the initial cellular events on a D-glucose-displayed surface, in the present study, we investigate the effect of displaying D-glucose on the attachment and morphology of human epithelial cells with the expression of GLUTs changed by adding insulin. Moreover, the fundamental mechanisms of cell and culture surface interaction are discussed with respect to the formation of the actin cytoskeleton and binding domain.

MATERIALS AND METHODS

Preparation of glucose-displayed surfaces Unless otherwise stated, a conventional plastic surface of 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 generate a template with a hydroxyl group on the surface, an aqueous solution of 50 $\mu mol/cm^3$ potassium *tert*-butoxide was poured into each well. The wells were incubated for 1 h under an ambient condition, and then irrigated three times with sterilized water. An aqueous solution of 360 $\mu mol/cm^3$ glutaraldehyde was introduced into the wells, which were then allowed to stand for 1 h and washed thoroughly with sterilized water. The wells were 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 each well and the wells were incubated for 2 h. The ratio of D- to L-glucose in the applied solution was changed so as to obtain 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 wells, and after allowing the wells to stand for 24 h, the wells were washed twice with phosphate buffered saline (PBS; Sigma-Aldrich, St. Louis, 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 culture surface were nearly the same as those 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 $\mu mol/cm^3$ D-glucose (Kurabo Industries, Osaka). For the experiments, the initial concentration of viable cells, determined by trypan blue exclusion, was fixed at $X_0 = 5.0 \times 10^3$ cells/cm^2, and the cells were cultured on the prepared surfaces using the medium with or without insulin (10 $\mu g/cm^3$ when added) under the same conditions as those described above.

For tracing dynamic variation in cell morphology, time-lapse observation of individual cells was conducted, and the images captured using a CCD camera system were processed in accordance with previous reports that described the details of tools (29, 30). The value of roundness, R_c, of each cell was determined using 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).

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

To evaluate cell attachment and apparent cell morphology 24 h after seeding, triplicate wells were selected, and the bottom surface images were captured at five different positions in each well 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 to seeded cells on five images, was determined in each well. The overall roundness R_{oc} of cells in each well was calculated using the following equation.

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

Here, the overall area and periphery, respectively A_{oc} and L_{oc}, of all cells projected on five images in each well were employed. X_{24}/X_0 and R_{oc} were recorded as means, represented by $\bar{X}_{24}/\bar{X}_0$ and $\bar{R}_{oc}$, respectively, which were determined from the measurements for triplicate wells.

Treatments of cells to block GLUTs and integrin For antibody treatments, cells were recovered from the subculturing flasks by trypsin digestion, and were preincubated with 0.5% Block Ace (Dainippon Sumitomo Pharma, Osaka) for 30 min at 37°C to mask 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, 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, Temecula, CA, USA) were adequately mixed with the medium, and the cells were treated with the appropriate antibodies for 1 h at 4°C with gentle mixing every 20 min. The antibody-treated or nontreated cells were cultured on the indicated surfaces to examine cell attachment and morphology.

Fluorescence microscopy for cytoskeletal and GLUT localization observation For the 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 the 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