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CHANGES IN CELL ATTACHMENT BY RSV TRANSFORMATION IN RAT LIVER EPITHELIAL CELLS

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SUMMARY: BRL, a non-malignant rat liver epithelial-like cell line, possessed the ability to adhere through fibronectin to a solid substrate. Oncogenical transformation of these BRL cells with RSV induced a significant decrease in the fibronectin molecules in the extracellular matrix and reduction in its ability to adhere to fibronectin. The α_5 and β_1 subunits of integrin (fibronectin receptor) were quantitatively diminished during RSV transformation in BRL cells. These results suggest that adhesive reduction of BRL cells to a substrate by RSV transformation may be caused by a decrease in cell surface fibronectin and fibronectin receptor molecules.

One remarkable aspect of transformed cells is reduction of adhesion to solid substrates. Since adhesion of cells to a substrate is under the control of molecules present in the extracellular matrix (ECM) such as fibronectin, collagen, laminin, *etc.* (1-3), changes in the ECM components associated with fibroblasts during transformation have been extensively studied. Fibronectin, which is a well-defined ECM molecule, was lost by transformation (4,5). The loss of cell surface fibronectin was explained by reducing biosynthesis (6-8) or degrading it with protease (6, 9-12), but there has not been a complete explanation for the loss of cell surface fibronectin. In contrast to the extensive data on ECM molecules, little information is available on changes in receptors for ECM molecules. There are some reports indicating that tyrosine phosphorylation occurred in both α_5 and β_1 subunits of RSV-transformed chicken embryo fibroblasts (13), or that both α_5 and β_1 subunits of integrin were diminished by oncogenical transformation in rat1, NRK, and Nil8 cells (14).

In this paper, we report changes in cell attachment during RSV-transformation in the liver epithelial cell line, BRL, focusing on fibronectin and its receptor (integrin α_5/β_1).

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MATERIALS & METHODS

Materials: Fibronectin and anti-rabbit IgG (Goat IgG) Fab'-HRP were from IBL (Fujioka, Japan). Anti-human fibronectin receptor (integrin $\alpha5/\beta1$) antiserum was from TOYOBO (Tokyo, Japan) and nitrocellulose membrane was from Schleicher & Schuell. All other chemicals were of the highest grade commercially available.

Cell culture: The non-malignant rat liver epithelial cell line BRL and Rous sarcoma virus-transformed malignant cell line RSV-BRL were cultured at 37°C in a Dulbecco's modified Eagle medium/Ham's F12 (1:1 mixture, DME/F12) medium in a humidified atmosphere of 5% CO_2 and 95% air (15). In most cases, cultures were supplemented with 10% fetal calf serum (FCS). The BRL cell line was a kind gift from Dr. Katsuzo Nishikawa, Kanazawa Medical University, Kanazawa Japan.

Cell Adhesion Assay: Cells were dissociated by 0.1% trypsin to single cells, collected by centrifugation, washed three times with BSS (Hank's Balanced Salt Solution), resuspended in BSS, and the cell number was counted. Tissue culture dishes (Falcon, 24-well plate) were coated with various concentrations of fibronectin or ECM in PBS for 2 h at 37°C. ECM was prepared from BRL or RSV-BRL cells by an incubation with 2 M urea-containing PBS for 2 h. After incubation, non-adherent cells in 2 M-urea PBS were removed by centrifugation and then the supernatant was collected and used as "ECM". Dishes were washed with PBS and incubated in 1 mg/ml of bovine serum albumin in PBS for 2 h at 37°C. After washing dishes twice with PBS, cells were added to the dishes (1×10^4 cells in 0.5 ml/dish) and incubated for 2 h. Adherent cells in a constant unit area were counted under a microscope. All data points were expressed as a fraction of maximum binding observed in the assay.

Immuno-blotting Analysis: BRL and RSV-BRL cells (about 1×10^7 cells) were harvested from dishes, washed three times with PBS, and homogenized in 100 µl of Buffer A (20 mM Tris-HCl buffer (pH 7.5), 0.5 M NaCl, 0.5% Triton X-100, 0.1% β-mercaptoethanol, 25 µg/ml phenyl methyl sulfonyl fluoride, and 1 mM benzeamide-HCl). After standing in ice for 30-60 min, centrifugation was carried out. The supernatant was used as the cell lysate. BRL and RSV-BRL cell lysates (about 20 µg of protein) were separated by 7.5% SDS-polyacrylamide gel electrophoresis. The proteins on the gel were transferred to a nitrocellulose membrane. Transferred membrane was blocked with PBS containing 1% bovine serum albumin (BPBS) for 1 h. Membrane was treated with anti-fibronectin-receptor antiserum (1:500 dilution) in TPBS (0.05% Tween 20 in PBS) at 4°C overnight. The membrane was washed with TPBS and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG antiserum (1:1000 dilution, IBL) for 2 h at room temperature. Antibody binding proteins were visualized using "Konica Immuno-staining HRP Kit IS-50B" (Seikagaku Kougyou, Tokyo Japan).

