SUPPLEMENTARY INFORMATION

Epithelial cell-derived a disintegrin and metalloproteinase-17 confers resistance to colonic inflammation through EGFR activation

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SUPPLEMENTARY MATERIALS AND METHODS

General assessment of colitis

The entire colon was dissected out and the length was recorded as previously described (Okayasu et al., 1990) and the ratio of the colonic length to the weight at day 0 was evaluated. The colon samples were fixed in 4% paraformaldehyde (PFA) and embedded in paraffin. Sections were stained with hematoxylin and eosin (HE). Severity of mucosal injury was graded as follows (Egger et al., 1997; Okayasu et al., 1990): Grade 0, normal; Grade I, partial distortion and/or destruction of the glands; Grade II, marked erosions/destruction of the glands or loss of the entire glands with remaining surface epithelium; and Grade III, loss of the entire glands (Supplementary figure 1). The proportion of the respective injured surface areas for each field was scored on a scale of 1 (10%) up to 10 (100%). A crypt damage score (CDS) was described as a product of the severity and proportion scores of the mucosal injury for each field (Egger et al., 1997). Means for all fields were calculated for each single animal. For assessment of mucus production, paraffin sections were subjected to Alcian-Blue staining. Histological scoring of sectioned tissues was performed in a blinded manner by expert pathologists (M.S. and A.S.).

Immunohistochemistry

Paraffin sections were subjected to immunohistochemistry for neutrophil antigen, F4/80, CD3, proliferation cell nuclear antigen (PCNA), epidermal growth factor receptor (EGFR) and phosphorylated EGFR (pEGFR) using rat anti-neutrophil antigen antibody (MCA771G; AbD Serotec), rat anti-F4/80 antibody (MCA497G; AbD Serotec), rat anti-CD3 antibody (MCA500G; AbD Serotec), mouse anti-PCNA (PC10; DAKO Cytomation), rabbit anti-EGFR (sc-03; Santa Cruz Biotechnology) and rabbit anti-pEGFR (sc-12351-R; Santa Cruz Biotechnology) antibody, respectively. They were followed by reactions with anti-rat Fab's fragments conjugated to horseradish peroxidase-labeled amino-acid polymer (Nichirei Biosciences), Envision system HRP-labeled polymer anti-mouse IgG (DAKO Cytomation) or Envision system HRP-labeled polymer anti-rabbit IgG (DAKO Cytomation). Immunostaining for mouse ADAM17 was performed using AMeX method (Sato et al., 1986). Briefly, tissues were fixed in acetone at -20°C overnight, then cleared in methyl benzoate and xylene, consecutively, and embedded in ordinary paraffin at 58-60°C. Paraffin sections were subjected to immunohistochemistry for ADAM17 with rabbit anti-ADAM17 (MBS240296; MyBioSource), followed by reactions with secondary antibody as described above. Color was developed with 3, 3'-diaminobenzidine

tetrahydrochloride (Sigma-Aldrich) . After immunohistochemistry, the sections were counterstained with hematoxylin. For assessment of inflammatory cell infiltrations in the colon, numbers of neutrophil antigen-, F4/80- or CD3-immunostained cells were counted by observing 6 different fields at x400 magnification without knowledge of the samples examined.

Western blot analysis

Tissues obtained from distal colon or cultured cells were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40) containing complete proteinase inhibitors (Roche Diagnostics), 1 mM Na_3VO_4 and 10 mM NaF, and equal amounts of homogenate supernatants were used for sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins were transferred to PVDF membranes, and the membranes were incubated with rabbit anti-ADAM17 antibody (AB39162; Abcam), rabbit anti-EGFR antibody (sc-03; Santa Cruz Biotechnology), rabbit anti-pEGFR antibody (sc-12351-R; Santa Cruz Biotechnology), rabbit anti-transforming growth factor (TGF)-α antibody (bs-0066R; Bioss) or mouse anti-β-actin (A5441; Sigma-Aldrich). Subsequently, the membranes were incubated with secondary antibodies, visualized by chemiluminescence and quantified by image analysis.

