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Biochimica et Biophysica Acta 1842 (2014) 635–645

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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Activation of focal adhesion kinase via M1 muscarinic acetylcholine receptor is required in restitution of intestinal barrier function after epithelial injury

Md Rafiqul Islam Khan [a,i], Takashi Yazawa [a], Abu Syed Md Anisuzzaman [i], Shingo Semba [a], Yanju Ma [k], Junsuke Uwada [c], Hisayoshi Hayashi [f], Yuichi Suzuki [f,g], Hiroki Ikeuchi [j], Motoi Uchino [j], Atsuo Maemoto [b,h], Ikunobu Muramatsu [c,d,e], Takanobu Taniguchi [a,*]

[a] Division of Cellular Signal Transduction, Department of Biochemistry, Asahikawa Medical University, Asahikawa, Japan
[b] Department of Gastrointestinal Immunology and Regenerative Medicine, Asahikawa Medical University, Asahikawa, Japan
[c] Division of Pharmacology, Department of Biochemistry and Bioinformative Sciences, University of Fukui, Fukui, Japan
[d] Organization for Life Science Advancement Programs, University of Fukui, Fukui, Japan
[e] Research Center for Child Mental Development, University of Fukui, Fukui, Japan
[f] Laboratory of Physiology, School of Food and Nutritional Sciences, University of Shizuoka, Shizuoka, Japan
[g] Division of Health and Nutrition, Sendai Shirayuri Women's College, Sendai, Japan
[h] Inflammatory Bowel Disease Center, Sapporo Higashi Tokushukai Hospital, Sapporo, Japan
[i] Department of Pharmacy, University of Rajshahi, Rajshahi, Bangladesh
[j] Inflammatory Bowel Disease Center, Hyogo College of Medicine, Nishinomiya, Japan
[k] Department of Medical Oncology, The First Hospital of China Medical University, Shenyang, China

ARTICLE INFO

Article history:
Received 16 July 2013
Received in revised form 26 November 2013
Accepted 16 December 2013
Available online 21 December 2013

Keywords:
Epithelial barrier function
Focal adhesion kinase
mAChR
IBD

ABSTRACT

Impairment of epithelial barrier is observed in various intestinal disorders including inflammatory bowel diseases (IBD). Numerous factors may cause temporary damage of the intestinal epithelium. A complex network of highly divergent factors regulates healing of the epithelium to prevent inflammatory response. However, the exact repair mechanisms involved in maintaining homeostatic intestinal barrier integrity remain to be clarified. In this study, we demonstrate that activation of M1 muscarinic acetylcholine receptor (mAChR) augments the restitution of epithelial barrier function in T84 cell monolayers after ethanol-induced epithelial injury, via ERK-dependent phosphorylation of focal adhesion kinase (FAK). We have shown that ethanol injury decreased the transepithelial electrical resistance (TER) along with the reduction of ERK and FAK phosphorylation. Carbachol (CCh) increased ERK and FAK phosphorylation with enhanced TER recovery, which was completely blocked by either MT-7 (M1 antagonist) or atropine. The CCh-induced enhancement of TER recovery was also blocked by either U0126 (ERK pathway inhibitor) or PF-228 (FAK inhibitor). Treatment of T84 cell monolayers with interferon-γ (IFN-γ) impaired the barrier function with the reduction of FAK phosphorylation. The CCh-induced ERK and FAK phosphorylation were also attenuated by the IFN-γ treatment. Immunological and binding experiments exhibited a significant reduction of M1 mAChR after IFN-γ treatment. The reduction of M1 mAChR in inflammatory area was also observed in surgical specimens from IBD patients, using immunohistochemical analysis. These findings provide important clues regarding mechanisms by which M1 mAChR participates in the maintenance of intestinal barrier function under not only physiological but also pathological conditions.
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1. Introduction

Epithelial integrity of the gut is essential for preventing the invasion of microorganisms and the development of inflammation in intestinal submucosa. The intestinal epithelium is a highly selective barrier that permits the absorption of nutrients from the gut lumen into the circulation and at the same time restricts the passage of harmful and potentially toxic compounds [1,2]. Disruption of intestinal barrier integrity (leaky gut) may lead to the penetration of luminal bacterial products into the submucosa to initiate local inflammation [1]. Mild form of intestinal epithelial injury commonly occurs in many diseases, which is rapidly repaired to reform the integrity of epithelial monolayers to prevent invasion of noxious compounds.

Findings on various tissues or cells reveal that focal adhesion kinase (FAK) is one of the key regulators for the maintenance and repair of barrier functions [3–7]. FAK is a non-receptor tyrosine kinase that modulates various cell functions, including survival, proliferation, and

* Corresponding author at: Division of Cellular Signal Transduction, Department of Biochemistry, Asahikawa Medical University, Asahikawa 078-8510, Japan. Tel.: +81 166 68 2340; fax: +81 166 68 2349.
E-mail address: takotago@asahikawa-med.ac.jp (T. Taniguchi).

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http://dx.doi.org/10.1016/j.bbadis.2013.12.007

migration [8,9]. On the other hand, the MAP kinase family makes up a group of important intracellular mediators of signal transduction to various stimuli. The classical MAP kinase, ERK1/2 (ERK) has been associated with the regulation of cellular proliferation and differentiation [10,11]. The role of MAP kinase pathway in the regulation of the paracellular permeability across epithelial cells has not been explored extensively. However, several studies have reported the positive regulation of epithelial barrier function as a result of the phosphorylation of ERK [12,13].

Carbachol (CCh), an agonist of muscarinic acetylcholine receptors (mAChRs), increased the phosphorylation of ERK and FAK in T84 cells [14]. Recent evidence implies the participation of mAChRs in the tightness of epithelial integrity in the proximal colon [15]. These findings suggest the crucial role of mAChRs in the intestinal epithelium to maintain and/or to repair barrier functions. However, the mechanisms and signaling molecules downstream from mAChRs in the regulation of barrier functions are still unknown.

The family of mAChRs belongs to a G-protein–coupled-receptor superfamily. Five subtypes of mAChRs (M1–M5), with difference in signal transduction, have been cloned [16,17]. Numerous studies revealed that intestinal infection and inflammation impaired the muscarinic cholinergic response to the gut epithelium [18]. The exact signaling mechanism of cholinergic hyporesponsiveness to the gut epithelium under inflammatory condition remains to be clarified. We reported previously that ERK and FAK are localized in the intestinal epithelial cells [19,20] and may be involved in the modulation of barrier functions under normal as well as inflammatory conditions. Impaired epithelial barrier function is a common feature of the inflammatory bowel diseases (IBD) and is caused, at least in part, by the elevated level of various cytokines. Treatment of T84 epithelial cell monolayers with interferon-γ (IFN-γ), one of those cytokines, has been shown to compromise their barrier integrity with a decrease in transepithelial electrical resistance (TER) and an increase in epithelial permeability [21,22]. Recently, it has been demonstrated that AMP-activated protein kinase and phosphatidylinositol 3′-kinase may be involved in IFN-γ-induced epithelial barrier dysfunction [23–25]. However, the precise mechanism of barrier dysfunction under the inflammatory condition is yet to be elucidated.

In this study, we aimed to uncover the role of mAChRs and the downstream signaling pathway in the maintenance and restitution of the barrier function in human intestinal epithelial cells. We also sought to investigate the cause of barrier dysfunction under inflammatory condition in T84 cell monolayers. Our data suggest that the IFN-γ-induced barrier dysfunction is associated at least in part, with the reduction of FAK phosphorylation presumably via the downregulation of M1 mAChR signaling. We also tested surgical specimens of colons from IBD patients in immunohistochemical analysis.