RESULTS & DISCUSSION

In this study, to understand the mechanism of reduction of cell adhesion to a solid substrate during transformation, we examined adhesive alteration using non-malignant the rat epithelial cell line, BRL, and the RSV transformed malignant cell line, RSV-BRL. We surveyed adhesion of BRL and RSV-BRL cells to an extracellular matrix fraction (ECM) extracted from both cells with 2 M urea. ECM from cells showed adhesion against both BRL and RSV-BRL cells (Fig. 1). While adhesion of BRL cells was dose-dependently increased and reached a maximum at about 0.5 mg/ml of the coating

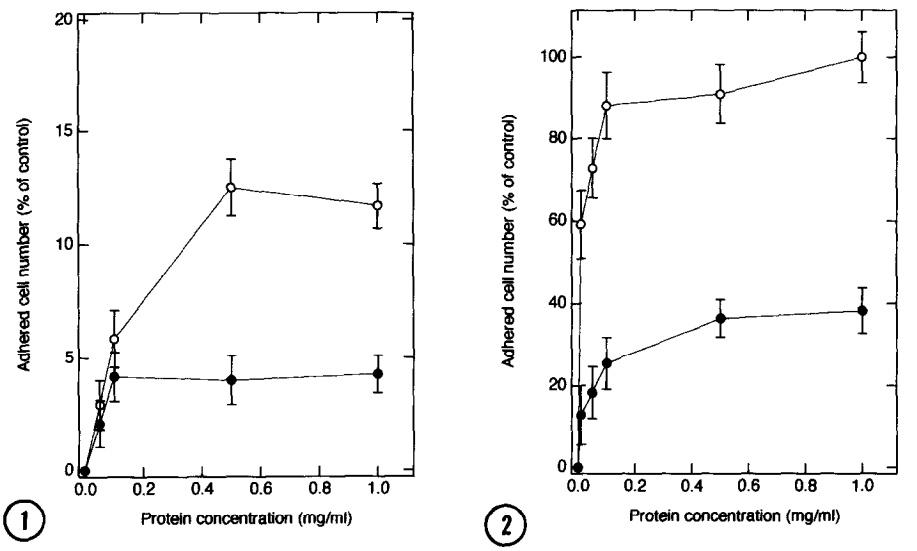


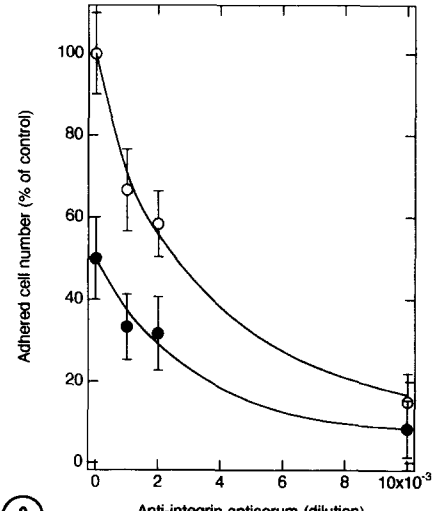
Fig. 1. Adhesion to ECM prepared from BRL cells was measured for BRL and RSV-BRL cells. The data against BRL (○) and RSV-BRL (●) represent the mean of three separate assays.

Fig. 2. Adhesion to fibronectin was measured for BRL and RSV-BRL cells. The data against BRL (○) and RSV-BRL (●) represent the mean of three separate assays.

concentration of ECM protein, adhesion of RSV-BRL cells was saturated at about 0.1 mg/ml of the protein concentration. Adhesion of BRL cells was about three-fold as much as that of RSV-BRL cells at 0.5 mg/ml of the protein concentration. ECM from RSV-BRL cells showed no adhesion against both BRL and RSV-BRL cells (data not shown). We confirmed the existence of a fibronectin molecule in ECM by an immunological analysis with anti-fibronectin antiserum. The fibronectin molecule of the BRL fraction was present, but not in the RSV-BRL fraction (data not shown). These results coincided with the report of Miyazaki *et al.* (12) in which the cell surface fibronectin of BRL cells was digested by proteinase and then disappeared during RSV transformation. The same experiment was carried out but replacing ECM with the purified fibronectin. The result is shown in Fig. 2, which was similar to the results in Fig. 1. Because the fibronectin concentration in ECM of BRL was low and then the amount of coated fibronectin was slight, adhesion of both cells to ECM was overall lower that of purified fibronectin, although adhesion of both cells to ECM had almost the same profile as that to purified fibronectin. Adhesion of BRL cells to both BRL-ECM- and fibronectin-coated dishes was about 3-fold as much as that of RSV-BRL cells during saturated conditions (0.5 mg/ml for ECM protein and 0.5 μg/ml for fibronectin). These results

