Two epithelial cell lines, MCF7 and HCC2998, dissociate after hyperactivation of p38 MAP kinase

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Abstract
We sought to determine how hyperactivation of p38 MAP kinase affects cell-cell adhesion in two epithelial cell lines. The first cell line, HCC2998/MKK6-DA, a human colon adenocarcinoma cell line containing inducible dominant-active MKK6, has been shown to disperse after hyperactivation of p38 MAP kinase. However, the mechanism of how these cells disperse has not been studied. Examination of adherens junction proteins, such as E-cadherin and β-catenin, revealed that adherens junctions were disrupted. HCC2998/MKK6-DA cells respond to heregulin β-1. MCF7, human breast cancer cells which also respond to heregulin β-1, were also examined for localization. MCF7/MKK6-DA-6-2-4 cells carrying the inducible dominant-active MKK6 gene were produced. These cells also dispersed after hyperactivation of p38 MAP kinase. As these cells grow as monolayers, observation of the cells was more convenient than in HCC2998 cells. Dissociation of MCF7/MKK6-DA-6-2-4 cells occurred 21–25 h after infection with adenovirus coding for Cre recombinase. F-actin, which associates with the adherens junction, disappeared before cells dispersed. Then, E-cadherin and occludin disappeared from cell-cell junctions to localize diffusely in the cytosol, which led to loss of cell-cell interaction. This was different from the case with HCC2998 cells, in which E-cadherin and β-catenin remained at the plasma membrane after dissociation. Treatment with SB202190, a p38 MAP kinase inhibitor, blocked dissociation of the cells, indicating that p38 MAP kinase activity was required for cell dispersal. Localization of adherens junction proteins was examined in MCF7/MKK6-6-2-4 cells. Cells were fractionated into the cytosol and membrane fractions. After dissociation of the cells, E-cadherin remained in the membrane fraction; however, α-catenin and β-catenin moved from the membrane fraction to the cytosolic fraction, suggesting that these proteins dissociate from E-cadherin. Finally, activation of Rac1, which has been shown to be important in dissociation of the cells was detected. These results suggest that hyperactivation of p38 MAP kinase causes dissociation of the cells through disruption of the adherens junction in these cell lines.

Keywords: p38 MAP kinase, adherens junction, cell-cell interaction, scattering

Background
The mitogen-activated protein (MAP) kinase pathways transduce a large variety of external signals, leading to a wide range of cellular responses including growth, differentiation, inflammation and apoptosis [1-3]. These pathways are characterized by a kinase cascade with specific effectors. In most cases, the MAPK cascade is activated by small monomeric GTPases including Ras, Rac and Rap1 [4,5]. However, it may be activated by other enzymes as well. In mammals, three major MAP kinase pathways have been identified: MAP kinase/ERK, SAP kinase/JNK, and p38 MAP kinase [6]. p38 MAP kinases are a class of MAP kinases which are responsive to stress stimuli — including cytokines, ultraviolet irradiation, heat shock, inflammation, and osmotic shock — and are involved in various cell responses including differentiation and apoptosis [7,8]. Four types of p38 MAP kinases, α, β, γ, and δ, have been identified [9]. p38 MAP kinases are activated by phosphorylation of two amino acids, Thr180 and Tyr182, as is the case with MAP kinases [7,8]. Their associated MAP kinase kinases are MKK3 or MKK6.

We found that in a highly differentiated colon adenocarcinoma line, HCC2998, activation of phosphatidylinositol 3-kinase induces dissociation of the cells to yield poorly differentiated adenocarcinoma cells similar to “signet ring” cell carcinomas [10]. p38 MAP kinase activity was required for this transition [11]. Introduction of dominant-active MKK6 into these cells also induced dissociation of the cells suggesting that strong activation of p38 MAP kinase can affect cell-cell interaction in these cells [11]. The p38 MAP kinase may be p38 MAP kinase-α, as this reaction was inhibited by SB202190, which is specific to p38 MAP kinase-α [12]. However, how this dissociation of the cells occurs was not studied.

After stimulation with HRG β-1, MCF7 cells undergo scattering and p38 MAP kinase is activated. Scattering
is inhibited by p38 MAP kinase inhibitors, suggesting that p38 MAP kinase is involved in scattering. However, whether activation of p38 MAP kinase alone is sufficient for scattering remains unclear. In our system expression of dominant active MKK6 is induced by infection of adenovirus coding for Cre recombinase by Cre-LoxP system. This system allows us to utilize MKK6 for exclusive activation of p38 MAP kinase, avoiding unspecific effects by other factors. We therefore used this system to see the specific effects of p38 MAP kinase activation.

Epithelial cells form adherens and tight junctions and interact with each other to connect neighboring cells [13-18]. It has been proposed that both adherens and tight junction complexes are associated with actin cytoskeleton [14,19]. E-cadherin is a transmembrane protein associated with the adherens junctions and interacts with intracellular proteins α-catenin, β-catenin and p120-catenin [17]. E-cadherin is important for recognition of neighboring cells and functions as a landmark. Tight junctions contain occludin and claudins as transmembrane proteins that interact with ZO-1, 2, or 3 [16].

