Table I. *Primers for real-time PCR*

Gene (Accession No.)	Sequence (5′ to 3′)
MFG-E8 (NM_008594)	
Forward	CGGGCCAAGACAATGACATC
Reverse	TCTCTCAGTCTCATTGCACACAAG
α_v Integrin (NM_008402)	
Forward	CTGGCTGTGTTGGTATTTGTAATGTAC
Reverse	AGCTGTTCTCGTTCTTGCTCTTC
β_3 Integrin (NM_016780)	
Forward	CGTCAGCCTTTACCAGAATTATAGTG
Reverse	TTTCCCGTAAGCATCAACAATG
OPN (NM_009263)	
Forward	TCTGATGAGACCGTCACTGC
Reverse	AGGTCCTCATCTGTGGCATC
FN (NM_010233)	
Forward	GAGGAGGGAGATGAACCACA
Reverse	GGGTCTACTCCACCGAACAA
VN (NM_011707)	
Forward	CTCTCTGTCAGCCGTGTTTG
Reverse	GTGGGATAAGGAGCCAGTGA
β-actin (NM_007393)	
Forward	CGTGAAAAGATGACCCAGATCA
Reverse	CACAGCCTGGATGGCTACGTA

Escherichia coli LPS (0111:B4 strain) and purified flagellin from *Salmonella typhimurium* (InvivoGen), mouse osteopontin (OPN) and dexamethasone (Sigma-Aldrich), recombinant integrin $\alpha_v\beta_3$ (R&D Systems), 6× His pTriEx-3hygro (Novagen), pNF-κB-Luc (Stratagene), pRL-TK (Promega), annexin V-Fluos (Roche), FITC-TUNEL (Promega), ELISA kits for IL-1β, TNF-α, and OPN (R&D Systems), and myeloperoxidase (MPO; Hycult Biotechnology) were acquired from their respective suppliers. The Abs used were: anti-mouse MFG-E8 (MBL International), PE-F4/80 (eBioscience), anti-His probe (H3), anti-p-IκB, anti-β_3 integrin, anti-focal adhesion kinase (FAK), and anti-pFAK (Santa Cruz Biotechnology), anti-IκB, anti-OPN, and anti-IgG (R&D Systems), anti-α_v integrin (Abcam), and anti-phospho-β_3 (pY759) integrin (BD Biosciences).

Preparation of recombinant MFG-E8

Mouse wild-type and arginine-glycine-aspartate (RGD) mutant MFG-E8 proteins were prepared as described previously (18). Briefly, coding regions excluding the signal peptide sequence of MFG-E8 (NM_008594) were cloned into the *Eco*RI and *Xho*I sites of a 6× His pTriEx-3hygro vector to generate pMFG-E8, and transfected into HEK293 cells using Lipofectamine 2000. At 48 h after transfection, cells were lysed in mild lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 1 mM (p-amidinophenyl)methanesulphonyl fluoride hydrochloride (pH 8.0)) by sonication on ice and purified using Ni-NTA columns, then the purity was checked by SDS-PAGE and Western blotting assays. For SDS-PAGE, purified proteins were run on a 12% polyacrylamide gel and stained with Coomassie brilliant blue, while for Western blotting the proteins were allowed to react with anti-6× His Ab, and the corresponding signals were detected using ECL (GE Healthcare). Similarly, RGD mutant MFG-E8 was produced by introducing a point mutation to generate D89E containing a p-mutant MFG-E8 expression vector, after which expression was confirmed and purification performed using the mammalian expression systems described above. The functional efficiency of recombinant MFG-E8 protein was evaluated based on its ability to enhance phagocytosis of apoptotic cells using fluorescent microscopy, as described previously (23).

Colitis induction and treatment of recombinant MFG-E8 in mice

Seven-week-old male specific pathogen-free BALB/c mice (Charles River Laboratories) were housed according to our institutional guidelines with the approval of the Ethics Committee of Shimane Medical University. Primarily, a single group of normal mice (n = 3) were euthanized to screen tissue-specific MFG-E8 expression using Northern and Western blotting methods. To produce a DSS colitis model, a group containing five mice was fed 2.5% DSS in drinking water for 9 days, while the control group received only normal drinking water throughout the experiment. Then, purified recombinant wild-type or mutant MFG-E8 was diluted in PBS and 300 μl of the solution (30 μg/kg) was injected i.v. through the tail vein, starting from 2 days before DSS administration, which continued until euthanasia. The parameters for colitis evaluation recorded in the experiments were body weight, colon length, and rectal bleeding, as determined by visual inspection. After stopping DSS treatment, the mice were euthanized and the colon was measured with a ruler on a nonabsorbent surface. For histology, 3-μm-thick formalin-fixed, paraffin-embedded colon tissues were stained with H&E and examined under a light microscope by two investigators in a double-blinded fashion.

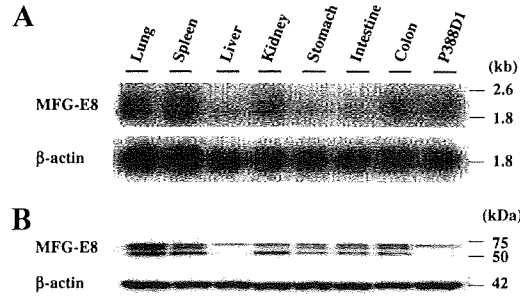
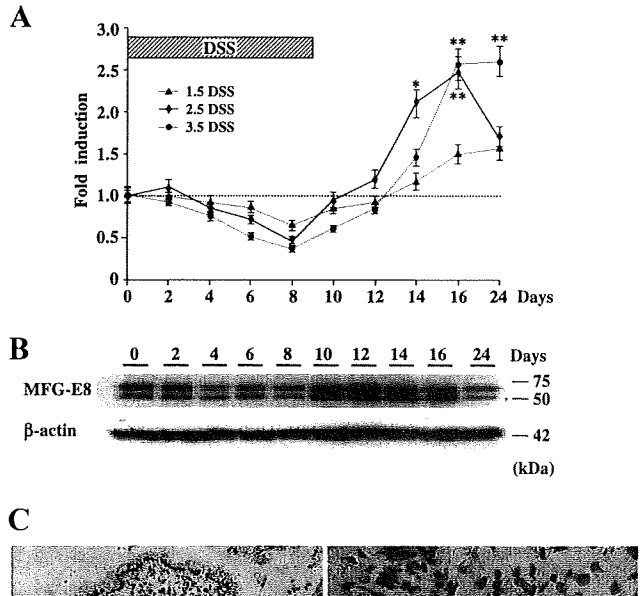


FIGURE 1. Tissue-specific MFG-E8 expression in mice. Northern and Western blots demonstrating the expression of MFG-E8 in different tissues of normal mice (n = 3) are shown.

Quantitative real-time PCR

Total RNA was extracted from colonic tissues and cultured cells using Isogen (Nippon Gene) and then equal amounts of DNase I (Ambion)-treated RNA were reverse transcribed into cDNA using a QPCR cDNA kit (Stratagene). A real-time fluorescence PCR assay based on SYBR Green (Applied Biosystems) was then performed using the primers described in Table I.



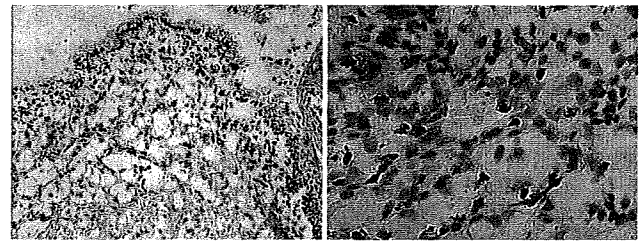


FIGURE 2. Differential expression of MFG-E8 in experimental colitis. Time course changes of MFG-E8 expression in inflamed colons (n = 90), as revealed by (*A*) real-time PCR using samples from 1.5%, 2.5%, and 3.5% DSS-treated mice, and (*B*) Western blotting with samples from 2.5% DSS-treated mice. *, $p < 0.05$ and **, $p < 0.01$ vs DSS (−). Error bars indicate the SEM values obtained from three independent experiments. *C*, Immunohistochemical localization of MFG-E8 in frozen colonic tissue sections from DSS-treated colitis mice shown with magnification. Left, ×200; right, ×400.