2. Materials and methods

2.1. Reagents

Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12, trypsin-EDTA solution and CCh (Sigma Aldrich, St. Louis, USA), fetal bovine serum (FBS) and Lipofectamine 2000 (Invitrogen, Life Technologies Corp., CA, USA), atropine sulfate, U0126 and human IFN-γ (Wako Pure Chemical Industries Ltd., Osaka, Japan), muscarinic toxin 3 (MT-3) and 7 (MT-7) (Peptide Institute, Inc., Osaka, Japan), PF-573228 (PF-228, Tocris Cookson Ltd., Bristol, UK), [^{3}H]-*N*-methyl scopolamine chloride (PerkinElmer, Boston, USA) were obtained from the sources noted. Antibodies against MAP kinases (ERK, p38 and JNK), phosphorylated MAP kinases, MAP2K1, FAK, phosphorylated FAK at tyrosine 397 (Y397), and β actin were from Cell Signaling Technology, Inc. (Massachusetts, USA) and those against mAChR M1 (H-120) and mAChR M3 (H-210) were from Santa Cruz Biotechnology, Inc. (CA, USA).

2.2. Cell culture

A human colon cancer cell line, T84, from the Health Protection Agency Culture Collection (Salisbury, UK) was grown in a humidified atmosphere with 5% CO_2 at 37 °C in DMEM and Ham's F12 medium supplemented with 2 mM glutamine, 15 mM HEPES (pH 7.2), 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were separated by trypsinization, and 1×10^6 cells were seeded onto 12-mm diameter semipermeable filter supports (Millicell-PCF, 0.4 μm pore size, Millipore, Ireland) for TER measurement and immunoblot analysis. Cells were cultured for at least 14–21 days prior to use. Human small intestine epithelial cells (PD015-F, DV Biologics, CA, USA) were grown in pro-conditioned medium (D-PRO-015, DV Biologics) in collagen coated culture plates or collagen coated transwell as above.

2.3. Receptor binding assay

After development of 100% confluent monolayers with complete differentiation, cells were scraped with rubber policeman and washed with modified Krebs–Henseleit solution (KHS, comprising NaCl, 120.7 mM; KCl, 5.9 mM; $MgCl_2$, 1.2 mM; $CaCl_2$, 2.0 mM; NaH_2PO_4, 1.2 mM; $NaHCO_3$, 25.5 mM; and (+)-glucose, 11.5 mM, pH 7.4), which had been bubbled with a mixture of 95% O_2 and 5% CO_2. The whole-cell suspension (1×10^6 cells/ml in KHS) was then incubated for 2 h with [^{3}H]-*N*-methyl scopolamine chloride (NMS) and appropriate chemicals in a final volume of 1 ml at 4 °C. The assays were performed in duplicate and a nonspecific binding was defined in the presence of 1 μM atropine. [^{3}H]-NMS concentrations ranging from 30 to 2500 pM were used in saturation binding experiments, while 600 pM was used in the competition binding experiment with the addition of increasing concentrations of the unlabeled drugs. The reactions were terminated by centrifuging the incubation solution at 700 ×*g* for 2 min. The cell pellets were then washed once with 1 ml KHS and were dissolved in 0.3 M NaOH. The radioactivity was measured by a liquid scintillation counter (Hitachi Aloka Medical Ltd., Mitaka, Japan). The mock incubation, in which cells were omitted, gave less than 40 counts per minute per tube and was similar to the background reading. Protein concentration was measured using a protein assay kit (Bio-Rad Inc., CA, USA).

2.4. Immunoblotting

Cells were cultured onto 12-mm Millicell-PCF for 14–21 days. The monolayers on filters were washed with KHS for three times and allowed to equilibrate in KHS for 30 min at 37 °C with or without inhibitors. Cells were then stimulated with 100 μM carbachol (CCh) for 5 min. For receptor activation or inhibition by muscarinic ligands, all drugs were added basolaterally as described previously [26]. Reaction was terminated by aspirating the medium and rinsed with ice-cold PBS twice. Cells were then lysed by adding pre-heated 1× SDS sample buffer (50 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 1% β-mercapto ethanol, 0.1% bromophenol blue) to the monolayers and the lysates were collected into Ependorf tubes and heated for 3 min at 100 °C. Proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. Membranes were probed with appropriate concentrations of primary antibody. The immunoreactive proteins were detected by horseradish-peroxidase-labeled secondary antibody with Amersham ECL advance Western Blotting Detection Kit (GE Healthcare, Buckinghamshire, UK). The ratio of intensities of signals was quantified by densitometry.

2.5. Knockdown of M1 and MAP2K1 by siRNA

We used predesigned siRNAs (Life Technologies) as follows: CHRM1 (5′–3′)-AGGUCAACACGGAGCUCAAtt and MAP2K1 (5′–3′) GGCCUGAC AUAUCUGAGGGtt. Transient transfections of siRNA into T84 cells were

performed using Lipofectamine 2000 as described previously [27]. Briefly, a total of 25 pmol of specific or scrambled siRNA in Lipofectamine/Opti-MEM was added to suspension cultures of T84 cells (1×10^6 cells/ml) in antibiotic-free FBS-containing culture medium. The cells were then seeded onto filter supports as described above. Following an overnight incubation, adherent cells were washed and transferred into antibiotic containing culture medium. After transfection, the cells were cultured for 4 days before experiments.

2.6. Measurement of TER

TER across the cell monolayers was measured using a Millicell ERS-2 epithelial volt–ohm meter (Merck Millipore, Darmstadt, Germany). The values ($\Omega \times cm^2$) were obtained by subtracting the resistance of blank filters without cells from the resistance of filters with cells multiplied by the effective membrane area of the filter insert. Inserts having >1000 $\Omega \times cm^2$ of TER values were used for the experiments.

2.7. Monolayer injury models

The modulation of barrier function in T84 cell monolayers was carried out as described previously [4,28]. Briefly, T84 cell monolayers were treated with ethanol (7%, bilaterally) and incubated at 37 °C for 15 min. After removal of ethanol, monolayers were incubated with fresh culture medium and the TER was measured at the times indicated. In the case of inflammation-induced epithelial injury, the monolayers were treated with IFN-γ (20 ng/ml, basolaterally) as described previously [23].

2.8. Immunohistochemistry

There were four IBD cases: case 1, a 53-year-old male; case 2, a 31-year-old male; case 3, a 40-year-old male; and case 4, a 52-year-old female. All of them were diagnosed as ulcerative colitis but were refractory to medical therapy and underwent total colectomy. Two specimens, one from non-inflammatory area and another from inflammatory area, were isolated from each total colectomy sample from IBD patients. They were fixed with 20% buffered formalin and embedded in paraffin, and cut into tissue sections. After deparaffinization, each section was incubated with 1:100 dilutions of FAK, P-FAK (Calbiochem, Darmstadt, Germany), ERK, P-ERK (Cell Signaling Technology) or mAChR M1 antibody (H-120) (Santa Cruz Biotechnology) for 1 h at room temperature and then washed three times with PBS. Bound primary antibody was detected by using anti-rabbit antibody and diaminobenzidine.

All patients provided written informed consent before surgery. The research protocol using human materials was approved at the ethical committee of Asahikawa Medical University.

2.9. Statistical analysis

Binding data were analyzed using PRISM software (Version 5.01, Graph Pad Software, La Jolla, USA), as described previously [19,29]. Briefly, the data from saturation binding studies were fitted by a one-site saturation-binding isotherm and the K_d values and the binding capacity were then calculated. The abundance of the mAChRs is indicated as the maximum binding capacity per mg of total cell protein (B_{max}). For the competition studies, the data were analyzed using the Binding–Competitive Equation of the PRISM software. A two-site model was adopted only when the residual sums of squares were significantly less ($p < 0.05$) for a two-site fit to the data than for a one-site by F test comparison. In immunoblots, the signal intensity was calculated using the Image J software. Statistical significance was evaluated using Student's t-test and was considered to be significant when p values were less than 0.05. Data are represented as the mean ± SEM with the number of experiments in parenthesis (n).

