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Focal adhesion kinase regulates intestinal epithelial barrier function via redistribution of tight junction

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ABSTRACT

Disruption of epithelial barrier function was identified as one of the pathologic mechanisms in inflammatory bowel diseases (IBD). Epithelial barrier consists of various intercellular junctions, in which the tight junction (TJ) is an important component. However, the regulatory mechanism of tight junction is still not clear. Here we examined the role of focal adhesion kinase (FAK) in the epithelial barrier function on Caco-2 monolayers using a specific FAK inhibitor, PF-573, 228 (PF-228). We found that the decrease of transepithelial resistance and the increase of paracellular permeability were accompanied with the inhibition of autophosphorylation of FAK by PF-228 treatment. In addition, PF-228 inhibited the FAK phosphorylation at Y576/577 on activation loop by Src, suggesting Src-dependent regulation of FAK in Caco-2 monolayers. In an ethanol-induced barrier injury model, PF-228 treatment also inhibited the recovery of transepithelial resistance as well as these phosphorylations of FAK. In a sucrose gradient ultracentrifugation, FAK co-localized with claudin-1, an element of the TJ complex, and they co-migrate after ethanol-induced barrier injury. Immunofluorescence imaging analysis revealed that PF-228 inhibited the FAK redistribution to the cell border and reassembly of TJ proteins in the recovery after ethanol-induced barrier injury. Finally, knockdown of FAK by siRNA resulted in the decrease of transepithelial resistance. These findings reveal that activation of FAK is necessary for maintaining and repairing epithelial barrier in Caco-2 cell monolayer via regulating TJ redistribution.

1. Introduction

Epithelial cells lining the lumens of gastrointestinal tract act as selective physical barriers that regulate transepithelial movement of solutes, ions, water and hostile substance. A number of recent studies have suggested that defective intestinal epithelial barrier function is an important pathogenic factor contributing to the development of intestinal inflammation [1–3]. Although the pathogenesis of ulcerative colitis (UC) and Crohn's disease, which are known as inflammatory bowel diseases (IBD), remains unclear, a number of studies suggest that the destruction of the gastrointestinal tract integrity by the alteration of epithelial barrier function was identified as one of pathologic mechanisms in IBD [4].

Epithelial barrier consists of various intercellular junctions, such as adherens junction, gap junction, desmosome and tight junction (TJ). The TJ is the most apical component of the epithelial junctional complex [5], acting as gates of the paracellular pathway by controlling the extent and selectivity of diffusion and as fences that keep the identity of plasma membrane domains by forming an apical–basolateral intramembrane diffusion barrier [6]. TJ consists of transmembrane proteins, such as claudin and occludin. These proteins form the paracellular seals by interaction of extracellular domain with each other, and the cytoplasmic plaque by interaction of intercellular domain and various adaptor proteins, such as ZO-1. These adaptor proteins are thought to be a linker connecting the transmembrane proteins and the actin cytoskeletons [7]. TJ also have been linked to various signaling molecules that are regulated by extracellular stimuli. Cytokines such as TNFα and interferon-γ, which are considered playing a key role in the pathogenesis of inflammatory disorders, disrupt TJ and barrier functions to increase epithelial permeability in various epithelial monolayers [8–10]. Furthermore, TNFα and interferon-γ synergized to induce TJ disruption and intestinal epithelial barrier dysfunction [11].

Although precise regulatory mechanism of TJ barrier functions is still not clear, recent studies have demonstrated an important role for focal adhesion kinase (FAK), which is a non-receptor protein tyrosine kinase, in modulating barrier function. Zhao et al. reported that

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FAK knockout mice exhibit increased permeability compared with wild type endothelial cell during embryonic development [12]. FAK activity is necessary for barrier enhancement in endothelia [13,14]. Siu et al. have identified that FAK is an integrated component of the occludin/ZO-1 complex that regulates cell adhesion by determining the phosphorylation status of occludin at the blood–testis barrier [15,16]. These results are suggesting that FAK is required for maintenance of TJ barrier functions via tyrosine phosphorylation of TJ protein complex. Furthermore, Golubovskaya reported that transcription of FAK was regulated by NF-κB, which is known as "first responder" against various cytokines [17]. After mucosal injury, FAK protein expression in gut epithelial cells was enhanced via Smad- and p38-mediated pathways [18]. We have also demonstrated that protein expression level of FAK increased in colonic epithelial cells by inducing chemical colitis [19]. These results suggest that FAK expression and functions may regulate by various cytokines in pathological conditions, however, the role of FAK in colonic epithelial cells remains unclear.