Assessment of proliferation of colonic epithelia

Cells in S phase were labeled by intraperitoneal administration of 5'-bromo-2'-deoxyuridine (BrdU; 30 μg/g body) in PBS. Intestines were excised at 24 hours post-injection and the distal colon was fixed in 4% PFA. Paraffin sections of the colon were immunostained with anti-BrdU antibody (Bu20a; DAKO Cytomation) as described above and they were counterstained with hematoxylin. To assess proliferative activity of colonic epithelia, the sections were also stained with anti-PCNA antibody. Number of BrdU-positive cells per colonic epithelial gland cells or ratio of PCNA-positive cells to total epithelial cells was quantified by counting number of cells in intact, well-oriented crypts, in which adjacent nuclei and lumen were visible, in 5 different fields at a magnification of x400 without knowledge of clinical data.

Colonic crypt isolation

The distal half of the colon was washed in Hanks' balanced salt solution (HBSS) and then treated with 30 mM EDTA in HBSS for 15 min. At the end of the incubation, the tissue was stirred and crypts were isolated from the stroma as described earlier (Tsukamoto et al., 2006).

Gene expression analysis

Gene expression of MUC2 was determined by quantitative real-time polymerase chain reaction (qPCR). RNA was isolated from isolated colonic crypts or cell lines using ISOGEN (Nippon Gene). RNA (1 μg) was reverse transcribed with ReverTra Ace Reverse Transcriptase (TOYOBO) as recommended by the manufacturer. Quantitative real-time PCR was performed using an ABI 7000 Cycler with SYBR Green Master Mix (Invitrogen). The primer sets for mouse MUC2, mouse β-actin, human MUC2, and human β-actin were as follows: forward primer 5-GCTGACGAGTGGTTGGTGAATG-3, reverse primer 5-GATGAGGTGGCAGACAGAGAC-3 for mouse MUC2; forward primer 5-AGGGAAATCGTGCGTGACAT-3, reverse primer 5-GAACCGCTCGTTGCCAATAG-3 for mouse β-actin; forward primer 5-CTGCACCAAGACCGTCCTCATG-3, reverse primer 5-GCAAGGACTGAACAAAGACTCAGAC-3 for human MUC2; forward primer 5-GGATGCAGAAGGAGATCACTG-3, reverse primer 5-CGATCCACACGGAGTACTTG-3 for human β-actin. Gene expression levels were

determined in triplicate and normalized to the expression of β-actin, which remained the same in the various groups.

Dot blot analysis

Tissues obtained from the distal colon or cultured cells were homogenized in lysis buffer containing complete proteinase inhibitors (Roche Diagnostics), 1 mM Na_3VO_4 and 10 mM NaF, and equal amounts of homogenate supernatants (2 μg) were spotted on PVDF membranes. After the membranes air-dried, they were incubated with primary antibodies for rabbit anti-MUC2 antibody (H-300; Santa Cruz Biotechnology) or GAPDH (Abcam), followed by incubation with secondary antibodies and visualization by chemiluminescence.

Time-lapse cell growth assay

Human colonic epithelial cell lines, Caco-2 and LS174T cells, were purchased from the American Type Culture Collection, and cultured in basal MEM media supplemented with 10% fetal bovine serum and L-glutamine, penicillin and streptomycin in a well-humidified incubator with 20% O_2 and 5% CO_2 at 37°C. For time-lapse cell growth assay, xCELLigence cell index (CI) impedance measurements were performed