demonstrated that fibronectin was indispensable for adhesion of both cells, and that the oncogenical transformation caused loss of cell surface fibronectin and a reduction in adhesion to fibronectin for these cells. Reduction in adhesive ability by RSV-transformation in BRL cells was not explained by only the disappearance of cell surface fibronectin, because the interaction between cells and fibronectin was diminished by RSV-transformation.

During the next step, alterations in the fibronectin receptor (integrin $\alpha5/\beta1$) of BRL cells were examined during RSV-transformation by an immunological method with the anti-fibronectin receptor (integrin $\alpha5/\beta1$) antiserum. After coating 1 μg/ml of fibronectin on dishes, the effect of anti-fibronectin receptor antiserum on the adhesion of BRL and RSV-BRL cells was determined (Fig. 3). Adhesion of BRL and RSV-BRL cells decreased under the influence of anti-fibronectin receptor antiserum. Adhesion of both cells was inhibited about 50% at a 1:500 dilution of this antiserum. It was confirmed that this antiserum recognized the fibronectin receptor of both cells. The fibronectin receptor molecules of BRL and RSV-BRL cells were then analyzed by immuno-blotting using this antiserum (Fig. 4). Two bands were detected as $\alpha5$ and $\beta1$ subunits of integrin in BRL



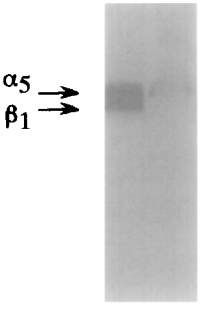


Fig. 3. Effect of anti-fibronectin receptor antiserum on adhesion of BRL and RSV-BRL cells. The data against BRL (○) and RSV-BRL (●) represent the mean of three separate assays.

Fig. 4. Immuno-blotting analysis of cell lysate using anti-fibronectin receptor antiserum. Cell lysates from BRL (lane 1) and RSV-BRL (lane 2) were separated on a 7.5 % SDS-PAGE under non-reducing conditions. "$\alpha5$" and "$\beta1$" on the left refer to integrin $\alpha5$ and $\beta1$ subunits.

cells, while bands of the α_5 and β_1 subunits were not detectable in RSV-BRL cells. We reached the conclusion that reduction in adhesive ability to a solid substrate during RSV-transformation was due to diminution of the fibronectin receptor molecules of BRL cells. There were some reports indicating alteration of fibronectin receptors by transformation in several cell lines. Study in fibronectin receptors of chicken embryo fibroblasts by RSV-transformation changed in the phosphorylation of both α_5 and β_1 subunits but no detectable change was observed in the amount or subunit composition of the receptor (13). Plantefaber *et al.* (14) reported other alterations of fibronectin receptor by oncogenical transformation. They indicated that the integrin α_5 and β_1 subunits of Rat1, NRK, and Nil8 cells were quantitatively decreased by transformation, and transformed cells required higher levels of fibronectin than do their normal counterparts. With respect to diminishing the fibronectin receptor by transformation, our results were similar to the report of Plantefaber *et al.* (14). But the adhesive ability of RSV-BRL cells was saturated at a low concentration of coating fibronectin in comparison with their parent cells, and adhesion of RSV-BRL cells could not reach that of BRL cells at high concentrations of fibronectin (>1 μg/ml). From this point of view, our result was different from theirs. In this paper, we showed that on epithelial cells adhesive reduction of the epithelial cell line, BRL, to a solid substrate resulted from loss of cell surface fibronectin and a decrease in adhesive ability by diminution of the fibronectin-receptor. Recently receptors for a number of ECM molecules have been identified on the surface of tumor cells (16), particularly GpIIb/IIIa (integrin α_{IIb}/β_3) was existence on several tumor cells which may serve as a multifunctional receptor for tumor cell attachment (17, 18). While few fibronectin receptor molecules were detected in RSV-transformed BRL cells, RSV-BRL cells had a slight adhesive ability for fibronectin. This suggested the possibility that the other integrin subunits including α_{IIb}/β_3 were expressed in RSV-BRL cells.

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