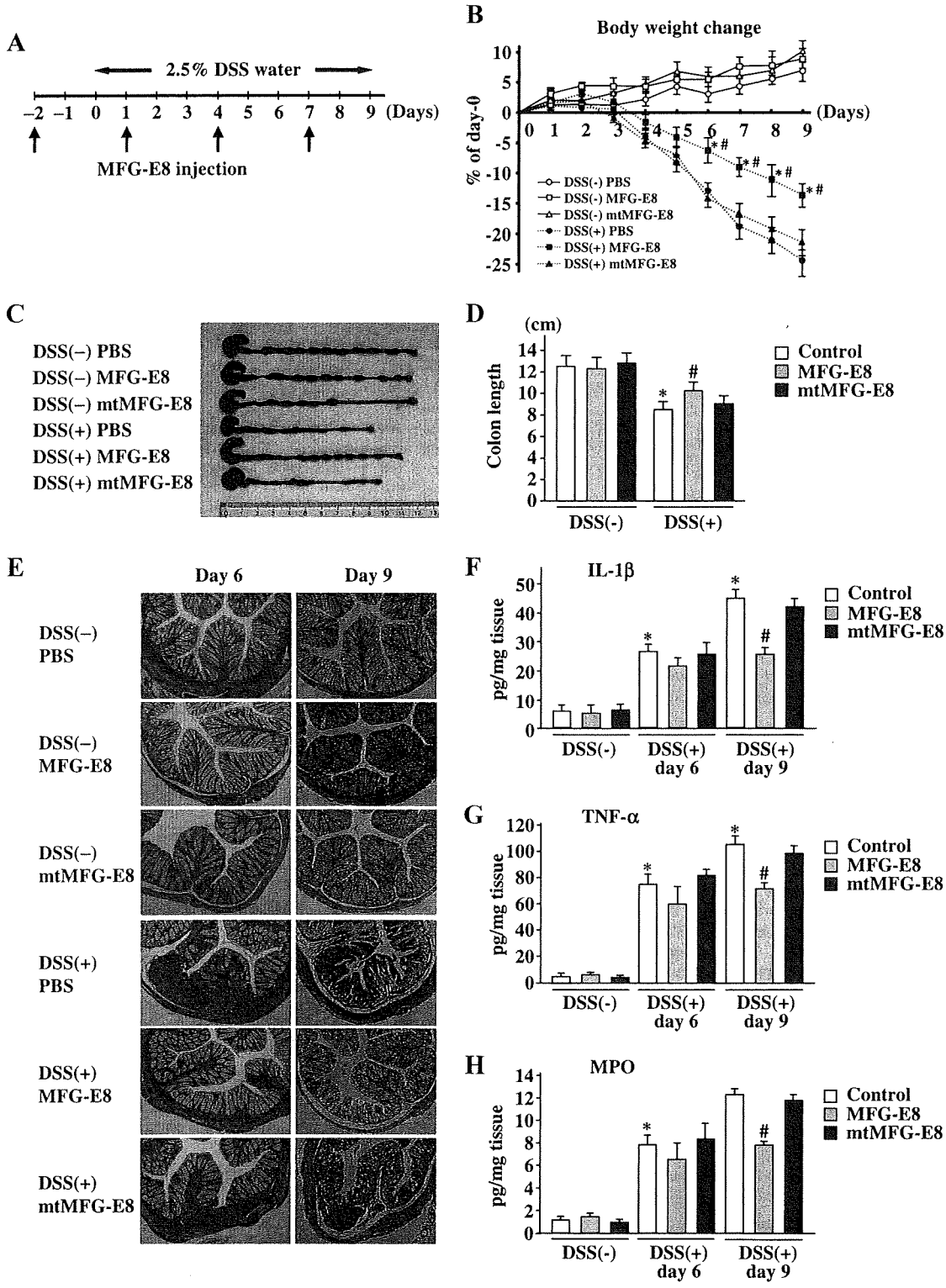


FIGURE 3. In vivo effects of recombinant MFG-E8 in DSS colitis. *A*, Protocol for experimental colitis and treatment with recombinant MFG-E8. *B*, Effects of MFG-E8 on body weight changes in 2.5% DSS-treated mice. Data are expressed as serial changes in percentage of weight change during DSS administration. *, $p < 0.05$ vs DSS (+) PBS; #, $p < 0.05$ vs DSS (+) mutant MFG-E8 (mtMFG-E8). *C* and *D*, Effects of MFG-E8 on colon length in 2.5% DSS-induced colitis. *, $p < 0.05$ vs DSS (−) PBS; #, $p < 0.05$ vs DSS (+) PBS. Error bars indicate the SEM values ($n = 5$ mice/group). *E*, Histological changes in distal colonic lesions on days 6 and 9 of DSS administration with or without MFG-E8 (original magnification ×100). *F–H*, Colonic tissue contents of proinflammatory cytokines and MPO at the same time points of DSS administration as above with or without MFG-E8. *, $p < 0.05$ vs DSS (−) PBS; #, $p < 0.05$ vs DSS (+) PBS. Error bars indicate the SEM values ($n = 5$ mice/group).

Protein extraction, Western blot, and immunoprecipitation

Protein extraction and Western blotting assays were performed as described previously (23). Briefly, after blocking with 10% skim milk (Difco), the membrane was reacted with anti-mouse MFG-E8 Ab at a concentration of 1 μg/ml for 1 h at room temperature and the resulting signals were visualized using ECL reagent. Similarly, Western blotting assays of IκB, p-IκB, α_v/β_3 integrin, and β-actin were performed using their respective Abs in optimized conditions. For immunoprecipitation, mouse

Table II. *Effect of recombinant MFG-E8 on histological parameters (day 9)*[a]

Sample	Inflammation	Depth of Injury	Crypt Damage	Total
DSS (−) PBS	0.17 ± 0.17	0.00	0.00	0.17 ± 0.17
DSS (−) MFG-E8	0.17 ± 0.17	0.00	0.00	0.17 ± 0.17
DSS (−) mtMFG-E8	0.00	0.00	0.00	0.00
DSS (+) PBS	3.75 ± 0.41	3.75 ± 0.45	6.50 ± 0.63	14.13 ± 0.93
DSS (+) MFG-E8	1.88 ± 0.35*#	2.88 ± 0.33	4.50 ± 0.46*	9.13 ± 0.67*#
DSS (+) mtMFG-E8	4.13 ± 0.48	4.25 ± 0.59	5.63 ± 0.75	13.50 ± 0.93

[a] During each histological examination, three different parameters were measured: severity of inflammation (based on polymorphonuclear neutrophil infiltration; 0–3: none, slight, moderate, severe), depth of injury (0–3: none, mucosal, mucosal and submucosal, transmural), and crypt damage (0–4: none, basal one-third damaged, basal two-thirds damaged, only surface epithelium intact, entire crypt and epithelium lost). The score for each parameter was multiplied by a factor reflecting the percentage of tissue involvement (×1, 0–25%; ×2, 26–50%; ×3, 51–75%; ×4, 76–100%), and all values were added to a sum, with a maximum possible score of 40. *, $p < 0.05$ vs DSS (+) PBS; #, $p < 0.05$ vs DSS (+) mtMFG-E8.

peritoneal macrophages were lysed in Nonidet P-40 buffer containing 150 mM Nacl, 1% Nonidet P-40, 50 mM Tris (pH 8.0), and 1 mM PMSF; after clarification, 200 μg of protein was immunoprecipitated with anti-β_3 integrin Ab and protein G-Sepharose (GE Healthcare). Immunoprecipitants were subjected to Western blotting using anti-phospho-β_3 and β_3 integrin Abs. Similarly, pFAK (phospho-Y397) and FAK assays were performed as described above.

Immunohistochemistry of MFG-E8

Frozen colonic tissue samples were sliced into 3-μm-thick sections and fixed in cold acetone for 20 min. After blocking endogenous peroxidase activity with 0.3% hydrogen peroxide in methanol, sections were incubated for 2 h at room temperature with 1 μg/ml MFG-E8 specific primary Ab and then processed with the corresponding protocols using an immunoperoxidase staining kit (Vectastain).

Transfection, luciferase assay, and RNA interference

For in vitro experiments, mouse macrophage-like cells P388D1 from American Type Culture Collection were grown and plated on 24-well plates (2.5×10^4 cells/well) in RPMI 1640 with 10% FBS in a humidified chamber. After 18–24 h the cells had reached 50% confluence and were transiently transfected with pNF-κB-Luc (200 ng/well) and pRL-TK-*Renilla*-Luc (20 ng/well) using Lipofectamine 2000 (2.5 μl/well), and then NF-κB activity was measured using a dual-luciferase reporter assay system (Promega). In another experiment, custom FlexiTube small interfering RNAs (siRNAs) for α_v/β_3 integrin (Qiagen) were transfected into P388D1 cells according to the manufacturer's protocol, and siRNA effects were checked by real-time PCR and Western blotting assays, as described above.

MFG-E8 binding assay

To investigate the binding of MFG-E8 to $\alpha_v\beta_3$ integrin, 96-well polyvinyl chloride microtiter plates were coated by pipetting 50 μl of recombinant $\alpha_v\beta_3$ integrin (20 μg/ml) diluted in coating buffer and incubated overnight at 4°C. Following incubation, the coating solution was removed and the plates were washed twice with PBS. The remaining protein-binding sites in the coated wells were blocked by adding 200 μl of 1% BSA. Recombinant MFG-E8 at different concentrations was then added to the $\alpha_v\beta_3$ integrin-coated wells and incubated for 2 h at room temperature. After subsequent washing, the wells were treated with HRP-labeled anti-mouse MFG-E8 Ab at a concentration of 1 μg/ml and the resulting absorbance was measured using a plate reader. Similarly, to measure the competitive binding of OPN and MFG-E8 to $\alpha_v\beta_3$, a fixed amount of exogenous OPN (500 ng/ml) was allowed to bind with $\alpha_v\beta_3$ integrin in coated wells in the presence of various concentrations of recombinant MFG-E8. After 2 h of incubation at

