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EGFR phosphorylation-dependent formation of cell–cell contacts by Ras/Erks cascade inhibition

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Abstract

Cell–cell contacts play important roles in the homeostasis of normal epithelium and in the steps of metastasis of tumor cells, although signaling mechanisms to regulate cell–cell contacts are unclear. In this study, we observed that phenotype of no cell–cell contacts in rat intestinal epithelial cell subline (RIE1-Sca) correlated with increased Erk1/2 signaling activity, compared to that of parental RIE1 cells growing in colonies. Furthermore, cell–cell contacts between RIE1-Sca cells were reformed by treatment with a specific MEK inhibitor (U0126), with translocation of ZO1 and β-catenin to cell–cell contacts, without changes of their expression levels. U0126 treatment also increased EGFR phosphorylation in a ligand-independent manner. Pretreatment with EGFR kinase inhibitor abolished U0126 treatment-mediated EGFR phosphorylation, and expression of dominant negative H-Ras N17 allowed EGFR phosphorylation and cell–cell contacts even without U0126 treatment. Furthermore, the expression of a nonphosphorylatable EGFR Y5F mutant abolished U0126-mediated cell–cell contacts. U0126 treatment also caused less efficient wound healing by keeping monolayer integrity intact, compared to control untreated cells. This U0126-mediated reduction in wound healing was further altered either by pretreatment of EGFR kinase inhibitor or expression of H-Ras N17 or EGFR Y5F. Taken together, this study supports a unique mechanism of cell–cell contact formation through MEK/Erks inhibition-mediated EGFR phosphorylation.

Keywords: Erk; EGFR; Cell–cell contacts; wound healing

1. Introduction

The architectural integrity of the adhesive epithelial monolayer is maintained by integrin engagement to extracellular matrix (ECM) proteins at basal membranes and cell–cell contacts between a cell and adjacent cells [1]. Disruption of this monolayer integrity not only impairs functions of normal epithelium but also allows the dissemination of cancerous cells from the primary tumor bodies during the early steps of cancer metastasis [2,3]. Moreover, the dissemination of individual cells from epithelial monolayer involves a transition of epithelial cell types with well-established cell contacts to mesenchymal-like cells (i.e., elongated spindle-type cells) with few or no cell contacts [4]. This loss of cell–cell contacts is important clinically, since disseminated tumor cells might be facilitated in cell migration and invasion leading to tumor metastasis [3,5]. Therefore, it is interesting to reveal the mechanisms of how cell–cell contact loss can involve the regulation of intracellular signal transduction and the suppression of E-cadherin [6].

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Cell–cell contacts at tight and adherence junctions are linked to intracellular actin filaments through diverse protein–protein interactions [1]. Therefore, epithelial monolayer integrity and intracellular actin organization influence each other bi-directionally. That is, the disruption of cell–cell contacts can cause alterations in actin organization, and conversely abnormal actin organization can cause changes in cell contact status [7]. In addition, several signaling molecules stimulated by integrin receptor, growth factor receptor, or cross-talks between these receptors are known to be involved in the regulation of cell–cell contact formation [6]. These molecules include focal adhesion kinase (FAK), c-Src family kinase [6], Rho GTPases [8], Akt/PKB [9], Ras/Raf1/MEK/Erks cascade [10,11], c-Met [12], and others. That is, the activation of these molecules disrupts cell–cell contacts, by releasing E-cadherin, β-catenin, and zonula occludens-1 (ZO1) from cell–cell contact sites [13].

Meanwhile, the opposite phenomenon, namely, cell–cell contact formation may also be clinically important, as normal epithelial cells maintain homeostasis at a monolayer integrity and tumor cells that have moved from primary tumor bodies to other distal sites through lymph nodes or blood vessels would undergo cell–cell contact formation and acquire growth for metastatic tumors [3]. However, the manner in which cells form cell–cell contacts via the regulation of intracellular signaling pathways beyond the regulation of E-cadherin expression is largely unknown. It has been shown that the disruption or inhibition of the signaling activities of the molecules responsible for cell–cell contact loss leads to the abolishment of the loss. However, it is not known whether such disruptions and inhibitions always cause formation of cell–cell contacts. Moreover, an understanding of the mechanisms of cell–cell contact formation could lead to the development of reagents or strategies not to allow dissemination of tumor cells from primary tumors and to deal with settle-down for metastatic tumors.

In this study, we used a normal rat intestinal epithelial subcell line growing in a scattered pattern (RIE1-Sca) to examine how scattered-growth patterns can be converted to colony-forming patterns. We observed that the inhibition of the Ras/Raf1/MEK/Erks cascade reverted the scattered to colony-forming patterns and this revert required phosphorylation of EGFR. Therefore, the Ras/Erks cascade inhibition-mediated phosphorylation of EGFR appears to be important for cell–cell contact formation in the normal rat epithelial cell system.

2. Materials and methods

2.1. Cells

RIE1-Sca cells growing in a scattered pattern were prepared by selection and enrichment from normal rat intestinal epithelial (RIE1) cells growing in a colony-forming pattern. The multiple clones with scattered-growing patterns were selected and mixed prior to enrichment. Subculture of the RIE1-Sca cells showed consistently scattered-growing patterns, while maintaining cells for 2 months by subculture every 2–3 days. Cells were maintained in DMEM-H (Gibco-BRL) culture media containing 10% (v/v) fetal bovine serum (FBS) at 37 °C and 5% CO_2.