In this paper, we described the effects of hyperactivation of p38 MAP kinase on cell–cell dissociation of HCC2998 and MCF7 cells by monitoring E-cadherin and F-actin.

Methods

Cells and culture condition

HCC2998 cells capable of inducing constitutively active MKK6 gene (HCC2998/MKK6DA) were cultured in RPMI1640 medium supplemented with 5% fetal calf serum [11,20]. MCF7 cells were cultured in DMEM supplemented with 5% fetal calf serum [21]. MCF7 cells capable of inducing constitutively activated MKK6 were established as previously described to yield MCF7/MKK6DA-6-2-4 [10,11]. The structure of the dominant active MKK6 was previously described [22]. The gene was introduced by infecting adenovirus carrying Cre recombinase (Cre-virus) [10].

Reagents used in this study

Anti-E-cadherin antibody was purchased from BD Biosciences (Franklin Lakes, NJ). Anti-p38 MAP kinase antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-phospho-p38 MAP kinase antibody was purchased from Cell Signaling Technology (Danvers, MA). TRITC-phalloidin was purchased from Sigma-Aldrich Corp. (St. Louis, MO). Anti-occludin antibody was purchased from Zymed Laboratory Inc. (South San Francisco, CA). Anti-E-cadherin, α-catenin, β-catenin primaries and the secondary antibodies conjugated with Alexa 488 were from GE Healthcare (London, UK). The p38 MAP kinase a inhibitor SB202190 was from Wako Co. Ltd. (Tokyo).

Immunofluorescence and staining of F-actin

Cells were fixed with 10% formaldehyde and permeabilized with PBS containing 0.2% Triton X100. The samples were treated with primary antibody for 1 h and then with secondary antibodies conjugated with Alexa 488 for 30 min. TRITC-phalloidin was used in place of antibodies for F-actin staining. The samples were observed under an inverted confocal microscope (Olympus FV300, Olympus, Tokyo).

Western blotting and fractionation of the cells

Western blotting was done using 1% skim milk as a blocking reagent and detected by an ECL system as described previously [23].

For cell fractionation, cells were suspended in a buffer containing 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, a proteinase inhibitor cocktail and then homogenized in a Dounce homogenizer. After removing the nuclei by low-speed centrifugation the cytoplasmic portion was further separated into the membrane and the cytosolic fractions by spinning at 45,000 rpm for 60 min.

Detection of activation of Rac1

GST-PAK-CRIB was expressed in E. coli strain BL21 and suspended in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM PMSF and 1 mg/ml lysozyme. After incubation on ice for 10 min the samples were sonicated and cell debris was removed by centrifugation. GST-PAK-Rac was bound to glutathione beads and washed with the buffer mentioned above. MCF7 cells were lysed in a buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X100 and 1 mM PMSF. After removal of cell debris, lysates were mixed with the GST-PAK-CRIB-loaded beads and incubated for 1 h. After washing with the lysis buffer three times, Rac1 bound to the beads was analyzed by western blotting with anti-Rac1 antibody.

Results and Discussion

Response of HCC2998/MKK6DA cells after induction of constitutively active MKK6

We reported in previous publications that HCC2998/MKK6DA cells were adherent with tight cell–cell interaction prior to infection. We observed rounding of the cells following a 36 hour incubation post infection. (Figure 1A). As dominant-active MKK6 is untagged, it is difficult to detect. We examined this by activation of p38 MAP kinase. Activation of p38 MAP kinase was clearly indicated by its phosphorylation (Figure 1B). The phosphorylation level was quite strong compared with levels seen after stimulation with growth factors (data not shown).

Adherens junctions of non-infected cells were detected by staining with anti-E-cadherin, anti-β-catenin antibodies or with TRITC-phalloidin (Figure 1C and D). The presence of F-actin was weak and difficult to detect. After induction of dominant-active MKK6, cells dissociated and no adherens junctions were observed. E-cadherin and F-actin remained at the plasma membrane (Figure 1C and D). These results suggest that rounding of the cells is related to loss of...
adherens junction. Levels of E-cadherin and β-catenin remained unchanged during dissociation of the cells (Figure 1E). It should be noted that tight junctions were not detected in these cells.

It has been shown that Rac1 is involved in dissociation of the cell [24]. We also tested whether Rac1 is activated in p38 MAP kinase superactivated cells. However no activation of Rac1 was observed (Figure 1F).