according to the instructions of the supplier. After seeding 2 x 10^3 human colonic epithelial cells in 100 μl of the cell suspensions into each well of the E-plate 96 (ACEA Biosciences), CI impedance was monitored every 15 minutes for a period of up to 120 hours by the xCELLigence system. After a 24-hour culture, cells were treated with ADAM17-selective inhibitor S-44029 (*N*-hydroxy-*N*-(1-(1-isobutylpiperidin-4-yl)-2-((4-((2-methylquinolin-4-yl)methoxy)phenyl)sulfonyl)ethyl)formamide; a gift from KAKEN PHARMACEUTICAL CO., LTD.; PATENT No.WO2003/022801) (20 μM) (Supplementary figure 5) (Kawasaki et al., 2006), EGFR inhibitor AG1478 (20 μM), MEK inhibitor (U0126) (10 μM), PI3K inhibitor (LY294002) (20 μM), STAT3 inhibitor (S3I) (100 μM) or negative control agent for MEK inhibitor U0124 (10 μM) and cultured for an additional period of up to 96 hours.

Alcian-Blue staining and assessment of mucus production

LS174T cells were plated on chamber slides (BD Biosciences) and cultured for 24 hours. After treatment with or without the above-mentioned inhibitors, they were cultured for additional 48 hours and subsequently fixed with 100% ethanol. Fixed cells were stained with Alcian-Blue solution (pH 2.5) for 20 minutes at room temperature,

followed by nuclear staining with Nuclear Fast Red. Ratio of Alcian-Blue-positive area in total cell area was determined using Adobe Photoshop CS4 software. Relative gene expression of MUC2 to β-actin in LS174T cells after treatment with the inhibitors was evaluated by quantitative real-time PCR as described above.

Knockdown of ADAM17 in colonic epithelial cells

Two pre-designed siRNAs for ADAM17 (S1004 and S1006) and non-targeting control siRNA (AllStars Negative Control siRNA) were obtained from Ambion. Caco-2 and LS174T cells (1.5×10^5 cells per well) were transfected with these siRNAs using Lipofectamine siRNAMAX (Invitrogen) and the Nucleofector™ Kit (Amaxa), respectively. They were plated in 6-well plates and cultured in basal MEM media supplemented with 10% fetal bovine serum and L-glutamine, penicillin, and streptomycin. After 24 hours, siRNAs were removed by changing the culture media with fresh media. The cells were cultured for additional 48 hours and then used for further analyses.

Clinical samples

Colon tissues were obtained from the patients with active UC (n=25) or under remission

(n=4) at colectomy. Diagnosis of the patients with UC was performed endoscopically, and all cases underwent surgery for severe inflammation or UC-associated dysplasia/carcinoma. As non-inflammatory controls, non-neoplastic colon tissues apart from tumors were obtained from the patients with colon cancer (n=30). The colon specimens were fixed with 10% formaldehyde, and HE-stained paraffin sections were analyzed by light microscopy. Histological inflammatory features of the UC samples were graded according to the Matts' classification (Matts, 1961) as follows: Grade 1, normal appearance; Grade 2, some infiltration of the mucosa or lamina propria with either round cells or polymorphs; Grade 3, much cellular infiltration of the mucosa, lamina propria, and submucosa; Grade 4, presence of crypt abscesses, with much infiltration of all layers of the mucosa; Grade 5, ulceration, erosion, or necrosis of the mucosa, with cellular infiltration of some or all of its layers. Inflammation was regarded as remission stage when a Matts' score of Grade 1 was found. Paraffin sections were subjected to Alcian-Blue staining or immunohistochemistry for ADAM17, EGFR, pEGFR and Ki-67 using rabbit anti-ADAM17 antigen antibody (AB19027; Chemicon), rabbit anti-EGFR (Santa Cruz Biotechnology), rabbit anti-pEGFR (Santa Cruz Biotechnology) and anti-Ki-67 antibody (MIB-1; DAKO Cytomation), respectively.