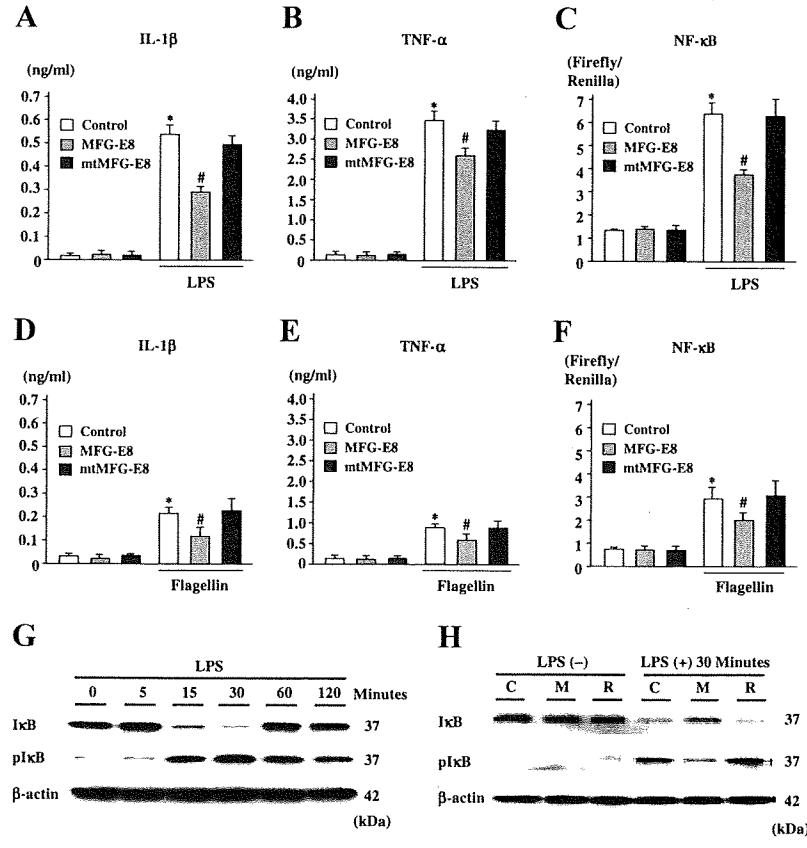


FIGURE 4. In vitro effects of recombinant MFG-E8. Peritoneal macrophages were pretreated with or without MFG-E8 (400 ng/ml) proteins for 2 h, followed by LPS (100 ng/ml) stimulation for 24 h. The culture supernatants were assessed for (*A*) IL-1β and (*B*) TNF-α using EIA. *C*, Assessment of NF-κB activity using a luciferase assay. Twenty-four hours after transfection, P388D1 cells were treated with recombinant MFG-E8 proteins for 2 h, followed by stimulation with LPS for 12 h, after which dual-luciferase assays were performed. *, $p < 0.05$ vs LPS (−); #, $p < 0.05$ vs LPS (+). Error bars indicate SEM values, which were obtained from three independent experiments. *D* and *E*, Peritoneal macrophages were pretreated with or without MFG-E8 (400 ng/ml) proteins for 2 h; following flagellin (100 ng/ml) stimulation for 24 h, the culture supernatants were assessed for IL-1β and TNF-α using EIA. *F*, In a 24-well plate, P388D1 cells (2.5×10^4 cells/well) were transfected with pNF-κB-Luc and pRL-TK-*Renilla*-Luc using Lipofectamine 2000. Twenty-four hours after transfection, the media was changed and pretreated with or without MFG-E8 protein (400 ng/ml) for 2 h; then, after 12 h of flagellin (100 ng/ml) stimulation, dual-luciferase assays were done. *, $p < 0.05$ vs flagellin (−); #, $p < 0.05$ vs flagellin (+). Error bars indicate SEM values obtained from three independent experiments. *G*, Time course changes of IκB degradation and phosphorylation in LPS-treated (100 ng/ml) mouse peritoneal macrophages. *H*, Effects of MFG-E8 on IκB degradation and phosphorylation in peritoneal macrophages (C, control; M, MFG-E8; R, RGD mutant MFG-E8) after 30 min of stimulation with LPS (100 ng/ml).

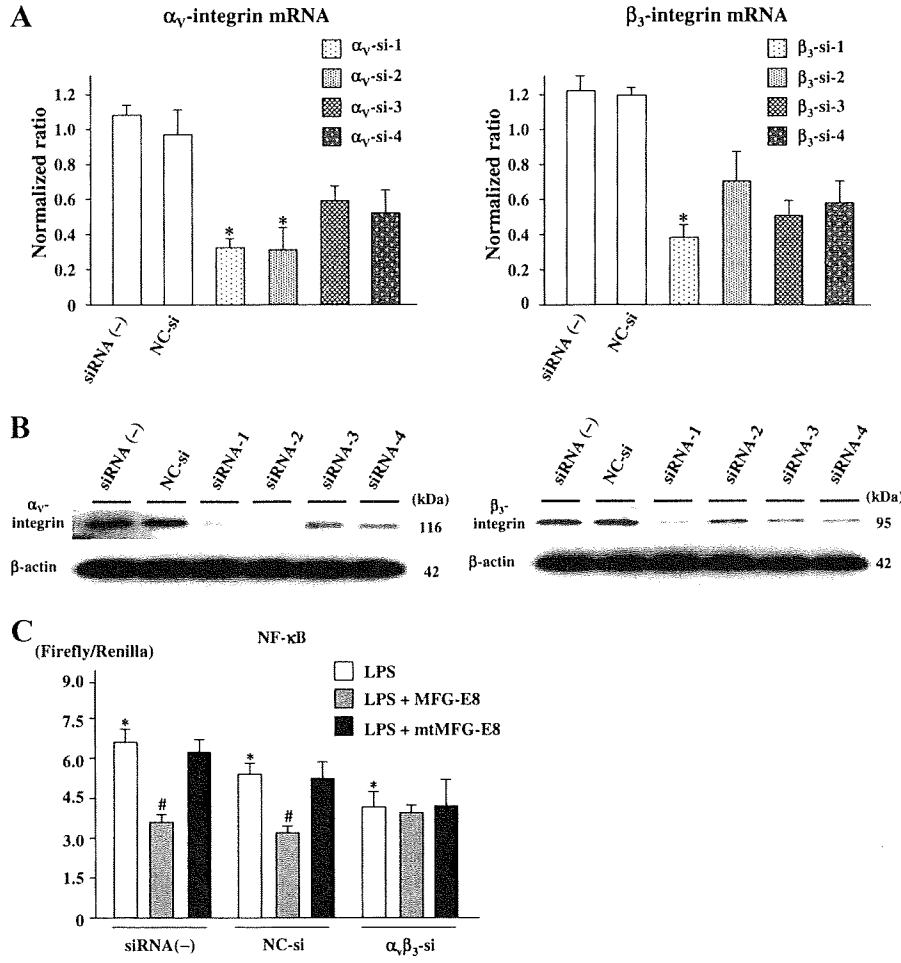


FIGURE 5. Involvement of $\alpha_v\beta_3$ integrin for MFG-E8-mediated NF-κB inhibition. *A* and *B*, Efficiency of four different sets of siRNAs used for knockdown of α_v/β_3 integrin at the mRNA and protein levels. *, $p < 0.05$ vs NC-si. Error bars indicate SEM values, which were obtained from three independent experiments. *C*, Twenty-four hours after transfection with respective custom siRNAs (33 nM/well), which inhibited the expression of α_v/β_3 integrin (α_v-si-2, β_3-si-1) by ~80%, P388D1 cells were treated with recombinant MFG-E8 for 2 h, followed by stimulation with LPS (100 ng/ml) for 12 h, after which dual-luciferase activities were measured. *, $p < 0.05$ vs LPS (−); #, $p < 0.05$ vs LPS (+). Error bars indicate SEM values, which were obtained from three independent experiments.

room temperature, the unbound materials were removed by washing with PBS, and HRP-labeled anti-mouse OPN Ab (1 μg/ml) was added to each well. Finally, the resulting signals were measured at a wavelength of 450 nm and analyzed using the CurveExpert 1.3 software package.

Enzyme immune assay (EIA)

Cytokines and MPO contents in colonic tissues and culture supernatants were estimated using EIA according to the manufacturer's protocol. Briefly, total proteins from distal colonic tissues were extracted using lysis buffer (200 mM NaCl, 5 mM EDTA, 10 mM Tris, 10% glycine, and 1 mM PMSF (pH 7.4)) and subjected to EIA using mouse IL-1β, TNF-α, and MPO EIA kits. In vitro culture supernatants from mouse peritoneal macrophages treated with or without LPS, flagellin, OPN, or recombinant MFG-E8 were also checked for IL-1β and TNF-α contents by EIA, as described above. In another experiment, OPN contents in LPS-treated peritoneal macrophage culture supernatants were assayed using an OPN EIA kit.

Statistical analysis

All quantitative data are expressed as the mean ± SE. Student's *t* test was used for statistical determinations. Values of p of <0.05 were considered statistically significant.

Results

Altered expression of MFG-E8 in acute colitis

We examined MFG-E8 expression in different tissues of normal BALB/c mice and observed the ubiquitous presence of MFG-E8 with its two transcript variants (24) in a tissue-specific manner (Fig. 1). Next, to assess the time course changes of MFG-E8 expression during colitis, mice were given 2.5% DSS in drinking water, after which MFG-E8 expression in colonic tissues was checked at various time points. As shown in Fig. 2, *A* and *B*, MFG-E8 was down-regulated in inflamed colons during the DSS induction period (days 1–9), while it gradually became up-regulated during the healing phase (days 10–24), when DSS was no

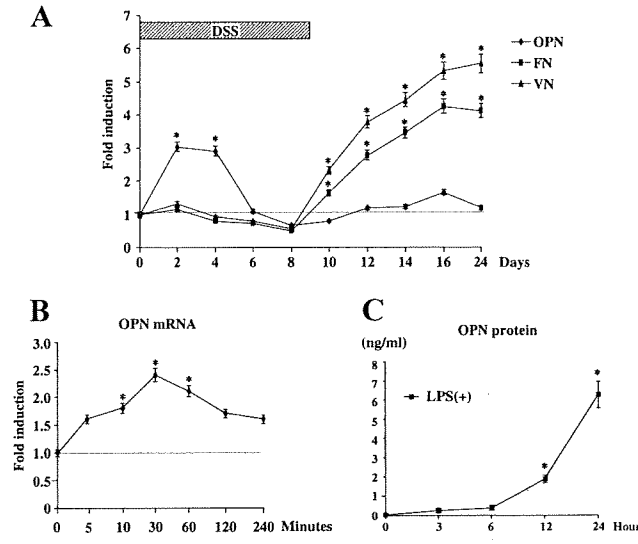


FIGURE 6. OPN expression during inflammation. *A*, Real-time PCR showing the time-dependent expression of OPN, FN, and VN in distal colonic tissues of 2.5% DSS-treated mice ($n = 30$). *, $p < 0.05$ vs DSS (−). *B* and *C*, LPS (100 ng/ml)-dependent time course changes of OPN expression by peritoneal macrophages using real-time PCR and EIA at various time points. *, $p < 0.05$ vs LPS (−). Error bars indicate the SEM values obtained from four independent experiments.