Response of MCF7/MKK6DA-6-2-4 cells after induction of constitutively active MKK6
Because HCC2998/MKK6DA cells dissociate after hyperactivation of p38 MAP kinase and because they dissociate after stimulation with heregulin β-1 in a p38 MAP kinase dependent manner [25], we tested MCF7/MKK6DA-6-2-4 cells, which also dissociate and scatter after stimulation with heregulin β-1. Hyperactivation of p38 MAP kinase was readily detectable after induction of dominant-active MKK6 (Figure 2A). MCF7/MKK6DA-6-2-4 cells started to disperse between 21 and 25 h after infection with Cre-virus. 48 h after infection, cells were almost entirely dispersed (Figure 2B). Addition of SB202190 at 21 h after infection completely blocked the dissociation of the cells (Figure 2B). This result suggests that p38 MAP kinase activity is required when cells lose cell-cell contact. Activation of p38 MAP kinase judged by its phosphorylation corresponded to dissociation of the cells (compare Figure 2A and B). These results suggest that hyperactivation of p38 MAP kinase is sufficient for dissociation of MCF7/MKK6DA-6-2-4 cells.

Localization of E-cadherin, occludin and F-actin was observed by cellular staining. The total amount of E-cadherin and occludin remained unchanged during dissociation of the cells (Figure 2C). Before induction of dominant-active MKK6, adherens and tight junctions were clearly visible (Figure 3A and B). At 25 h after induction of dominant-active MKK6 (36H), cells were rounded up and no cell-cell interaction was observed. (E) Levels of E-cadherin and β-catenin in the cells were examined by Western blotting. (F) HCC2998/MKK6DA cells were infected with Cre-virus, incubated for 24 hrs and analyzed for the level of activated Rac1. Input shows the levels of Rac1 used in the assay. Cre+SB, SB202190 was added 15 min before harvesting the cells.
the cell-cell junction (Figure 3A and B). Together with the fact that F-actin disappears prior to E-cadherin or β-catenin, these results suggest that F-actin’s regulation mechanism is different from those of E-cadherin and occludin.

Localization of E-cadherin, α-catenin, and β-catenin was examined. The cytosolic and membrane fractions of the cells before and after scattering were prepared and distribution of these proteins was monitored. As E-cadherin is a transmembrane protein, it was always found in the membrane fraction, even after internalization (Figure 3C). In contrast, β-catenin was found in the cytosol even before scattering. This cytosolic β-catenin may serve as a messenger for Wnt signaling. After scattering, cytosolic β-catenin increased, suggesting that β-catenin dissociated from E-cadherin and was released from the plasma membrane into the cytosol (Figure 3C). β-catenin also moved from the membrane fraction to the cytosolic fraction (Figure 3C). These results suggest that the complex of adherens junctions dissociates following scattering.

As Rac1 activation is important for scattering activation of Rac1 was examined. Rac1 was activated in a p38 MAP kinase dependent manner. Therefore, the p38 MAP kinase-Rac1 pathway may be important for scattering of MCF7 cells. In HCC2998 cells, Rac1 was not activated. The fact
that E-cadherin and F-actin remain at the cell membrane in HCC2998 cells, but were distributed in the cytosol in MCF7 cells might be due to this difference.

In this paper, we showed that specific hyperactivation of p38 MAP kinase can cause loss of cell-cell interaction in HCC2998/MKK6DA and MCF7/MKK6DA-6-2-4 cells (Figure 3D). It has been shown that MCF7 cells undergo scattering after heregulin β-1 stimulation in a p38 MAP kinase dependent manner [26]. However, before the current study only enhancement of cell motility has been discussed. Our results show that p38 MAP kinase also plays a role in cell dissociation. Therefore, p38 MAP kinase plays a central role in the scattering of these cells. We have also examined MKN28 cells, which are epithelial cells expressing ErbB2 and ErbB3. However, a similar effect was not observed in these cells (data not shown). Therefore, the effect of hyperactivation seen in this paper is not found in all cells. Further study may be necessary to understand the mechanism of various types of cell-cell dissociation.

Conclusions
In this paper, we demonstrated that hyperactivation of p38 MAP kinase in epithelial cells resulted in dissociation of two epithelial cell lines. Adherens junction complexes were disrupted after hyperactivation of p38 MAP kinase. These results suggest that p38 MAP kinase is one of the major pathways for dissociation in these epithelial cell lines.

Competing interests
The authors declare that there are no competing interests.

Authors’ contribution
R.O. performed biochemical experiments.
C.L.S. performed cell culture. YF did staining of the cells and wrote the manuscript.

Acknowledgement
This work was supported by grants-in-aid from the Ministry of Education, Science, Sports, and Culture of Japan, #20013044 (to Y. F.), a grant from the National Institute of Biomedical Innovation, Japan (http://www.nibio.go.jp/) (to Y. F.), and a grant from National Health Research Institutes, Taiwan (to Y. F.) We thank A. Kobayashi and M. Yamakawa for their assistance in performing experiments.

Publication history
Received: 30-Oct-2012 Revised: 5-Dec-2012 Accepted: 12-Dec-2012 Published: 22-Dec-2012

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Citation:
Okoshi R, Shu C-L and Fukui Y: Two epithelial cell lines, MCF7 and HCC2998, dissociate after hyperactivation of p38 MAP kinase. HOAJ Biology 2012, 1:12. http://dx.doi.org/10.7243/2050-0874-1-12