2.2. Cell lysates preparation and Western blots

Whole cell lysates from cells treated with DMSO or 20 μM U0126 (LC Laboratories®, Woburn, MA) for the indicated periods were prepared as described in previous studies [14, 15]. In cases of EGFR kinase inhibition, AG1478 (LC Laboratories®) at 1 μM was treated for the indicated period or pretreated 30 min or 2 h before U0126 treatment for 2 or 14 h. In cases, cells were infected with retrovirus expressing human H-Ras V12 (active form, a gift from Dr. In-San Kim, Kyungpook National University, Daegu, Korea), H-Ras N17 (dominant negative form), or EGFR Y5F for 12 h. After additional incubation for 22 h, cells were treated with DMSO or 20 μM U0126 for 14 h. In cases, cells were preincubated with neutralizing anti-EGF (10 μg/ml, Upstate Biotechnology) antibody or serum-starved overnight prior to U0126 treatment for additional 14 h. After incubations, cell lysates were prepared and their protein amounts were normalized using bicinchoninic acid (BCA) Protein Assay Reagent Kits (Pierce, Rockford, IL). Standard Western blots were performed by using antibodies against phospho-Y^{397}FAK, phospho-Y^{577}FAK, phospho-Y^{861}FAK, phospho-Y^{925}FAK (BioSource International Inc., Camarillo, CA), phospho-Y^{416}Src, c-Src, pY^{1173}EGFR, (Santa Cruz Biotech., Santa Cruz, CA), Erk1/2, phospho-Erk1/2, phospho-S^{473}Akt/PKB, phospho-Y^{992}EGFR, phospho-Y^{1045}EGFR, phospho-Y^{1068}EGFR (Cell Signaling Tech., Beverly, MA), E-cadherin, phospho-Tyrosine, FAK, α-tubulin (BD Transduction Lab., San Jose, CA), vimentin (Sigma, Saint Louis, MI), Desmoplakin (Serotec Ltd., Oxford, UK), ZO1 (Zymed Lab., South San Francisco, CA), or EGFR (Upstate Biotech., Lake Placid, NY).

2.3. Preparation of retroviruses

Human H-Ras V12 in pLNCX vector was used to make dominant negative H-Ras N17 (DN H-Ras N17) via a PCR approach. pRC/CMV-EGFR-Y5F construct in which the sites of tyrosine autophosphorylation (tyrosines 992, 1068, 1086, 1148, and 1173) have been mutated to phenylalanines [16] was also subcloned into a modified pLNCX retroviral vector as a *Hin*dIII and *Xba*I insert. Sequences of mutants were confirmed by direct sequence analyses. Retroviral vectors were separately transfected into PT67 packaging cells by using Lipofectamine 2000® (Life Technologies, Gaithersburg, MD) following the manufacturer's protocol. Two days after separate transfections, cells were selected with G418 (250 μg/ml). The culture soup of the stable PT67 cells was used for retrovirus infections, and reproducible infection rates for similar expressions of target proteins were confirmed by Western blots.

2.4. Immunofluorescence microscopy

Cells were replated on normal culture media-precoated glass coverslips and incubated overnight at 37 °C to achieve typical cell adhesion and spreading, as described previously [17]. In certain cases, cells were pretreated with AG1478 (1 μM) 30 min or 2 h before U0126 (20 μM) treatment for 14 h. Pretreatment of AG1478 either 30 min or 2 h prior to U0126 treatment resulted in similar results. In cases, cells were infected with pLNCX-H-Ras V12 or N17 or pLNCX-EGFR Y5F retroviruses for 12 h. The cells were then normally incubated for an additional 22 h. Alternatively, cells were preincubated with normal rabbit IgG or neutralizing anti-EGF (10 μg/ml) 60 min before DMSO or U0126 treatment. U0126 at 20 μM was then treated for an additional 14 h to cells infected with H-Ras N17 or EGFR Y5F. Wounds through cell monolayer on cover glasses were made as below. Cell images were then taken using a phase contrast microscope or cells were fixed with 3.7% formaldehyde in PBS for 10 min, permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature, and washed three times with PBS (3 times × 10 min). Cells were then incubated with primary antibody or TRITC-conjugated phalloidin (Molecular Probes, Eugene, OR) for 1 h and washed with PBS as above. The primary antibody used included anti-β-catenin (Santa Cruz Biotech.) and ZO1 (Zymed Lab. Inc.). Cells were then incubated with anti-rabbit or mouse IgG-conjugated TRITC or FITC (Chemicon International, Inc., Temecula, CA) in a dark and humidified chamber for 1 h Alternatively, cells were stained for actin by using phalloidin-conjugated with TRITC, as described earlier [17]. After washings three times with PBS as described above, cells on glass coverslips were mounted with mounting solution (DakoCytomation, Germany) and visualized by fluorescent microscopy (BX51TR, Olympus, Japan).