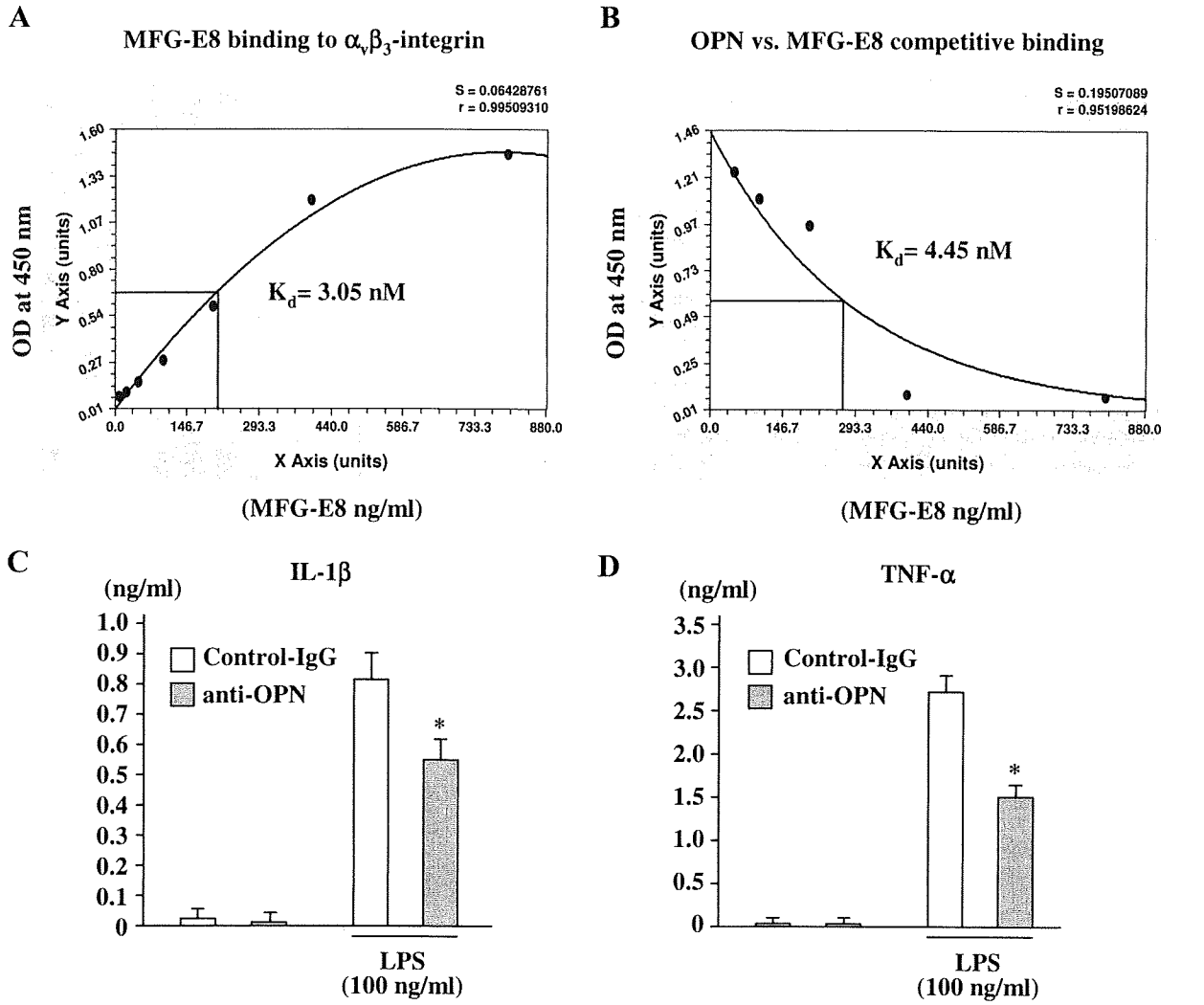


FIGURE 7. MFG-E8 relative binding assay and autocrine effects of OPN. *A*, MFG-E8 binding to $\alpha_v\beta_3$ integrin was determined by adding serially diluted recombinant MFG-E8 (0–800 ng/ml) to $\alpha_v\beta_3$-coated plates, after which K_d was determined as the amount of MFG-E8 required to saturate to half of the optimum binding point. *B*, Competitive binding of MFG-E8 with OPN for $\alpha_v\beta_3$ integrin was assessed by adding a fixed amount of recombinant OPN (500 ng/ml) with varying concentrations of MFG-E8 (0–800 ng/ml) to $\alpha_v\beta_3$-coated wells. OPN binding to $\alpha_v\beta_3$ was decreased with increasing concentrations of recombinant MFG-E8. Relative K_d was determined as the amount of MFG-E8 required to desaturate to half of the optimum amount of OPN needed to bind to $\alpha_v\beta_3$. The corresponding K_d value was determined from results of four independent experiments. *C* and *D*, Mouse peritoneal macrophages were cultured with either the IgG isotype control (5 μg/ml) or anti-mouse OPN Ab (5 μg/ml) and then stimulated for 12 h with LPS, after which EIA was performed for IL-1β and TNF-α using culture supernatants. *, $p < 0.05$ vs LPS (+) anti-IgG.

longer added to the drinking water. Moreover, to reveal any link between MFG-E8 down-regulation and DSS-induced colitis characterized by intestinal inflammation, we performed a dose-dependent experiment using low (1.5%) and high (3.5%) concentrations of DSS, and assessed MFG-E8 expression at different time points. Notably, in mild colitis shown by a low grade of inflammation, MFG-E8 expression was not reduced to the same extent as in severe colitis (Fig. 2*A*). Thus, we concluded that the level of MFG-E8 down-regulation caused by different doses of DSS may be related to inflammation severity. Additionally, to confirm MFG-E8 localization, we performed immunohistochemical analyses using colonic tissue sections, which revealed its presence in lamina propria mononuclear cells that had become infiltrated during colitis (Fig. 2*C*).

MFG-E8 protects mice from DSS-induced colitis

Mice treated with 2.5% (w/v) DSS in their drinking water showed clinical, histological, and immunological signs of colitis (25). Recombinant wild-type and RGD mutant MFG-E8 were separately injected into mice during the DSS period, while other mice were injected with PBS instead of MFG-E8 and received normal drinking water and served as controls (Fig. 3*A*). As shown in Fig. 3*B*, in the mice who received DSS with PBS, body weight loss of ~23% commenced on day 4 and continued to day 9, while mice injected with the wild-type MFG-E8, as opposed to those with mutant MFG-E8, showed significant improvement in body weight loss from day 4, which reached only 14% on day 9. Representative specimens from the dissected colons from the MFG-E8-treated group showed increased length as compared with those from the PBS- or mutant MFG-E8-treated DSS groups (Fig. 3, *C* and *D*). Histological examinations also showed that lamina propria infiltration by mononuclear cells as well as crypt epithelial damage were markedly decreased in the MFG-E8-treated colitic mice (Fig. 3*E*). Furthermore, total histological scores in the distal parts of colon samples from MFG-E8-treated mice after 9 days of DSS administration were significantly lower than in those from the other groups (Table II).

To investigate the effects of MFG-E8 on proinflammatory cytokine production, protein was extracted from colonic tissues, and IL-1β and TNF-α contents were measured. Notably, they were decreased in MFG-E8-treated, but not in the PBS- or mutant MFG-E8-treated, DSS groups (Fig. 3, *F* and *G*). Furthermore, MFG-E8 treatment significantly down-regulated the tissue content of MPO as compared with that in PBS- or mutant MFG-E8-treated DSS mice (Fig. 3*H*). With the present experimental system, we assessed the effects of recombinant MFG-E8 using body weight change as a parameter of colitis, which was apparent at the beginning of day 6 of DSS treatment. Therefore, we also performed histology and immunology experiments using samples from day 6 as an additional time point. As shown in Fig. 3*E–H*, although the effects of MFG-E8 on day 6 samples were evident, they were more distinct in samples from day 9. Taken together, these results demonstrate that recombinant MFG-E8 has a protective role in colitis.

We also noticed that the colitis was refractory in mice treated with the RGD mutant form of MFG-E8. In a previous study of apoptotic cell phagocytosis by Asano et al., the RGD mutant MFG-E8 (D89E) was shown to be a dominant negative form of MFG-E8 (18). While performing the present experiment to evaluate the functional efficacy of our purified recombinant proteins, we noticed inhibition of phagocytosis of apoptotic cells following addition of purified recombinant RGD mutant MFG-E8 to cocultured cells (macrophage and apoptotic thymocytes) (data not shown), whereas we did not observe any dominant negative effects on DSS-induced colitis or in subsequent in vitro studies. Therefore, it is possible that our RGD mutant protein was nonfunctional rather than having any dominant-negative effects on colitis.