3. Results

3.1. ERK-dependent FAK phosphorylation regulates the barrier function in T84 cell monolayers

To investigate the function of ERK and FAK on the barrier function of T84 cell monolayers, we evaluated TER by using inhibitors for ERK (U0126) and FAK (PF-228). We first examined the effect of inhibitors on the maintenance of epithelial barrier function. Monolayers were treated with 10 μM U0126 or 10 μM PF-228 and the change of TER was measured. As shown in Fig. 1A, U0126 or PF-228 caused a significant reduction of TER within 30 min. Immunoblot analysis showed that U0126 significantly inhibited the phosphorylation of both ERK and FAK but PF-228 inhibited the phosphorylation of FAK alone (Fig. 1B). We next investigated the effect of U0126 and PF-228 on the recovery of epithelial barrier function after damage. We employed an ethanol-induced damage model, in which the treatment with low noncytotoxic dose of ethanol produced a reversible change of barrier function [4]. As shown in Fig. 1C, 15-min ethanol treatment caused approximately 60% reduction of TER. Ethanol-induced reduction of TER was recovered to about 75–85% 3 h after ethanol removal but the recovery was impaired up to 50–60% in the presence of either U0126 or PF-228. As shown in Fig. 1D, ethanol treatment significantly reduced the basal phosphorylation levels of both ERK and FAK and these phosphorylation levels were recovered again after ethanol removal. The recovery of ERK and FAK phosphorylation was concordant with the recovery of TER. We also observed that U0126 inhibited the recovery of both ERK and FAK phosphorylation levels but PF-228 inhibited the recovery of FAK phosphorylation alone. These results suggest that ERK-dependent FAK phosphorylation regulates the maintenance and recovery of barrier function in T84 cell monolayers.

3.2. Pharmacological characterization of mAChRs in T84 cells

We have characterized mAChRs in T84 cells by binding methods using [^{3}H]-NMS as a radioligand. We incubated cells as a whole-cell suspension for 2 h at 4 °C because we observed stable and saturable bindings at this time point ($T_{1/2} = 11.8 \pm 0.7$ min). The total receptor density was 151.1 ± 13.2 fmol/mg of total cell protein with K_d, 153.1 ± 5.3 pM (Fig. 2A). We examined the pharmacological profiles of the [^{3}H]-NMS binding sites in the cells in competition binding experiments using several subtype selective drugs. MT-7, an M1 selective antagonist, showed a simple high affinity competition ($pK_i = 9.6 \pm 0.3$). However, there was a part insensitive to MT-7 of the [^{3}H]-NMS binding sites (63.2 ± 3.2% of the total specific binding), which was not displaced by MT-7 even at high concentrations (Fig. 2B). Darifenacin, an M3 selective antagonist, gave a shallow competition curve which fitted better to a two-site model in computer analyses (Fig. 2C) with pK_i values, 8.8 ± 0.2 and 7.4 ± 0.1 for the high and low affinity components, respectively. The proportion of the darifenacin high affinity component, which was presumably the M3 subtype, was 65.4 ± 4.6% of the total specific binding sites. This proportion showed good agreement with that of the MT-7 resistant component. Furthermore, in order to verify the identity of the MT-7-insensitive component as M3 subtype, displacement by darifenacin was examined in the presence of 0.3 μM MT-7, which masked more than 95% of the MT-7-sensitive site. As shown in Fig. 2D, the MT-7-insensitive site was displaced completely by darifenacin in a monophasic manner with a pK_i value of 8.8 ± 0.1, which is compatible to that of the M3 subtype. AF-DX 116, an M2 selective antagonist showed monophasic competition with low affinity (pK_i 5.9 ± 0.2) (Supplementary Fig. S1A) and MT-3, an M4 selective antagonist did not show any displacement (Supplementary Fig. S1B). Therefore, we assumed that the M2, M4 or M5 subtype would be none or very minor population if at all as reported previously [18,30]. Based on these data, we concluded that T84 cells express mAChRs, M1 and M3 with the proportion of approximately 35% and 65%, respectively.

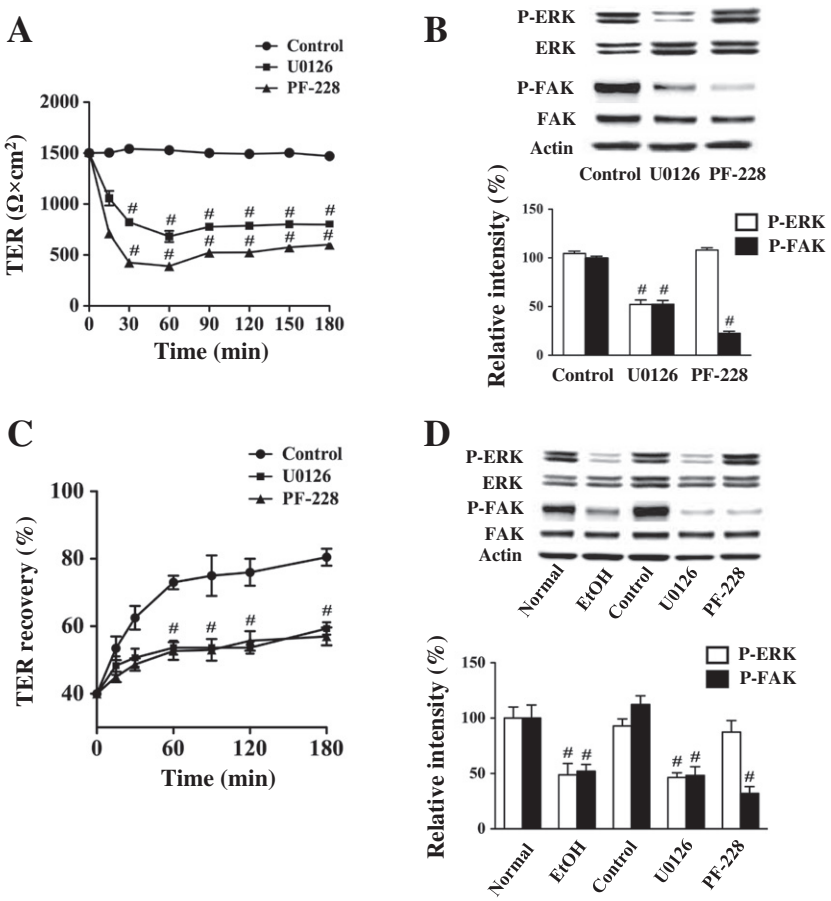

Fig. 1. ERK-dependent FAK phosphorylation maintains and recovers the barrier function of T84 cell monolayers. (A) Effects of U0126 and PF-228 on T84 cell monolayer barrier function. Treatment of monolayers with 10 μM U0126 or 10 μM PF-228 significantly (# $p < 0.05$) reduced the TER (n = 3). Each data point represents the mean of quadruplicate measurements of TER with SEM. (B) Effects of U0126 and PF-228 on the levels of ERK and FAK phosphorylation. Monolayers were incubated with fresh culture medium for 60 min without (Control) or with 10 μM U0126 or 10 μM PF-228. The monolayers were lysed, separated in SDS-PAGE, blotted and probed with anti-phospho-ERK (P-ERK) or anti-ERK (ERK) antibodies (n = 3). The same membranes were stripped and reprobed with anti-phospho-FAK (P-FAK), anti-FAK (FAK), and anti-β actin antibodies as a loading control. U0126 significantly (# $p < 0.05$) inhibited the phosphorylation of both ERK (white column) and FAK (black column) while PF-228 inhibited the phosphorylation of FAK alone. The ratio of intensities of signal was quantified by densitometry and was normalized to that without treatment as 100%. (C) Time course of barrier recovery after ethanol injury. T84 cell monolayers were treated with 7% ethanol for 15 min. After ethanol removal, monolayers were incubated with fresh culture medium without (Control) or with 10 μM U0126 or 10 μM PF-228 (n = 3). (D) Immunoblot analyses of the phosphorylation of ERK and FAK in the ethanol injury model (n = 3). Cell lysates were obtained before the addition of ethanol (Normal), 15 min after the addition (EtOH), 60 min after the removal without (Control) or with 10 μM U0126 (U0126) or 10 μM PF-228 (PF-228). The phosphorylation levels of ERK and FAK were assessed by immunoblot as described in (B).

The immunoblot results (Fig. 2E) also supported the presence of M1 and M3 subtype in T84 cells.