The purpose of this study was to examine the role of FAK on the epithelial barrier function on Caco-2 monolayers. We investigated the effect of FAK inhibitor, PF-573, 228 (PF-228) on barrier function in Caco-2 monolayers. PF-228 is a small molecule FAK inhibitor, which interacts with FAK in the ATP-binding pocket, and effectively blocks the catalytic activity of FAK [20]. This compound is a useful tool to dissect the role of FAK in regulation of cell adhesion signaling and dynamics [20], in signaling pathways in human platelet [21], and in directional motility of cells [22]. In this manuscript, we sought to find the role of FAK in intestinal epithelial barrier function by measuring changes of transepithelial electrical resistance (TEER), paracellular permeability and phosphorylation of FAK by PF-228 treatment and by knockdown with siRNA. Furthermore, we determined the effect of PF-228 on distribution of TJ proteins, ZO-1 and occludin. Our findings indicate that activation of FAK is necessary for maintaining and repairing epithelial barrier in Caco-2 cell monolayer via regulating TJ redistribution.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM) and trypsin–EDTA solution were purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS) was from Invitrogen Life Technologies Corp. (Carlsbad, CA, USA). The focal adhesion kinase (FAK) inhibitor PF-573, 228 (3,4-dihydro-6-[[4-[[[3-(methyl-sulfonyl)phenyl]methyl]amino]-5-(trifluoromethyl)-2-pyrimidinyl]amino]-2(1H)-quinolinone) was from Tocris (Bristol, UK). Antibodies against total FAK, phosphorylated FAK at Tyr397, at Tyr576/577, Src and claudin-1 were from Cell Signaling Technology (Danvers, MA). For immunofluorescence staining, anti-total FAK was purchased from Calbiochem (Billerica, MA), anti-phosphorylated FAK at Tyr397 was from Abcam (Cambridge, UK) and anti-ZO-1 and anti-occludin antibodies were from Invitrogen Life Technologies Corp.

2.2. Cell culture

Caco-2 cells were purchased from the RIKEN Cell Bank (Tsukuba, Japan). The cells were cultured in DMEM supplemented with 10% FBS, 1% nonessential amino acids, 10 mM HEPES (pH 7.2), 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were grown in a humidified atmosphere containing 5% CO_2 at 37 °C. Culture medium was changed every 2 days. For TEER study, Caco-2 cells were seeded in cell culture inserts (Millicell-PCF, 0.4 μm pore size, 12 mm diameter, Millipore, Ireland) in 24-well plates at a density of 1×10^5 cells/cm^2. For immunofluorescence staining, Caco-2 cells were plated in 8-well Millicell EZ slide (Merck Millipore, Billerica MA, USA) at 1.5×10^5 cells/well in growth medium.

2.3. Measurement of Caco-2 monolayer epithelial resistance and paracellular permeability

Caco-2 cells were cultured for 3–4 weeks on Millicell culture inserts to form a monolayer. TEER values across the cell monolayers were measured using a Millicell ERS-2 epithelial volt-ohm meter (Merck Millipore). For resistance measurements, both apical and basolateral sides of the epithelia were bathed in culture medium. Inserts having over 400 $\Omega\cdot cm^2$ of TEER values were used for further experiments. Each experiment was repeated at least 3 times to ensure reproducibility. The modulation of Caco-2 monolayer paracellular permeability by ethanol treatment was carried out as established [23]. Briefly, Caco-2 monolayers were incubated in DMEM containing 7.5% ethanol at 37 °C for 30 min. After removal of ethanol, monolayers were incubated with culture medium and measured TEER at indicated time points. For determination of lucifer yellow permeability, Caco-2 monolayers were incubated for 4 h in the indicated concentration of PF-228. After washed with HBSS, lucifer yellow was added to the apical compartment at a final concentration of 1 mM. The monolayers were placed on a shaker in a 37 °C incubator with a humidified atmosphere containing 5% CO_2 for 1 h. Fluorescence leakage was determined for lucifer yellow by 430 nm excitation and 535 nm emission using a fluorescence spectrophotometer.

2.4. Immunoprecipitation and Western blot

Immunoprecipitation was performed as described [24] with some modifications. Caco-2 cells were lysed in immunoprecipitation buffer (20 mM Tris–HCl (pH 7.5), 0.5 M NaCl, 1% TritonX-100, 2 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 0.1% sodium dodecyl sulfate, 0.5% Nonidet P-40, 1% Triton X-100 and 10% glycerol (vol/vol)), containing protease inhibitor cocktail (Roche, Mannheim, Germany). After 4 cycles of freeze–thaw and sonication, lysates were centrifuged at 12,000 *g* for 10 min. The supernatant was incubated with anti-FAK antibody overnight. The immunocomplex was then precipitated with protein G-Sepharose beads (GE Healthcare). The immunocomplex was washed five times with the immunoprecipitation buffer, eluted by boiling in sample buffer for SDS-PAGE and Western blot was performed as described previously [19]. About 1.4 mg protein in approximately 2 ml was used per sample tube for immunoprecipitation.

2.5. Immunofluorescence and confocal microscopy

Caco-2 cells that were grown on 8-well glass slide chambers to 100% confluence for 3–4 weeks were used. After treatment as indicated in the figures, cells were rinsed once with PBS, and fixed with 3.7% formaldehyde in phosphate buffered saline (PBS) for 15 min at room temperature. Then cells were rinsed three times with PBS and permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature. After washing, cells were blocked in Image-IT FX signal enhancer (Invitrogen Life Technologies Corp.) for 1 h at room temperature. And then cells were incubated with respective primary antibodies to total FAK (1:100), phosho-FAK at Y397 (1:100), ZO-1 (1:100), and occludin (1:100) diluted in 0.5% Triton X-100 in PBS overnight at 4 °C. Subsequently the secondary antibodies, goat anti-rabbit IgG-Alexa 555 and goat anti-mouse IgG-Alexa 488 (Invitrogen Life Technologies Corp.) were diluted at 1:100 with PBS containing 0.5% Triton X-100 and incubated with cells for 1 h at room temperature in the dark. Slides were mounted in ProLong Gold antifade reagent with 4′, 6′-diamidino-2-phenylindole (DAPI) (Invitrogen Life Technologies Corp.) to stain nucleus. Cells were observed with confocal laser scanning microscope, FV1000-D (Olympus, Tokyo, Japan) using a ×100 oil immersion objective and FV10-ASW software (Ver 3.0, Olympus).