MFG-E8 down-regulates proinflammatory cytokines through $\alpha_v\beta_3$ integrin-mediated NF-κB inhibition

To elucidate the detailed mechanism of the MFG-E8-dependent antiinflammatory effect on experimental colitis, we used bacterial LPS, which is a potent inducer of inflammatory signals in cultured murine peritoneal macrophages treated with or without MFG-E8 in vitro. As expected, LPS markedly induced IL-1β and TNF-α in culture supernatants, while cells pretreated with recombinant MFG-E8 significantly down-regulated the effects of LPS on cytokine production, whereas mutant MFG-E8 had no such effects on LPS-stimulated macrophages (Fig. 4, *A* and *B*). We then examined the effects of recombinant MFG-E8 on NF-κB status in LPS-treated P388D1 cells. Following LPS treatment, NF-κB activity became considerably elevated, while cells exogenously pretreated with recombinant MFG-E8 showed significant down-regulation of the LPS-induced NF-κB activity, whereas mutant MFG-E8 had no significant effects on this event (Fig. 4*C*). Other than LPS, several pathogen-associated molecular patterns are also known to activate the innate immune responses in immune-reactive cells via TLRs, and therefore it is of interest to check whether recombinant MFG-E8 inhibits the flagellin (as an additional stimulant)-induced effects on mononuclear cells. Interestingly, we noticed an MFG-E8-dependent significant down-regulation of proinflamatory cytokine production, as well as NF-κB activity in flagellin-treated macrophages, although the RGD mutant protein had no such effects in these events (Fig. 4*D–F*). According to these findings, macrophage cells are less responsive to flagellin compared with LPS stimulation, and we therefore conducted our subsequent in vitro studies using LPS as one of the stimulants for the initiation of inflammation in macrophages. We also observed that the optimum degradation and phosphorylation of IκB occurred at 30 min after LPS treatment, and that treatment with the wild-type MFG-E8, but not RGD mutant MFG-E8, considerably reduced the effects of LPS on NF-κB at that time point (Fig. 4, *G* and *H*).

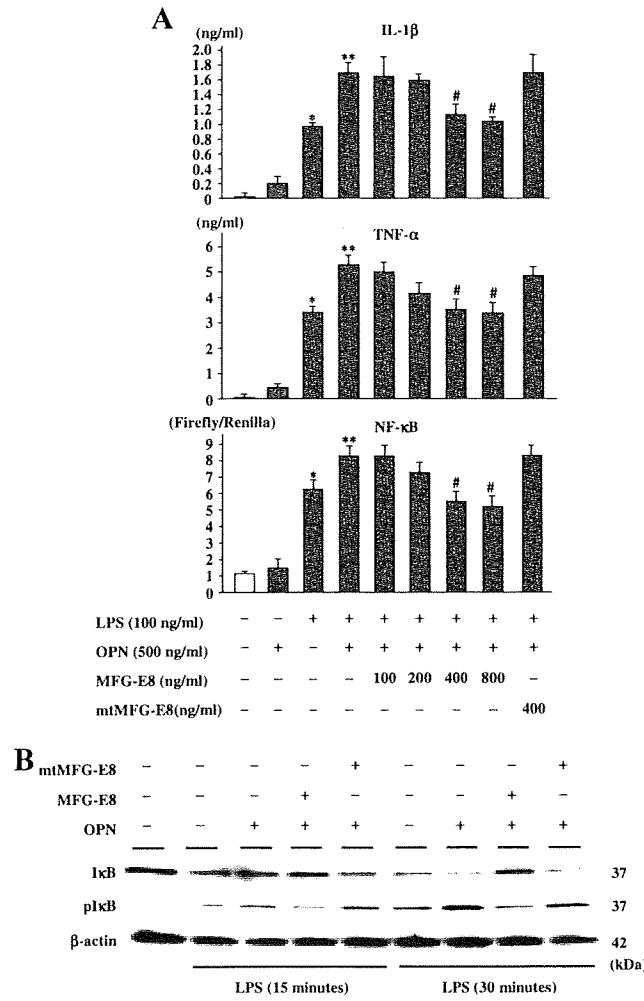


FIGURE 8. Paracrine effects of OPN. *A*, IL-1β and TNF-α in culture supernatants were treated with a fixed amount of recombinant OPN (500 ng/ml) or varying amounts of MFG-E8 as well as LPS (100 ng/ml) for 12 h and then measured using EIA. NF-κB in P388D1 cells was examined using a dual-luciferase assay. Transfected cells were treated with recombinant OPN, as well as MFG-E8 proteins at various concentrations, followed by stimulation with LPS for 12 h, after which dual-luciferase assays were performed. *, $p < 0.05$ and **, $p < 0.01$ vs LPS (−); #, $p < 0.05$ vs LPS (+) OPN. Error bars indicate the SEM values obtained from three independent experiments. *B*, Effects of OPN and MFG-E8 on IκB degradation and phosphorylation in peritoneal macrophages after 15 and 30 min of stimulation with LPS (100 ng/ml). Blots shown represent one of three independent experiments.

Next, to confirm the involvement of $\alpha_v\beta_3$ integrin in MFG-E8 functions, we utilized $\alpha_v\beta_3$ integrin siRNA in P388D1 cells and assessed NF-κB activity in LPS-treated conditions, after validating the siRNA efficiency of corresponding gene knockdown at both the mRNA and protein levels (Fig. 5, *A* and *B*). In P388D1 cells transfected with the control siRNA, MFG-E8 inhibited LPS-induced NF-κB activation, which was similar to the results observed in cells without siRNA transfection. On the other hand, in the presence of both α_v and β_3 integrin siRNAs, MFG-E8 did not show a significant inhibitory effect on NF-κB activation in LPS-treated cells (Fig. 5*C*). These results suggest that the antiinflammatory role of MFG-E8 via inhibition of NF-κB is dependent on $\alpha_v\beta_3$ integrin signaling in P388D1 cells. Based on these observations, we speculated that MFG-E8 may competitively bind to $\alpha_v\beta_3$ integrin with certain potential ligands existing in the culture medium and inhibit $\alpha_v\beta_3$ integrin-mediated NF-κB activation in macrophages.

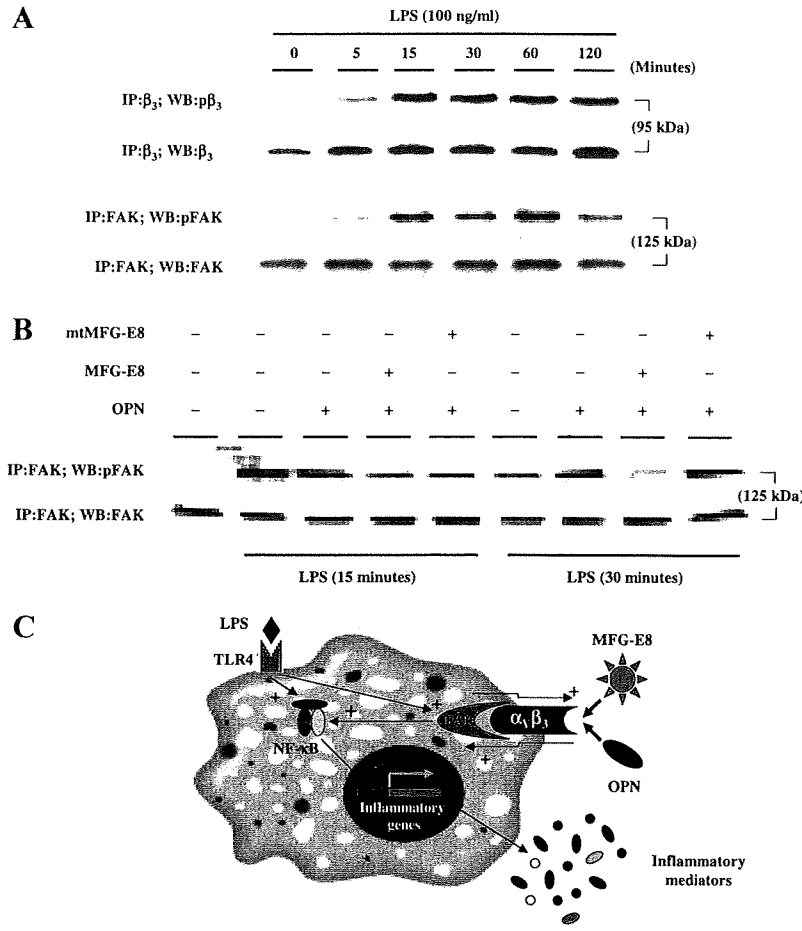


FIGURE 9. Effects of MFG-E8 on focal adhesion kinase in LPS-treated peritoneal macrophages. *A*, Western blotting was performed using cultured peritoneal macrophages treated with LPS (100 ng/ml) for various time points with the anti-pβ_3 integrin (pY759) Ab from β_3 integrin immunoprecipitated samples and anti-pFAK Ab from FAK immunoprecipitated samples. *B*, Western blotting results showing pFAK among total FAK in peritoneal macrophages treated with LPS, OPN, or MFG-E8 proteins for the indicated time periods. Blots shown are representative of three independent experiments. *C*, Proposed model of MFG-E8-mediated antiinflammatory effects in immune-reactive cells. An external stimulus factor, such as LPS, activates inflammatory cascades via TLR-4-mediated pathways by utilizing a variety of signaling events. Although LPS-mediated NF-κB induction is common, it also activates FAK and subsequently $\alpha_v\beta_3$ integrin to generate inside-out signaling for enhanced binding of its potential ligands. OPN, which becomes activated during colitis or in an LPS-treated condition, recognizes activated $\alpha_v\beta_3$ integrin and performs outside-in signaling via FAK phosphorylation. Exogenous MFG-E8 interferes with OPN binding to $\alpha_v\beta_3$ integrin and affects downstream signaling for NF-κB activation by modulating FAK phosphorylation.