3.3. Stimulation of M1 mAChR leads to phosphorylation of ERK and FAK in human intestinal epithelial cells

In order to investigate the muscarinic cholinergic response in T84 cells, we stimulated the T84 cell monolayers with 100 μM CCh. The stimulation of cell monolayers with CCh resulted in an increase in phosphorylation of ERK (Fig. 3A) and FAK (Fig. 3B), approximately 60–70% and 50–60%, respectively above the baseline. The other MAP kinases p38 and JNK were also phosphorylated by CCh approximately 55–65% and 80–100%, respectively above the baseline (Supplementary Fig. S2A, B). These increases in phosphorylation were completely inhibited by the addition of either 10 μM atropine or 1 μM MT-7 prior to the stimulation. These results suggest that the phosphorylation of MAP kinases and FAK is elicited by M1 mAChR. To determine whether the phosphorylation of FAK by CCh in T84 cells is mediated through an ERK-dependent pathway, the cell monolayers were stimulated with CCh under the presence of different concentrations of U0126 or PF-228. U0126 inhibited the phosphorylation of both ERK and FAK, but PF-228 inhibited the phosphorylation of FAK alone in a dose-dependent manner (Fig. 3C), suggesting that the M1-elicited FAK phosphorylation is a downstream of ERK phosphorylation. In addition, in the human intestine epithelial cells, we detected M1 mAChR (Fig. 2E) that also elicited the similar ERK-dependent FAK phosphorylation as shown in Fig. 3D.

3.4. Ablation of M1 mAChR or MAP2K1 reduces ERK-dependent FAK phosphorylation in T84 cells

Genetic ablation of M1 mAChR or MAP2K1 (an upstream activator of ERK) in T84 cells with specific siRNA reduced their protein expression levels compared to those in the cells treated with nonspecific control siRNA. As a result of these knockdown of M1 (65.5 ± 3.6% significant reduction) or MAP2K1 (61.8 ± 1.6% significant reduction) caused a significant reduction of CCh-induced ERK and FAK phosphorylation in T84 cells. There were 4.5 ± 0.4 and 1.8 ± 0.2 fold increase in the CCh-induced phosphorylation of ERK and FAK, respectively in the monolayers transfected with control siRNA. In contrast, knockdown of M1 caused 2.0 ± 0.3 and 1.2 ± 0.1 fold increase (Fig. 4A), and that of MAP2K1 caused 1.6 ± 0.4 and 1.2 ± 0.2 fold increase (Fig. 4B) in the phosphorylation of ERK and FAK, respectively. These findings are consistent with those in pharmacological analyses, supporting the conclusion that FAK is phosphorylated via M1 mAChR-mediated ERK activation.

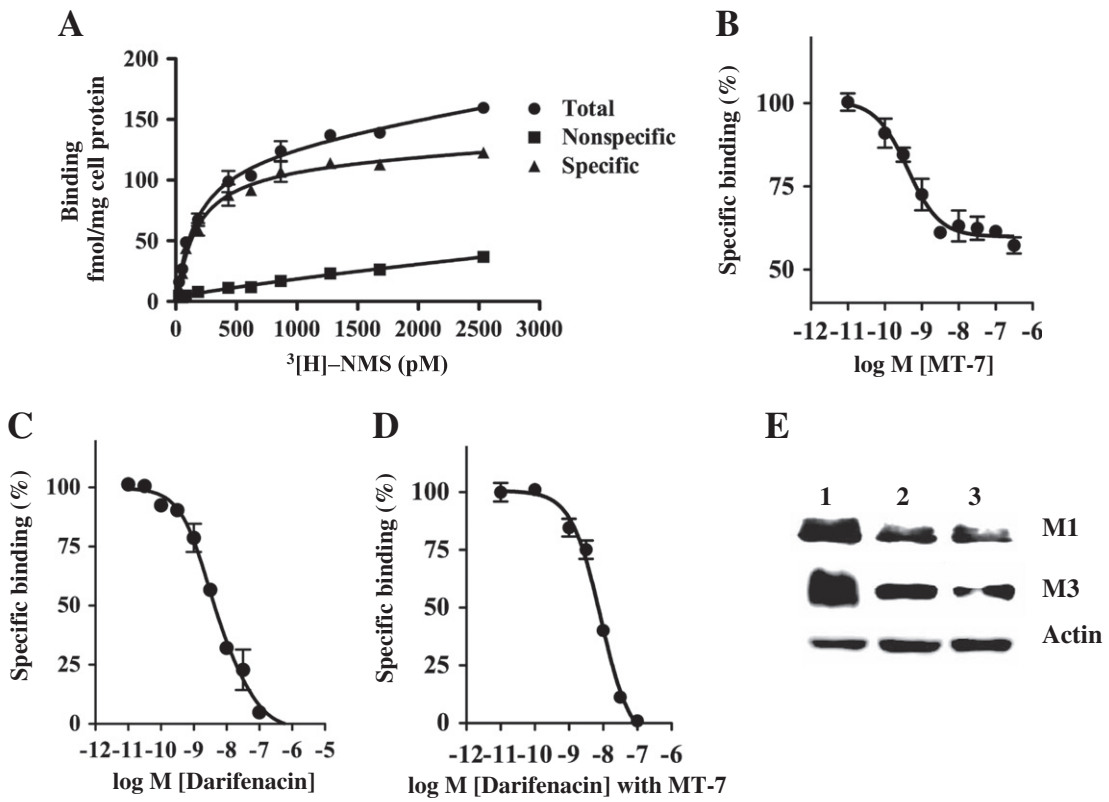

Fig. 2. Pharmacological characterization of mAChRs in T84 cells. (A) Representative saturation curve of the four independent experiments was shown. Specific binding was determined by subtracting the binding of [^{3}H]-NMS in the presence of 1 μM atropine (nonspecific binding) from the total binding. (B) Competition by MT-7 against 600 pM [^{3}H]-NMS was monophasic with high affinity but was incomplete; there were binding sites insensitive to MT-7. (C) Darifenacin showed a shallow competition curve fitted to a two site-model. (D) Competition by darifenacin against the MT-7-insensitive sites was examined in the presence of 0.3 μM MT-7, showing complete displacement in a monophasic manner with high affinity. The competition experiments were repeated at least three times. Each data point represents the mean of duplicate determinations with SEM. (E) Immunoblot analysis detected M1 and M3 mAChRs in the mouse brain (lane 1), T84 cells (lane 2) and human small intestine epithelial cells (lane 3). The molecular size of the observed band was 52 kDa (M1) or 75 kDa (M3). These experiments were repeated at least three times.

3.5. Stimulation of M1 mAChR enhances the restitution of barrier function after ethanol-induced epithelial injury

We assumed that the stimulation of M1 mAChR might have positive effects on the restitution of epithelial barrier function after ethanol injury because M1 also elicited ERK-dependent FAK phosphorylation as shown in Figs. 3 and 4. To verify this hypothesis, cell monolayers bathed in fresh culture medium were stimulated by CCh after ethanol-induced epithelial injury. As shown in Fig. 5, addition of CCh significantly augmented the recovery of TER as compared to control. The CCh-induced increase in TER was canceled in the presence of either atropine, or MT-7 (Fig. 5A). The CCh-induced increase in TER was also canceled in the presence of either U0126 (Fig. 5B) or PF-228 (Fig. 5C) in a dose-dependent manner, suggesting that the stimulation of M1 mAChR facilitates the restitution of barrier function through ERK-dependent FAK phosphorylation pathway after ethanol injury. However, there was no significant change of TER after addition of either CCh or mAChR inhibitors in the confluent monolayers without injury (data not shown), presumably because of the maximum barrier function that is already established.

3.6. Attenuation of TER and mAChR signaling after IFN-γ treatment

Treatment of monolayers with IFN-γ (20 ng/ml) decreased the TER in a time-dependent manner as shown in Fig. 6A. Since IFN-γ treatment compromised the epithelial barrier function, we have investigated the signaling aspect of mAChRs in the IFN-γ treatment. The monolayers were treated with IFN-γ for 48 h and were then stimulated with CCh, and the phosphorylation levels of ERK (Fig. 6B) and FAK (Fig. 6C) were compared with those of cells without IFN-γ treatment. There was approximately 50 ± 3.7% and 35 ± 2.7% significant reduction of CCh-induced increase in the phosphorylation levels of ERK and FAK, respectively after IFN-γ treatment. As shown in Fig. 6B, there was slight increases (but not significant) in the basal levels of ERK and ERK phosphorylation after IFN-γ treatment. In contrast, IFN-γ treatment significantly decreased the basal level of FAK phosphorylation 44.2 ± 3.9% as compared to the control monolayers without any change of total FAK levels (Fig. 6C). These results suggest that downregulation of FAK phosphorylation participates in the IFN-γ-induced reduction of TER.