Statistics and repeatability of experiments

Data were reported as mean ± s.d. Results between the two independent groups were determined by the two-sided Student's *t*-test. Comparisons among more than three groups were determined by one-way ANOVA followed by Bonferroni's post-hoc testing. For survival analysis, Kaplan–Meier test was evaluated. For *in vivo* experiments, the minimum number necessary to achieve statistically significant differences between groups was estimated based on our pilot experiments by power analysis calculation. This calculation was made assuming a significance level of 0.05 and a power level of 0.80. *P* values less than 0.05 were considered significant. Figures 1b,c,g, 2c,e, 3c and 7a and Supplementary figures 2 and 3 are representative images from three independent experiments. Figures 2a,b, 3a,b,d,f, 4a,c,f, 5a,c and 6c and Supplementary figure 4 are representative images from two independent experiments. The experiments were not randomized. No samples or animals were excluded from the analysis and the investigators were not blinded to allocation during the animal experiments.

SUPPLEMENTARY REFERENCES

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FIGURE LEGENDS for supplementary figures

Supplementary figure 1. Histological grade of colitis. Severity of mucosal injuries was scored as Grades 0, I, II and III. Grade 0, normal; Grade I, distortion and/or destruction of the bottom third of the glands; Grade II, erosions/destruction of bottom two thirds or loss of the entire glands with remaining surface epithelium; Grade III, loss of the entire glands. Scale bar, 20 μm.

Supplementary figure 2. Infiltrating leukocytes in colon tissue. Representative images of neutrophil antigen-, F4/80- or CD3-immunostained cells in colon sections from control and *Adam17*$^{flox/flox}$*Mx1-Cre*$^{+}$ (*ΔAdam_Mx1*) mice after DSS administration are shown. Scale bar, 20 μm.

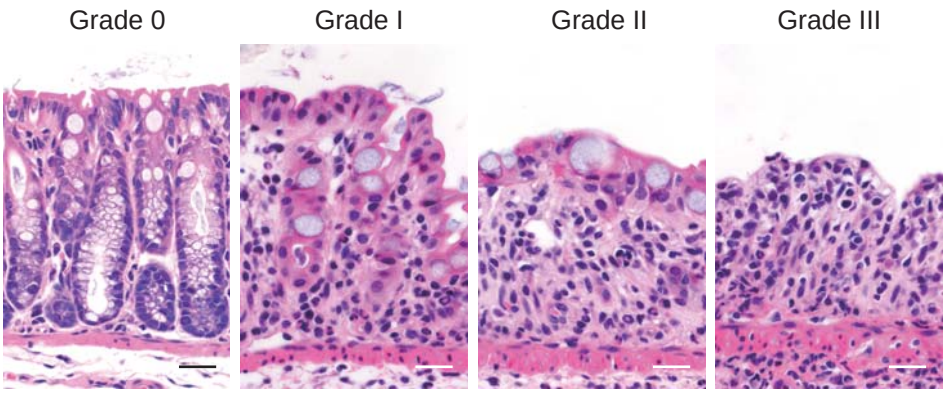
Supplementary figure 3. Histological presentation of cell proliferation markers in colonic tissue. Representative images of BrdU- or PCNA-stained colon tissue sections from control and *Adam17*$^{flox/flox}$*Mx1-Cre*$^{+}$ (*ΔAdam_Mx1*) mice at day 8 after DSS challenge are shown. Scale bars, 50 μm.

Supplementary figure 4. Expression of ADAM17, EGFR and TGF-α in human colonic epithelial cell lines. Immunoblots of ADAM17, EGFR and TGF-α in human colonic epithelial cell lines, Caco-2 and LS174T cells are presented. β-Actin was used as a loading control.

Supplementary figure 5. Molecular structure of S-44029 (*N*-hydroxy-*N*-(1-(1-isobutylpiperidin-4-yl)-2-((4-((2-methylquinolin-4-yl)methoxy) phenyl)sulfonyl)ethyl)formamide).