MFG-E8 interrupts OPN effects for NF-κB activation during inflammation

Previous studies have shown that several extracellular matrix proteins (ECMs), including OPN, vitronectin (VN), and fibronectin (FN), are potential ligands for $\alpha_v\beta_3$ integrin and regulate a variety of physiological and pathological functions in various organs (26, 27). To explore potential ligands related to the association of $\alpha_v\beta_3$ integrin with the antiinflammatory role of MFG-E8, we initially examined the expression of colonic ECMs during experimental colitis. As shown in Fig. 6*A*, a robust induction of OPN was observed in the early phase of DSS-induced colitis, while other ligands (VN and FN) were increased during the regeneration phase of colitis after the end of DSS administration. Consistent with these in vivo results, we also noticed an increased production of OPN by peritoneal macrophages in an LPS-dependent manner (Fig. 6, *B* and *C*). Recently, several studies have shown that OPN is one of the potential ligands used by $\alpha_v\beta_3$ integrin to participate in innate immune responses during colitis or other autoimmune diseases via the activation of NF-κB (5, 28–31). In consideration of those findings, we focused on OPN function to evaluate the antiinflammatory role of MFG-E8 in macrophages and designed several kinds of in vitro experiments. In this regard, we first confirmed MFG-E8 binding to $\alpha_v\beta_3$ integrin and investigated its relative binding affinity as compared with OPN based on a dose-response curve of varying MFG-E8 amounts with a fixed amount of OPN. As shown in Fig. 7*A*, MFG-E8 exhibited a strong binding affinity to $\alpha_v\beta_3$ integrin with an apparent K_d value of ~3.05 nM. Moreover, we also found that OPN binding to $\alpha_v\beta_3$ become decreased with increased concentrations of recombinant MFG-E8. In contrast, when the relative K_d value was ~4.45 nM, it inhibited OPN by competitive binding to $\alpha_v\beta_3$ integrin (Fig. 7*B*).

To explore the possible role of OPN in production of proinflammatory cytokines by macrophages, we blocked the function of secreted OPN using a neutralizing Ab with LPS-treated cells. Treatment with the neutralizing Ab for OPN significantly decreased the production of IL-1β and TNF-α (Fig. 7, *C* and *D*), indicating that OPN secreted by macrophages induces cytokine production via $\alpha_v\beta_3$ integrin in an autocrine-dependent manner. These results also support our speculation that MFG-E8 may inhibit $\alpha_v\beta_3$ integrin signaling mediated by other ligands (OPN) existing in culture medium treated with LPS. To precisely elucidate the antiinflammatory role of MFG-E8, we used recombinant OPN in various in vitro experiments (Fig. 8). Exogenous stimulation with recombinant OPN induced the production of IL-1β and TNF-α in LPS-treated macrophages, which was clearly related to NF-κB activation and IκB phosphorylation. As expected, treatment with MFG-E8 significantly decreased OPN-induced cytokine production and NF-κB activation in the cells in a dose-dependent manner (Fig. 8*A*), whereas no effect was observed in cells treated with mutant MFG-E8. Taken together, these results clarify the potential role of OPN to induce inflammatory cascades via $\alpha_v\beta_3$ integrin-mediated NF-κB activation, whereas those are interrupted in the presence of MFG-E8.

MFG-E8 modulates FAK via $\alpha_v\beta_3$ integrin

Although our in vitro results indicate that OPN induces proinflammatory cytokine production via $\alpha_v\beta_3$ integrin signaling, its effect was clearly observed only in the LPS-treated conditions. Tyrosine

phosphorylation of the β_3 integrin subunit is an essential event for $\alpha_v\beta_3$ integrin-mediated functions (32, 33), as it facilitates the binding of its potential ligands and subsequently activates FAK to mediate several downstream signaling pathways (34–36). Thus, we examined whether time course changes of LPS stimulation alter the status of β_3 integrin and FAK status in peritoneal macrophages. Following 15–60 min of stimulation with LPS, increased phosphorylation of the β_3 integrin subunit as well as FAK were clearly observed in the cells (Fig. 9*A*), suggesting that LPS-induced activation of the integrin further promotes the binding of its potential ligands to induce integrin-dependent cellular responses. To further investigate whether OPN binding to activated $\alpha_v\beta_3$ integrin generates the phosphorylation of FAK, cells were treated with LPS then exposed to recombinant OPN with or without MFG-E8 proteins, after which the phosphorylation of FAK was examined in total FAK pools. As shown in Fig. 9*B*, OPN stimulation induced abundant phosphorylation of FAK (*lanes 3* and *7*), while that was markedly inhibited by wild-type MFG-E8 (*lanes 4* and *8*), but not by mutant MFG-E8 (*lanes 5* and *9*). These findings indicate the possible role of MFG-E8 to interfere with $\alpha_v\beta_3$ integrin-mediated OPN functions by modulating FAK phosphorylation. Interestingly, phosphorylation of FAK was also observed in LPS-treated cells without OPN stimulation (*lanes 2* and *6*).

Discussion

MFG-E8 was originally identified in the process of phagocytic clearance of apoptotic cells (17), while the present novel findings indicate its antiinflammatory function via the modulation of NF-κB activity during acute colitis. Our results showed a decreased production of MFG-E8 during the initiation of colitis, whereas it was up-regulated during the healing phase to restore homeostasis mechanisms. In the present study, we investigated whether down-regulation of MFG-E8 is dependent on the severity of inflammation. Our results were consistent with those of other studies of atherosclerotic mice characterized by severe inflammation (37), and they indicate a reduced level of suppressive activity by regulatory T cells and enhanced atherosclerotic plaque formation. We also noted that MFG-E8 effectively ameliorates the development of experimental colitis by attenuating inflammation and disease status. Using an in vitro model, we further confirmed that the antiinflammatory effects of MFG-E8 are mediated via $\alpha_v\beta_3$ integrin by modulating the family of protein tyrosine kinases, which finally inhibit NF-κB activation.

Recent reports have revealed the essential role of MFG-E8 to maintain normal tissue homeostasis by clearing apoptotic cells from the body, and preserving the balance between pro- and antiinflammatory cytokines during inflammation (18, 19). Because the pathogenesis of IBD is mainly due to a disorder of intestinal immune homeostasis, a therapeutic role of MFG-E8 without notable side effects is an important finding. Based on our speculation, we treated mice with recombinant MFG-E8 and observed its benefits to control intestinal inflammation, which led us to consider that the antiinflammatory role of recombinant MFG-E8 might be generated via enhanced clearance of apoptotic cells during acute colitis. However, previous studies that used an MFG-E8 knockout murine model reported no increase in accumulated apoptotic cells in small intestinal crypts (22), while apoptotic B cells were found infiltrated only in the germinal centers in spleens of MFG-E8-null mice (19). Those results suggest that the in vivo molecular mechanisms used by MFG-E8 to remove apoptotic cells vary depending on tissue type. Thus, we speculated that MFG-E8-mediated antiinflammatory effects are facilitated not only by removal of apoptotic cells, but there may be some other mechanisms by which recombinant MFG-E8 performs an antiinflammatory role during colitis.

Expression of proinflammatory cytokines is under the control of the potent transcription factor NF-κB and is increased in intestinal lamina propria of patients with IBD (38, 39). Recently, we reported that decoy oligodeoxynucleotides targeting NF-κB attenuate intestinal inflammation in murine experimental colitis, indicating that blockade of NF-κB-mediated signaling is a potent therapeutic strategy for IBD (25). In the present study, lower levels of proinflammatory cytokines in inflamed colonic mucosa were observed in MFG-E8-treated mice, and thus we speculated that MFG-E8 may inhibit intestinal inflammation via modulation of NF-κB-related pathways. To reveal a plausible antiinflammatory mechanism of MFG-E8, we established an in vitro model by utilizing LPS, one of the potent inducers of the inflammatory cascade in immune-reactive cells, and observed that treatment with recombinant MFG-E8 significantly down-regulated LPS-induced proinflammatory cytokines by modulating NF-κB. Once the involvement of NF-κB in this event was confirmed, we investigated how MFG-E8 inhibits NF-κB activation in LPS-dependent conditions. Several reports have shown that RGD mutant MFG-E8 protein decreases phagocytosis of apoptotic cells by macrophages due to the inability of binding to $\alpha_v\beta_3$ integrin (18, 19). In the present study, RGD mutant MFG-E8 protein did not show any inhibitory effects toward NF-κB activation in LPS-treated cells, suggesting that the antiinflammatory role of MFG-E8 may depend on $\alpha_v\beta_3$ integrin-mediated intracellular signaling. To explore this, we investigated the relevance of the $\alpha_v\beta_3$ integrin using an RNA interference technique and noticed that blocking of $\alpha_v\beta_3$ integrin reversed the potential effects of MFG-E8 for inhibiting NF-κB in LPS-treated in vitro conditions. These findings clarify the two major points of our study: first, the involvement of $\alpha_v\beta_3$ integrin to generate MFG-E8 effects; and second, the possibility of the presence of certain ligands that competitively share the same $\alpha_v\beta_3$ integrin with MFG-E8.

OPN is an extracellular matrix phosphoprotein and contains the RGD domain, which is predominantly expressed in macrophages, activated T cells, osteoblasts, and epithelial cells (40) and induces the production of NF-κB-mediated inflammatory cytokines after binding to $\alpha_v\beta_3$ integrin (29). In the present study, we observed increased OPN expression in DSS-induced inflamed colonic tissues, and our in vitro experiments with the neutralizing Abs for OPN and recombinant OPN clearly showed that OPN is a potent mediator to induce proinflammatory cytokines in peritoneal macrophages via NF-κB activation. Recently, Zhong et al. reported that OPN-null mice demonstrated significantly inhibited disease activity of DSS-induced colitis as compared with wild-type mice, as shown by reduced levels of rectal bleeding, weight loss, and histological intestinal injury (41), which supports our speculation that OPN plays an important role in the development of intestinal inflammation. Additionally, the present findings clarified that wild-type MFG-E8 protein but not the RGD mutant significantly inhibited OPN-induced production of proinflammatory cytokines in both autocrine (Fig. 7, *C* and *D*) and paracrine (Fig. 8) models using cultured macrophages. Taken together, these results suggest the possibility that MFG-E8 interferes with OPN binding to $\alpha_v\beta_3$ integrin for downstream signaling toward NF-κB activation.