We further investigated whether IFN-γ alters the expression levels of mAChRs that elicit the phosphorylation of ERK and FAK. As shown in Fig. 6D, there was 51.3 ± 2.3% and 14.4 ± 5.0% reduction of the signal intensity for the M1 and M3, respectively. The reduction of receptor subtypes was also confirmed in binding studies as shown in Fig. 6E. The densities of mAChRs in the control T84 cells were 160.8 ± 3.8, 63.6 ± 1.8 and 97.2 ± 2.3 fmol/mg of total cell protein for total, M1 and M3 subtypes, respectively, whereas in the IFN-γ treated cells the densities were 82.1 ± 3.8, 23.0 ± 2.5 and 59.1 ± 1.3 fmol/mg of total cell protein for total, M1 and M3 subtypes, respectively. There was 63.9 ± 1.8% and 39.2 ± 2.3% reduction of the density of [^{3}H]-NMS binding sites, for the M1 and M3 subtypes, respectively in the cells from IFN-γ treated monolayers compared to those from the control monolayers (Fig. 6F). Thus the IFN-γ treatment caused a reduction of mAChR density and the reduction was significantly higher in M1 subtype as compared to that of M3. These data suggest that the reduction of M1 mAChR under inflammatory condition might be relevant to the attenuation of ERK/FAK phosphorylation and to barrier dysfunction as well.

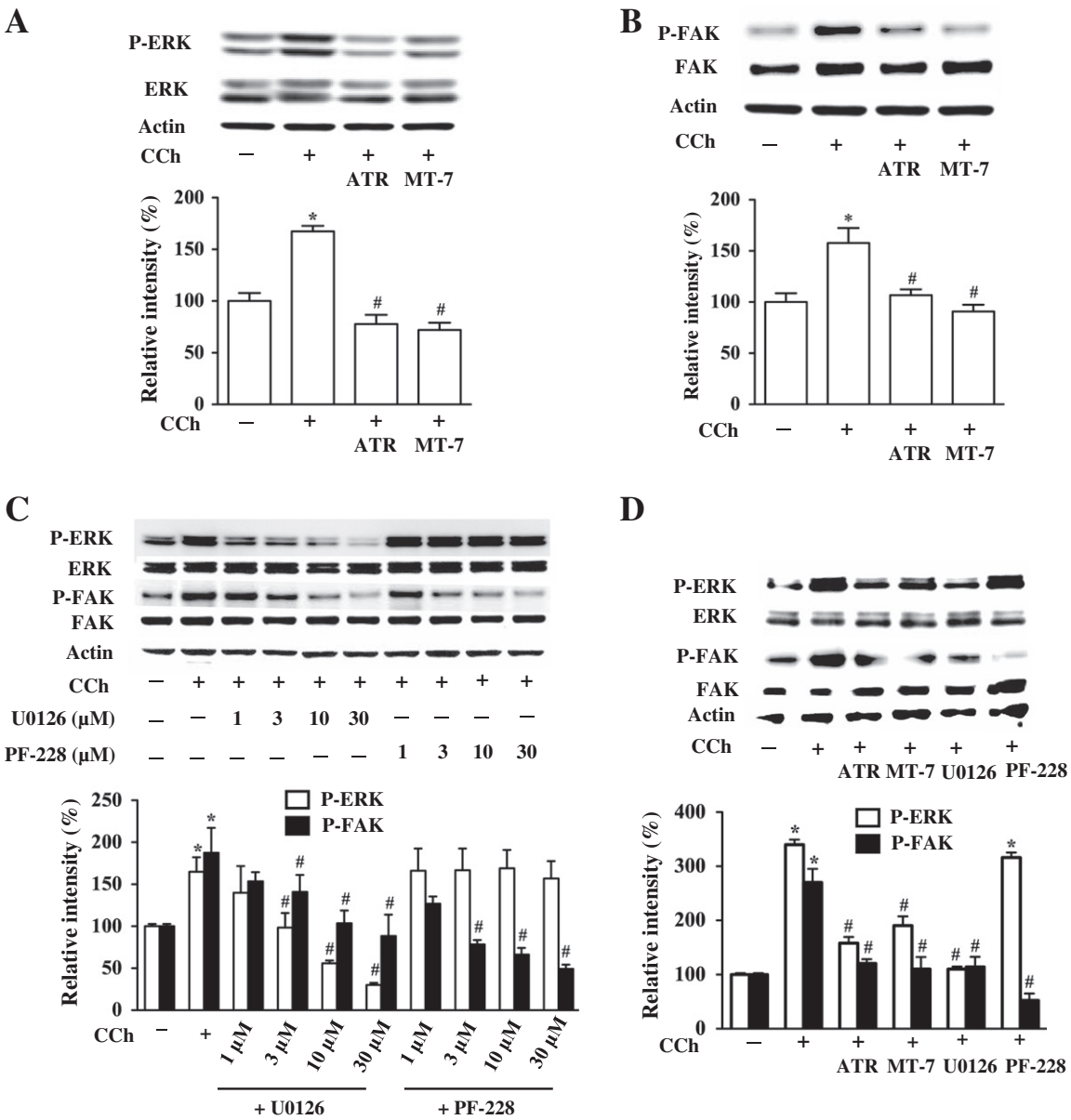

Fig. 3. Phosphorylation of ERK and FAK via M1 mAChR stimulation in human intestinal epithelial cells. (A) M1-elicited ERK phosphorylation. T84 cell monolayers were stimulated for 5 min by none (−), by 100 μM CCh (+), plus 10 μM atropine (+ATR), or 1 μM MT-7 (+MT-7). The monolayers were lysed, separated in SDS-PAGE, blotted and probed with anti-phospho-ERK (P-ERK) and anti-ERK (ERK) antibodies (n = 4). (B) M1-elicited FAK phosphorylation. The same membranes of ERK phosphorylation experiments were reprobed with anti-phospho-FAK (P-FAK) and anti-FAK (FAK) antibodies. There was a significant increase in the phosphorylation of ERK and FAK by CCh (*$p < 0.05$), which was completely inhibited by either ATR or MT-7. (C) ERK-dependent FAK phosphorylation. The monolayers were stimulated by none (−), by 100 μM CCh (+), plus U0126, or PF-228 at various concentrations (1–30 μM) as indicated in the figure. U0126 inhibited the phosphorylation of both ERK and FAK, but PF-228 inhibited the phosphorylation of FAK alone in a dose-dependent manner (n = 4). PF-228 even at the highest concentration (30 μM) did not interfere the phosphorylation of ERK. (D) M1-elicited ERK-dependent FAK phosphorylation in human small intestine epithelial cells. The cells were stimulated by CCh in the absence or presence of inhibitors as described above. The CCh-induced phosphorylation of both ERK and FAK was suppressed by ATR, MT-7 and U0126, and that of FAK alone was suppressed by PF-228 (n = 3). The ratio of intensities of signal was quantified by densitometry and was normalized to that without stimulation as 100%.