Supplementary figure 6. A schematic model representing epithelial ADAM17 as a rate-limiting determinant for activation of EGFR signaling, which contributes to epithelial regeneration and barrier maintenance.

Supplementary figure 1 (*Shimoda et al.*)



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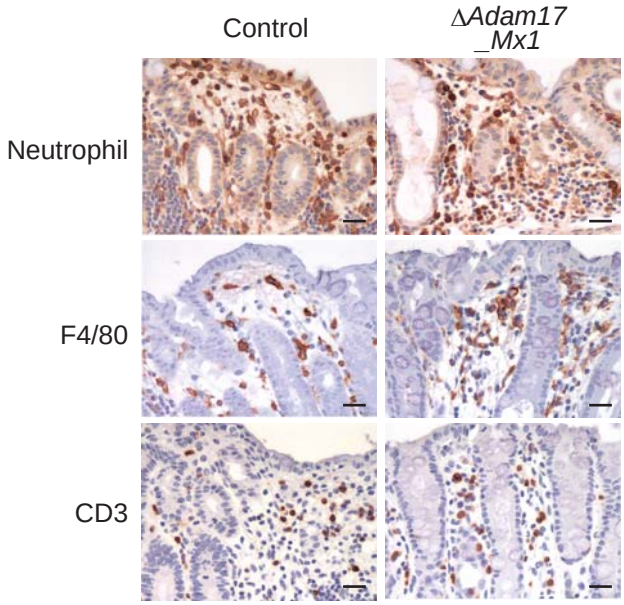

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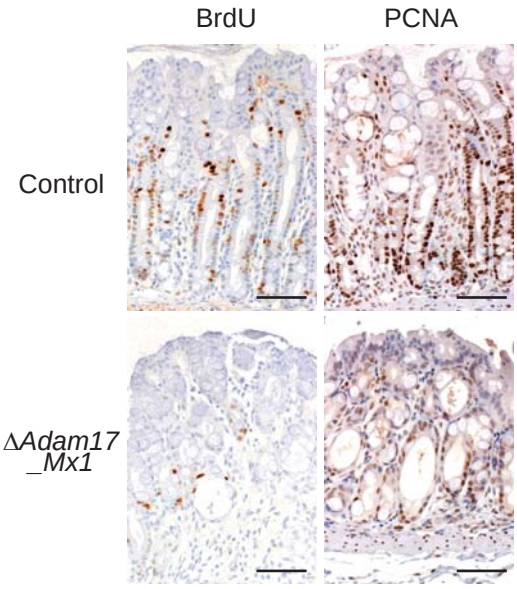

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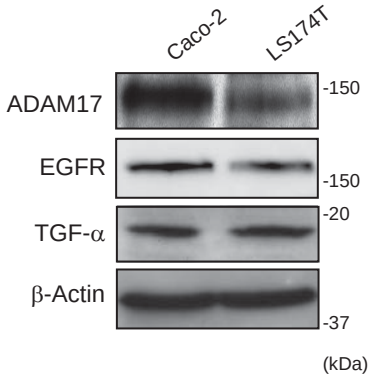
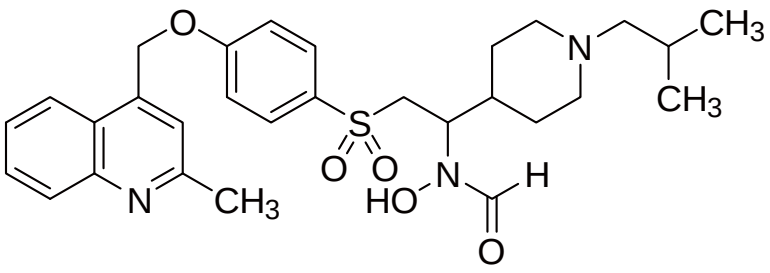


Supplementary figure 5 (*Shimoda et al.*)



Supplementary figure 6 (*Shimoda et al.*)