In the present in vivo and in vitro experiments, the effects of recombinant MFG-E8 were evident only in DSS colitis or LPS-treated conditions, which indicates that an inflammatory environment is required for effective MFG-E8 functioning. Although the essential pathway of TLR-4-mediated signaling for NF-κB activation has been widely recognized (42), there may also be other

surface molecules that play a complementary role in TLR-4-induced events. Integrins are a class of receptors that have been linked to LPS signaling, and a recent study revealed that TLR-4 signaling mediates FAK phosphorylation, with subsequent phosphorylation of integrin leading to increasing binding of its ligands (43). After ligand binding, the activation of integrin also results in recruitment of phosphorylated FAK (pFAK), leading to NF-κB activation (35, 36). Consistent with the above reports, we found that in vitro treatment of macrophages with LPS resulted in increased phosphorylation of FAK and β_3 integrin to generate "inside-out signaling" (TLR-4 signaling $\rightarrow$ pFAK $\rightarrow$ $\alpha_v\beta_3$ integrin activation), and such treatment facilitated enhanced binding of exogenous OPN to generate downstream "outside-in signaling" (OPN binding to activated $\alpha_v\beta_3$ integrin $\rightarrow$ pFAK $\rightarrow$ NF-κB activation) by augmenting FAK phosphorylation. By targeting this pathway, we found that MFG-E8 markedly reduced LPS-induced NF-κB activation by blocking OPN binding and modulated $\alpha_v\beta_3$ integrin-dependent and FAK-mediated downstream signaling (Fig. 9*C*).

Recent studies have reported that high levels of both plasma and tissue OPN were observed in patients with IBD (44, 40, 29), suggesting that OPN, which regulates proinflammatory and T cell-mediated immune responses, may be part of a new therapeutic strategy for the disease. Although our present findings showed a beneficial antiinflammatory role of recombinant MFG-E8 in acute murine model of colitis, there are several points that require further clarification before clinical use. Since IBD refers to a chronic, relapsing form of intestinal disorder, the role of MFG-E8 in chronic models of colitis must be addressed further. Additionally, although we assessed the effects of MFG-E8 with noncolitic healthy mice, it was given for only a short period, and the long-term effects of MFG-E8 in regard to physiological, immunological, and clinical aspects should be evaluated in the future. The present findings also suggest the possibility of a downstream pathway initiated by activation of the family of protein tyrosine kinases, which implicates the need of future studies to determine the nature of the downstream pathway that links the LPS-induced activation of $\alpha_v\beta_3$ integrin and outside-in-mediated signaling for NF-κB during inflammation. Furthermore, LPS-mediated integrin activation leads to induction of other signaling pathways, including the MAPK family. Herein, we evaluated NF-κB as one of the LPS-dependent integrin-mediated signaling events and also focused on other pathways.

In summary, we investigated the antiinflammatory effects of MFG-E8 against experimental colitis in mice and report for the first time that MFG-E8 attenuated intestinal inflammation, indicating the potential of targeting NF-κB-mediated proinflammatory cytokines in the development of new therapies for IBD.

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Disclosures

The authors have no financial conflicts of interest.

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Expression of IL-24, an Activator of the JAK1/STAT3/SOCS3 Cascade, Is Enhanced in Inflammatory Bowel Disease

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IL-24 is a member of the IL-10 family of cytokines. In this study, we investigated IL-24 expression in the inflamed mucosa of patients with inflammatory bowel disease (IBD), and characterized the molecular mechanisms responsible for IL-24 expression in human colonic subepithelial myofibroblasts (SEMFs). IL-24 expression in the IBD mucosa was evaluated by immunohistochemical methods. IL-24 mRNA and protein expression was determined by real-time PCR and ELISA, respectively. AP-1 and C/EBP DNA-binding activity and IL-24 promoter activity were assessed by EMSA analysis and a reporter gene assay, respectively. IL-24 mRNA expression was significantly elevated in active lesions from patients who have ulcerative colitis and Crohn's disease. Colonic SEMFs were identified as a major source of IL-24 in the mucosa. IL-1β, but not IL-17A, TNF-α, or IFN-γ, significantly enhanced IL-24 mRNA and protein expression in isolated colonic SEMFs. The IL-1β-induced IL-24 mRNA expression was mediated by the activation of the transcription factors, AP-1 and C/EBP-β. Induction of IL-24 mRNA stabilization was also involved in the effects of IL-1β. IL-24 induced JAK1/STAT-3 phosphorylation and SOCS3 expression in HT-29 colonic epithelial cells. IL-24 did not modulate the proliferation of HT-29 cells, but significantly increased the mRNA expression of membrane-bound mucins (MUC1, MUC3, and MUC4). IL-24 derived from colonic SEMFs acts on colonic epithelial cells to elicit JAK1/STAT-3 activation and the expression of SOCS3 and mucins, supporting their suppressive effects on mucosal inflammation in IBD. ***The Journal of Immunology,*** **2009, 183: 687–695.**

Inflammatory bowel disease (IBD),[3] ulcerative colitis (UC), and Crohn's disease (CD) are characterized by chronic inflammation, in which a dysfunction of the host immune response against common Ags such as dietary factors or bacteria may be involved (1, 2).

IL-24, a member of the IL-10 family of cytokines (together with IL-10, IL-19, IL-20, IL-22, IL-26, IL-28, and IL-29), was discovered by the subtraction hybridization of cDNA libraries prepared from melanoma cells treated with IFN-β and a protein kinase C inhibitor (mezerein) (3, 4). It was originally termed melanoma differentiation-associated protein 7 (3), and was renamed IL-24 (5). The human *il24* gene is located in chromosome 1, within a 195-kb cytokine cluster containing the IL-10, IL-19, IL-20, and IL-24 genes (6). IL-24 shares a 20–30% amino acid homology with IL-10, IL-20, and IL-22 and interacts with two different heterodimeric receptor complexes, IL-20R1/IL-20R2 and IL-22R1/IL-20R2 (7–9). Binding to both receptors leads to the activation of STAT-3, similar to other members of the IL-10 family of cytokines (7–10). Immune cells do not express IL-24 receptors (11), suggesting that IL-24 cannot stimulate the acquired immune response (12). In contrast, the restricted expression of the IL-24 receptor components IL-20R1 and IL-22R1 in nonimmune tissues suggests the innate immune response as the selective target of IL-24 (8, 11).

Under the appropriate stimulation, IL-24 expression has been identified in certain cell types (11, 13), such as cultured melanocytes (3), dermal keratinocytes (12), LPS-stimulated monocytes (13), and Th2-polarized T cells (13). Treatment with IFN-β plus mezerein induced a transient expression of IL-24 mRNA in some cancer cell lines (6). However, the precise cytokine regulation and molecular mechanisms of IL-24 induction still remain unclear.

IL-24 can function either as an intracellular cell death-inducing factor to cancer cells, or as a classical cytokine through its cell surface receptors. With respect to its anticancer actions, adenoviral vector-mediated expression of IL-24 selectively and efficiently induces cell death in a vast variety of cancer cells, especially melanoma cells, independent of receptor expression and JAK/STAT signaling (14–18). In contrast, through receptor binding IL-24 has been reported to induce the expression of proinflammatory cytokines from monocytes (5). In vivo, IL-24 is predominantly expressed by skin tissue cells during inflammatory conditions, such as psoriasis (12). IL-24 gene expression was also greatly increased at the edge of excisional skin wounds (19). Thus, the available data on IL-24 suggest a role as a cytokine during inflammation and tissue repair.

In this study, we investigated IL-24 expression in the inflamed mucosa of IBD patients. Furthermore, to characterize the molecular mechanisms responsible for IL-24 expression in the colonic mucosa, we analyzed IL-24 expression in nontransformed human colonic subepithelial myofibroblasts (SEMFs).

Materials and Methods

Reagents

Recombinant human cytokines were purchased from R&D Systems. Inhibitors of p42/44 MAPKs (PD98059 and U0216), and an inhibitor for p38 MAPK (SB203580) were purchased from Cell Signaling Technology. An inhibitor of JNK was purchased from Calbiochem. C/EBP-specific,

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[3] Abbreviations used in this paper: IBD, inflammatory bowel disease; SEMF, subepithelial myofibroblast; UC, ulcerative colitis; CD, Crohn's disease; siRNA, small interfering RNA; SMA, smooth muscle actin; SOCS, suppressors of cytokine signaling.

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Table I. *Oligonucleotides used to determine IL-24 expression in IBD*

Gene Name	Primers	Ref.
IL-24	sense 5′-GACTTTAGCCAGCAGACCCTT-3′	3
	antisense 5′-GGTTGCAGTTGTGACACGAT-3′	
IL-22R1	sense 5′-CTGTCCGAGATCACCTACTTAGG-3′	29
	antisense 5′-GCACATTTGGGTCAGATGTTCTGTC-3′	
IL-20R1	sense 5′-GCTCAGCCTTCTGAGAAGCAGTG-3′	9
	antisense 5′-CGCACAAATGTCAGTGGTTCTGAC-3′	
IL-20R2	sense 5′-GCTGGTGCTCACTCACTGAAGGT-3′	9
	antisense 5′-TCTGTCTGGCTGAAGGCGCTGTA-3′	
MUC1	sense 5′-AGTTCAGGCCAGGATCTGTG-3′	30
	antisense 5′-CAGCTGCCCGTAGTTCTTTC-3′	
MUC2	sense 5′-GAACTACGCTCCTGGCTTTG-3′	31
	antisense 5′-CCTGGCACTTGGAGGAATAA-3′	
MUC3	sense 5′-TGTCAGCTCCAGACCAGATG-3′	32
	antisense 5′-CCTGCTCATACTCGCTCTCC-3′	
MUC4	sense 5′-CCTCAGGAGAGACGACAAGG-3′	33
	antisense 5′-CAGAGTGTGGGTCTGGGTTT-3′	

SOCS3-specific, and control small interfering RNA (siRNA) were purchased from Santa Cruz Biotechnology.