3.7. Immunohistochemical analyses of M1 mAChR, ERK/FAK and their phosphorylation in the colonic epithelium from IBD patients

We compared the expression levels of M1 mAChR, ERK and FAK and the phosphorylation levels of the kinases in between a relatively non-inflammatory area and an inflammatory area of each colon resected from IBD patients by using immunohistochemistry method as shown in Fig. 7. Although one out of four cases had severe inflammation (case 3) so that the colonic epithelium was denuded almost completely, there was a discernible reduction of M1 mAChR density in the inflammatory area of the epithelium as compared to the relatively non-inflammatory area (Fig. 7). These results are concordant with the in vitro experiment of IFN-γ-induced inflammation in T84 cell monolayers. In contrast, the expression levels of the kinases were increased slightly and the phosphorylation levels of the kinases were also increased considerably in the epithelial cells in inflammatory areas as compared to in non-inflammatory areas (Fig. 7). These increases, especially that of FAK phosphorylation disagreed with the IFN-γ-induced decrease in a cell culture system (Fig. 6C). However, there must be not only a pro-inflammatory system but also an anti-inflammatory system as well functioning at the same time in vivo. We speculated that these increases were a part of compensatory cellular mechanisms which reinforce the barrier function in the inflammatory condition. However, we have to be very careful to interpret these results in clinical samples, not only because a diverse range of inflammatory mediators and cytokines participated in the pathology of IBD, but also

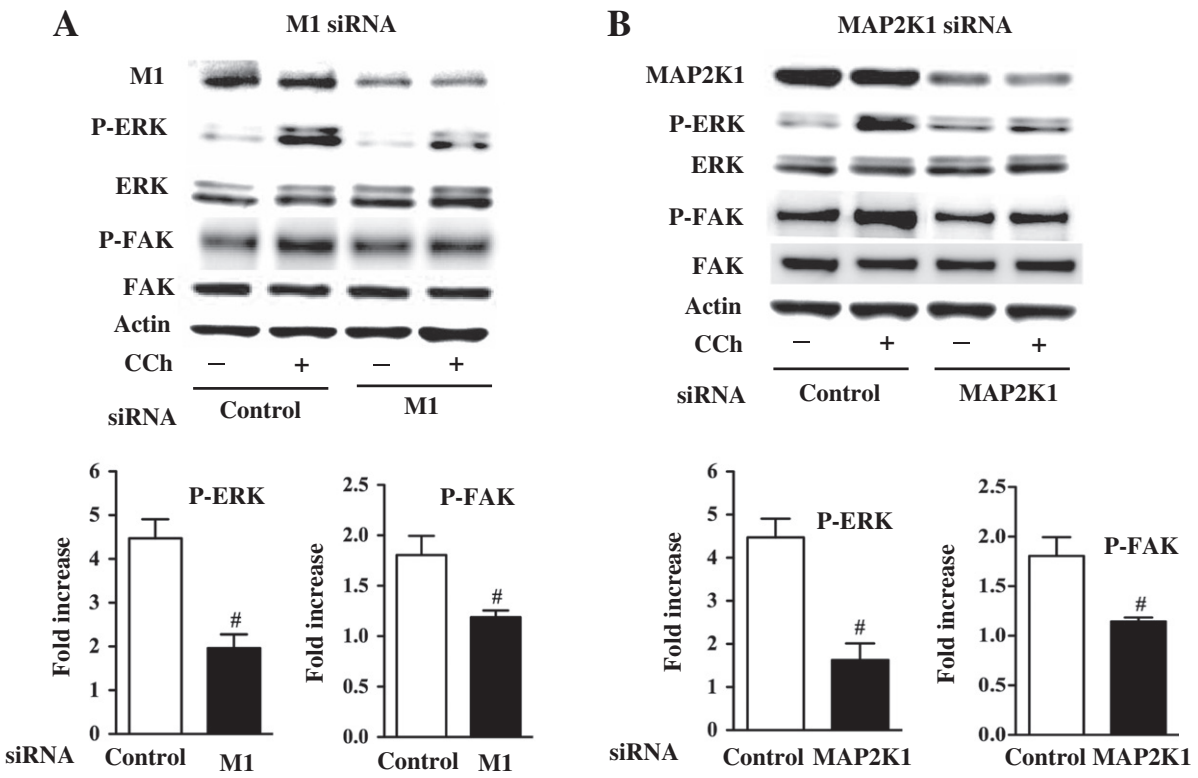

Fig. 4. Knockdown of M1 mAChR or MAP2K1 suppresses the phosphorylation of ERK and FAK in T84 cells. (A) Knockdown by siRNA targeting M1 mAChR significantly (# $p < 0.05$) reduced the CCh-induced phosphorylation of ERK and FAK in T84 cells (n = 3). (B) Knockdown by siRNA targeting MAP2K1 significantly (# $p < 0.05$) reduced the CCh-induced phosphorylation of ERK and FAK in T84 cells (n = 3).

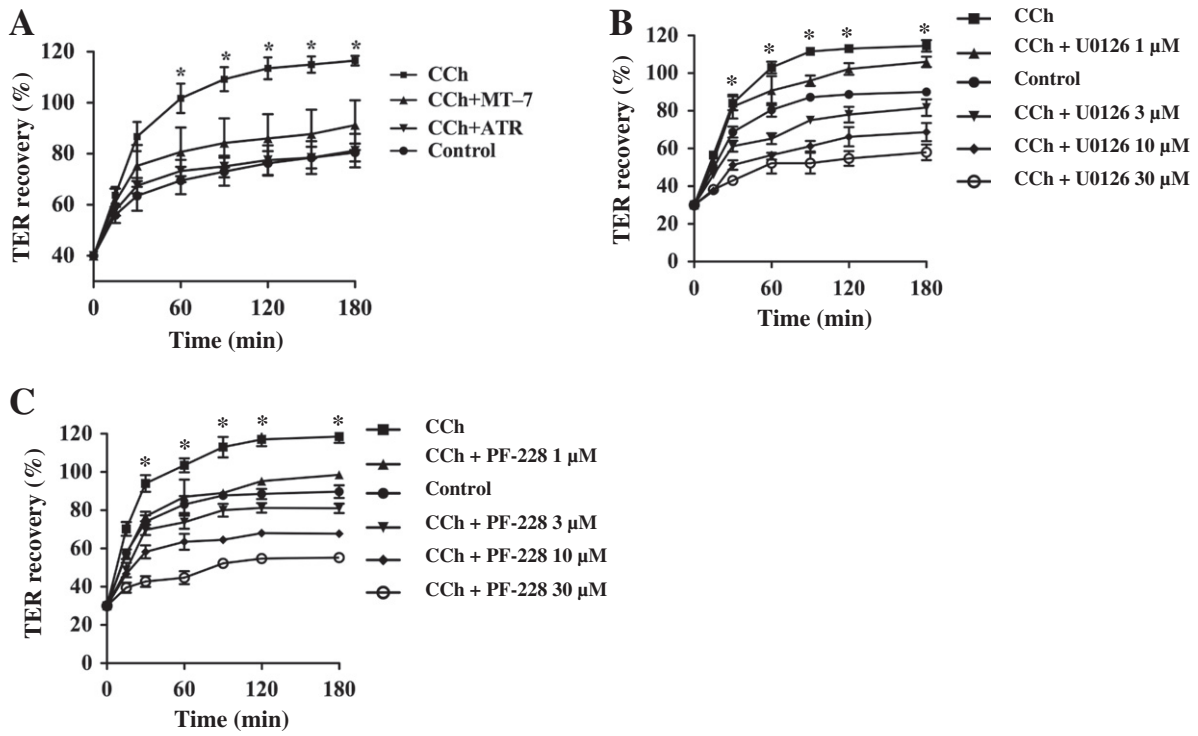

Fig. 5. Stimulation of M1 mAChR enhances the TER recovery after ethanol-induced barrier injury. (A) T84 cell monolayers were treated with ethanol for 15 min, and then incubated with fresh culture medium without (Control), or with 100 μM CCh (CCh), plus 10 μM atropine (CCh + ATR), or 1 μM MT-7 (CCh + MT-7). There was a significant increase in the recovery of barrier function by CCh (* $p < 0.05$), which was canceled by either ATR or MT-7 (n = 5). (B) U0126 or (C) PF-228 (1–30 μM) canceled the CCh-induced increase in the recovery of barrier function in a dose-dependent manner (n = 3). Each data point represents the mean of quadruplicate measurements of TER with SEM.

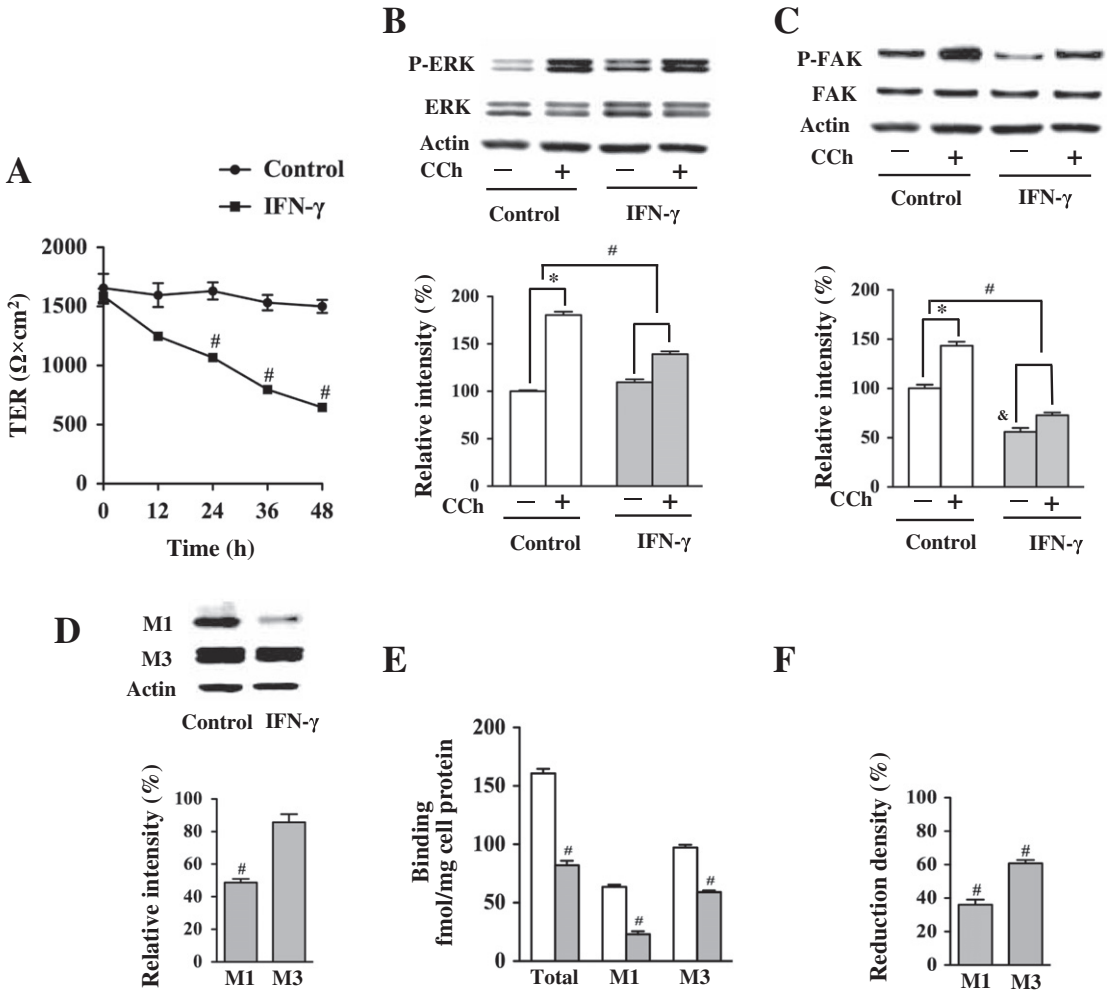

Fig. 6. Attenuation of TER and mAChR signaling after IFN-γ treatment. (A) Treatment of monolayers with IFN-γ (20 ng/ml) decreased the TER in a time-dependent manner. The significant reduction of TER (# $p < 0.05$) was observed from 24 h after treatment (n = 5). (B) Attenuated phosphorylation of ERK. Monolayers were treated with IFN-γ for 48 h and then stimulated by none (−) or 100 μM CCh (+). The phosphorylation levels and the ratio of intensities of ERK were assessed by immunoblot as described in Fig. 1. (C) Attenuated phosphorylation of FAK. The same membranes of ERK phosphorylation experiments were reprobed with anti-phospho-FAK (P-FAK) and anti-FAK (FAK) antibodies. The basal FAK phosphorylation was decreased after IFN-γ treatment as compared to control (& $p < 0.05$). CCh-induced phosphorylation of both ERK and FAK was significantly (# $p < 0.05$) attenuated in the IFN-γ treated monolayers as compared to control (n = 3). (D) Representative immunoblots of M1 and M3 levels after IFN-γ treatment. A 48-h IFN-γ treatment significantly (# p < 0.05) reduced the levels of M1 but not much of M3 as compared to control (n = 3). (E) Reduction of the total, M1 and M3 mAChRs density after IFN-γ treatment. A 48-h IFN-γ treatment significantly (# p < 0.05) reduced the density of total, M1 and M3 mAChRs as compared to control (n = 3). (F) The reduction of M1 was significantly higher than that of M3. The percentages were calculated taking the B_{max} value of control as 100%.

because multiple factors such as a phase of disease, chemotherapy regimens, etc. may be involved in the modulation of ERK/FAK signaling in the IBD patient in vivo.

4. Discussion

In this study, we have shown that treatment of T84 cell monolayers with U0126 or PF-228 significantly reduced the TER with the concurrent reduction of ERK/FAK phosphorylation levels. Our data exhibited that U0126 inhibited the phosphorylation of both ERK and FAK but PF-228 inhibited the phosphorylation of FAK alone not only in the steady phase but also in the recovery phase (Fig. 1). These findings indicate that both ERK and FAK activities are required in the maintenance and recovery of the barrier function in T84 cell monolayers and suggest that the activation of FAK is dependent on ERK activity.

We, next, exhibited that human intestinal epithelial cells including primary culture and T84 cells express M1 and M3 mAChRs (Fig. 2), based on pharmacological profiling and on immunoblot analyses. We have demonstrated that stimulation of T84 cells by CCh increased the phosphorylation of MAP kinases (ERK, p38 and JNK) and FAK. The CCh-induced increase in phosphorylation of both MAP kinases and FAK was completely inhibited in the presence of either subtype-nonselective mAChR antagonist (atropine) or M1 selective mAChR antagonist (MT-7). These results suggest that the phosphorylation of MAP kinases and FAK in human intestinal epithelial cells was mediated by M1 mAChR.

We have, then, focused on ERK and FAK in relation to the modulation of barrier function. We have found that the CCh-induced phosphorylation of FAK in human intestinal epithelial cells is a downstream of ERK phosphorylation, because U0126 inhibited the CCh-induced phosphorylation of both ERK and FAK but PF-228 inhibited the phosphorylation of FAK alone (Fig. 3). In addition, genetic ablation of M1 mAChR or MAP2K1 by specific siRNA in T84 cells significantly suppressed the CCh-induced phosphorylation of both ERK and FAK (Fig. 4A and B), supporting the conclusion drawn from pharmacological analyses. Liu et al. [31] demonstrated that ERK-mediated HGF-induced phosphorylation of paxillin resulted in the recruitment and activation of FAK in mIMCD-3 epithelial cells. Similar results of ERK-dependent FAK activation have also been reported in various other tissues or cell models [32,33]. However, there are also some contradictory reports. Flinder et al. [34] showed that EGF induced FAK-dependent ERK activation in hepatocytes through Rac1-NADPH oxidase pathway. These

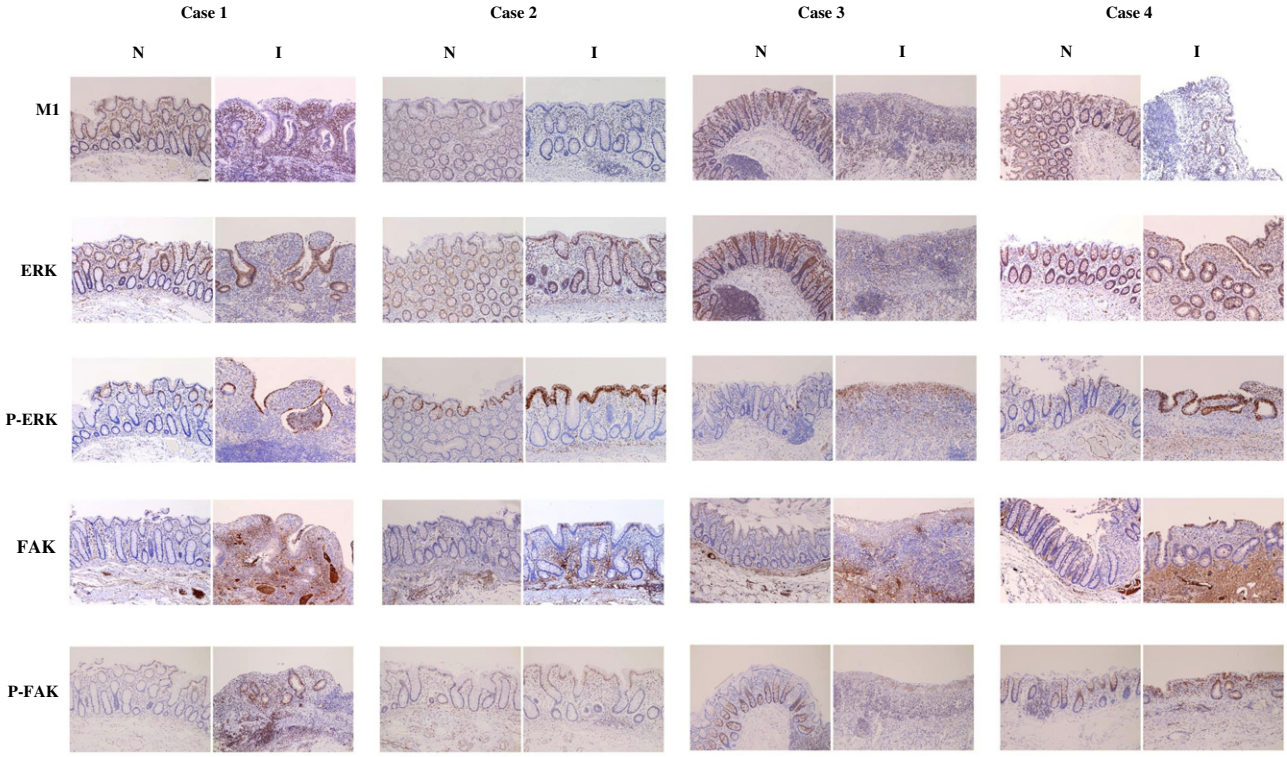

Fig. 7. Immunohistochemical analyses of colonic epithelium from IBD patients. Two specimens, one from non-inflammatory area (N) and another from inflammatory area (I), were analyzed in each total colectomy sample from four IBD patients. They were stained with anti-M1, ERK, P-ERK, FAK or P-FAK antibodies as described in the "Methods" section (*scale bar* in the top left panel shows 100 μm).

discrepancies could be due to the conditions of the experiments or different cell types used in the respective studies.

ERK is believed to be involved in the regulation of barrier function and paracellular permeability. Several researchers reported an enhancement of the epithelial barrier functions in T84 cell monolayers via MAP kinase signaling pathway by up-regulating the tight junction proteins [12,13]. On the other hand, several studies implicate a significant role of FAK signaling pathway in the barrier function and paracellular permeability. Recent studies have demonstrated that FAK activity is necessary for the barrier enhancement [6,7] and that FAK is an integrated component of the occludin/ZO-1 complex [5,35]. In this study, we also showed that FAK participated in the augmentation of the barrier recovery by CCh in T84 cell monolayers (Fig. 5). These data including ours suggest that the enhancement of TER recovery is mediated by M1 mAChR, via the activation of ERK-dependent FAK signaling pathways.

A leaky intestinal barrier is considered an important contributor to the pathology of IBD, which includes Crohn's disease and ulcerative colitis [36,37]. Indeed, a decline in barrier function of the intestinal epithelium has been shown to correlate positively with the degree of mucosal inflammation in IBD patients [38]. Participation of pro-inflammatory cytokines in the pathophysiology of IBD is well recognized and IFN-γ, one of those cytokines, has been implicated in epithelial barrier dysfunction [39]. In our study, we have shown that IFN-γ treatment decreased the TER of T84 cell monolayers (Fig. 6A). We identified that IFN-γ treatment decreased the basal phosphorylation levels of FAK at the same time (Fig. 6C). These results are consistent with the findings by Leeb et al. [40], which showed that IFN-γ treatment decreased the phosphorylation levels of FAK in colonic lamina propria fibroblast (CLPF). They also demonstrated the reduction of FAK phosphorylation levels in CLPF obtained from the active Crohn's disease patients. On the other hand, we found that the phosphorylation level of ERK was increased slightly in IFN-γ treatment (Fig. 6B). We speculate that under inflammatory conditions, FAK phosphorylation is downregulated via ERK-independent pathway and the reduction of FAK phosphorylation may be relevant to the mechanism of IFN-γ-induced barrier dysfunction in T84 cell monolayers. Further studies are necessary to uncover the complex mechanism of these signal transductions.

The ERK/FAK signaling via mAChRs was also significantly attenuated in IFN-γ treatment (Fig. 6B and C). Cholinergic hyporesponsiveness is a well-known phenomenon in colitis. Dextran sulfate sodium (DSS)-induced colitis in mice results in a profound hyporesponsiveness of the colonic epithelium to prosecretory agents and a complete loss of response to mAChR activation [41]. Immunological results revealed that the IFN-γ treatment caused a significant reduction of M1 mAChR but not much of M3 (Fig. 6D). In addition, binding experiments also supported the reduction of the density of M1 subtype significantly higher than that of M3 in the T84 cells after treatment with IFN-γ (Fig. 6E and F). We have speculated that the reduction of M1 mAChR density on the cell surface of colonic epithelium may be one of the mechanisms in the cholinergic hyporesponsiveness in inflammatory conditions.

Finally, immunohistochemical staining of colonic epithelium from IBD patients has revealed that there was apparent reduction of M1 mAChR density in inflammatory area as compared to the relatively non-inflammatory area of the colonic epithelium (Fig. 7). Recently, we reported that colitis induction in mouse model resulted in the reduction of epithelial mAChRs where M1 subtype was highly susceptible to inflammation than M3 [19]. Although the mechanism of this reduction is not clarified yet, our data provide direct evidence that the reduction of mAChRs could be a fundamental mechanism of inflammatory cholinergic hyporesponsiveness. Our data also revealed that the expression levels of ERK and FAK were slightly increased in the inflammatory areas and the phosphorylation levels of the kinases were also increased considerably (Fig. 7). These results are concordant with the previous studies, in which, using cell cultures or isolated crypts in biopsies from IBD patients, ERK was not only over-expressed but also highly phosphorylated during the active phase of IBD [42,43]. In the case of FAK, this is the first report showing the upregulation in the colonic epithelium of IBD patients. In a mouse model also, we reported the

increase in the expression level of FAK [20]. Our results in this manuscript suggest that FAK functions in the maintenance and recovery of the barrier function in colonic epithelium. This is concordant with recent findings by Owen et al. which have shown that the FAK knockout mice exhibited earlier onset and increased severity of DSS-induced colitis as compared to control animals. The colonic epithelial repair was also impaired significantly in the absence of FAK [44]. Since the patients with IBD represent a heterogeneous spectrum of pathological features with the participation of a diverse range of inflammatory mediators, it is difficult to conclude how FAK is involved in the pathology. However, we could speculate that the activity of FAK might be relevant with the episode of inflammation. In some phase of inflammation, FAK expression and activity may be upregulated to compensate the epithelial damage. Further studies are required in the future to elucidate the involvement of FAK in the pathology of IBD.

In conclusion, our data show that human intestinal epithelial cells express M1 mAChR that positively regulates the barrier function through ERK-dependent FAK phosphorylation pathways. In a pathophysiological aspect, reduction of FAK phosphorylation in T84 cells may be relevant to the IFN-γ-induced barrier dysfunction. The attenuated phosphorylation of ERK and FAK upon muscarinic stimulation after IFN-γ treatment is presumably due to the reduction of M1 mAChR density on the cells. Although further studies are necessary, these findings could implicate an important role of M1 mAChR for the maintenance and restitution of intestinal epithelial barrier function in pathophysiological conditions.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

This work was supported in part by National Project “Knowledge Cluster Initiative” (2nd stage, “Sapporo Biocluster Bio-S”) and Grant-in-Aid for Scientific Research (C) and Grant-in-Aid for Young Scientists (B) granted by the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Smoking Research Foundation, and the fund for Asahikawa Medical University Creative Research in the Field of Life Science.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2013.12.007.

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