Abs analysis

Goat anti-human IL-24 Abs (R&D Systems), mouse anti-α-smooth muscle actin (SMA) Abs (Sigma-Aldrich), goat anti-JAK1 and anti-JAK2 Abs, anti-MAPK, and anti-STAT-3 Abs (Cell Signaling Technology), goat anti-human c-Jun, c-Fos, and C/EBP-β Abs (Santa Cruz Biotechnology), rabbit anti-human IL-22R1 and IL-20R1 Abs (Abcam) were purchased from commercial suppliers.

Tissue samples

Diagnosis for IBD was based on conventional clinical and endoscopic criteria. Surgically rejected or biopsy specimens from 24 patients who have UC and 21 patients who have CD were used with informed consent. The ethics committee of Shiga University of Medical Science approved this project.

During sample collection, all patients were clinically and endoscopically active with colitis activity index for UC (20) and CD activity index (21). Five patients who have UC and six patients who have CD received surgical operation due to resistance to medication or due to other complications (e.g., massive bleeding, fistula formation, or perforation). Histological examinations were performed in macroscopically and microscopically non-affected (n = 13 in UC; and n = 11 in CD) or affected (n = 11; and n = 10 in CD) areas from each patient. All patients were treated with salicylates, and 12/24 UC and 10/21 CD patients received treatment with corticosteroids. Seven patients with UC and seven patients with CD were treated with azathioprine. Biopsy samples derived from infectious and ischemic colitis (n = 7) were obtained by colonoscopy. Normal colorectal tissues were obtained by the surgical resection of colon cancer at distal tumor sites (n = 7 samples).

Immunohistochemistry

Immunohistochemical analyses were performed according to the method described in our previous report (22). Images were then obtained with a digital confocal laser scanning system MRC-600 (Bio-Rad).

Culture of human colonic SEMFs and colonic epithelial cell lines

Primary colonic SEMF cultures were prepared according to a method reported by Mahida et al. (23). The cellular characteristics and culture conditions have also been described in our previous report (24). The human colon cancer cell lines HT-29 (25), Caco-2 (26), and SW480 (27) were obtained from the American Type Culture Collection.

Quantification of human IL-24

Antigenic IL-24 in all samples was quantified by sandwich ELISA kits purchased from R&D Systems.

RT-PCR and real-time PCR

The expression of mRNA in the samples was assessed by RT-PCR and real-time PCR analyses. RT-PCR was performed according to the methods described in our previous report (28). The oligonucleotide primers used in this study are shown in Table I (3, 9, 29–33). Real-time PCR was performed using a LightCycler 2.0 system (Roche Applied Science). The PCR was conducted using a SYBR Green PCR Master Mix (Applied Biosystems). The data were normalized vs β-actin for human IL-24.

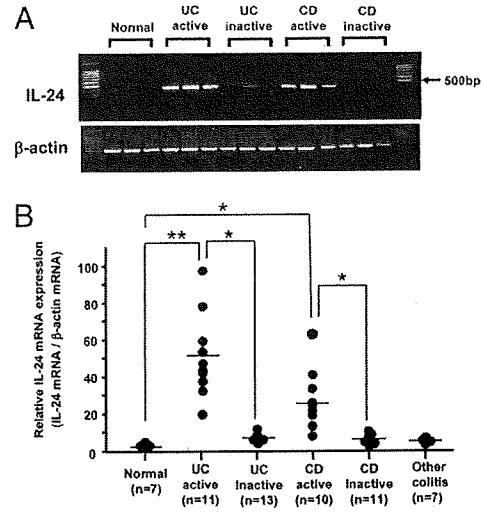

FIGURE 1. IL-24 mRNA expression in the colon. Total RNA was extracted from biopsy or surgical samples, and IL-24 mRNA expression was evaluated by either RT-PCR (*A*) or real-time PCR (*B*) analyses. The data from the real-time PCR were normalized vs β-actin for human IL-24. Data are expressed as mean ± SD (number of samples (n) = 5). *, $p < 0.05$; **, $p < 0.01$.

Nuclear extracts and EMSA analysis

Nuclear extracts were prepared from cells exposed to the cytokines for 1.5 h by the method of Dignam et al. (34). The consensus oligonucleotides for AP-1 (5′-CGCTTGATGAGTCAGCCGGAA) and C/EBP (5′-TGCAGATTGCGCAATCTGCA) were purchased from Promega and Santa Cruz Biotechnology, respectively. The oligonucleotides were 5′ end-labeled with T4 polynucleotide kinase (Promega) and [γ-^{32}P]ATP (Amersham Biosciences). The binding reactions were performed according to previously described methods (35).

IL-24 promoter luciferase reporter constructs and cell transfection

Three different regions of the human IL-24 promoter extending to −1280 bp upstream of the transcription start site were amplified by PCR using human genomic DNA as a template. This region contains consensus binding sites for AP-1 (at bp −1023, −714, and −109) and C/EBP (at bp −1132, −1032 and −114) (36). The following primers were used: IL-24 (−1280 bp) TATGGTACCAGTCACAACTACT CATCT; IL-24 (−784 bp) CATGGTACCATC- TAGAGCTGAGT GCCT; IL-24 (−550 bp) TCAGGTACCACTCCTCAA- CTTCCTG GCCC; and IL-24 (+31 bp) AAGTCTAGACAGAAGTAAAGGTTTGCA (GenBank accession no. NM_006850.2; http://www.ncbi.nlm.nih.gov/sites/entrez?Db=gene&Cmd=ShowDetailView&TermToSearch=11009). The 5′ sequence of each primer was modified for a *Kpn*I restriction site (underlined), and the 5′ region of IL-24 (+31) was modified for a *Bgl*II restriction site (underlined), respectively. These were ligated into *Kpn*I-*Bgl*II sites of the luciferase reporter plasmids pGL3-Basic (Promega) and termed plasmids IL-24(−1280), IL-24(−784), and IL-24(−550). Transient transfections were performed using Lipofectamine Plus reagent (Life Technologies) according to the manufacturer's protocol. The detailed procedures were described in our previous report (37).

Western blot analyses

The stimulated cells were lysed in SDS sample buffer containing orthovanadate. Western blots were then performed according to a method previously described (35). The detection was performed using the ECL Western blotting system (Amersham Biosciences).

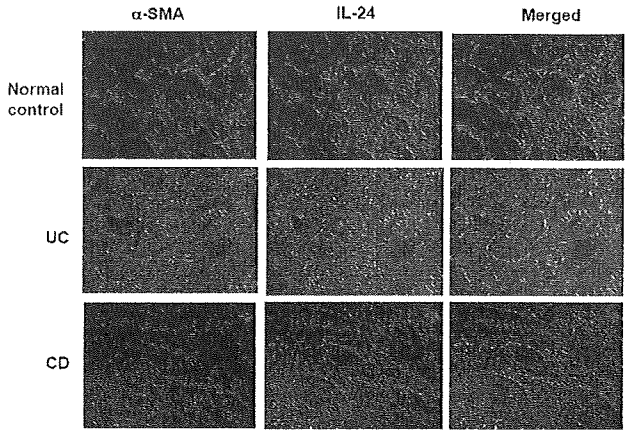

FIGURE 2. Immunohistochemical analyses of IL-24 protein expression in the normal and active IBD mucosa. Dual-colored immunofluorescence was used to localize α-SMA (Cy3-positive, red fluorescence) and IL-24 (Cy2-positive, green fluorescence). Double positive immunostaining can be seen as yellow fluorescence in the merged images.

Adenovirus-mediated gene transfers

We used a recombinant adenovirus expressing a dominant negative mutant of c-Jun (Ad-DN-c-Jun), and a recombinant adenovirus containing bacterial β-galactosidase cDNA (Ad-LacZ). The dominant negative mutant c-Jun (TAM67) lacks the transactivational domain of aa 3–122 of the wild-type c-Jun (38), but retains the DNA binding domain. The detailed procedures were described in our previous report (39).

C/EBPβ mRNA interference experiments

The siRNA for human C/EBPβ and a control siRNA were used. Human colonic SEMFs were cultured in complete medium that did not contain antibiotics for 4 days. The cells were then seeded onto a 6-well plate 1 day before the transfection, and cultured to 60–70% confluence on the following day. For the RNA interference experiments, Lipofectamine LTX and Lipofectamine PLUS Reagent (Invitrogen) were used.

[^{3}H]Thymidine incorporation

Subconfluent SEMF cultures (70–90%) were grown in 24-well plates, washed, and incubated in DMEM containing 0.2% FBS for 24 h to induce growth arrest. Agonists were added for 24 h. [^{3}H]Thymidine (1 μCi/well) was added for the final 12 h of the incubation, as described in our previous report (40).

Statistical analysis

The statistical significance difference was determined by the Mann-Whitney *U* test (Statview version 4.5). Differences resulting in p values less than 0.05 were considered to be statistically significant.

Results

IL-24 expression in IBD mucosa

To evaluate the expression of IL-24 mRNA in the mucosa, IL-24 mRNA expression was analyzed by RT-PCR and real-time PCR in the IBD mucosa. As shown in Fig. 1*A*, IL-24 mRNA expression was clearly detected in the samples from the active lesions of patients who have UC and CD. Similarly, real-time PCR analysis revealed a significant increase in IL-24 mRNA expression in samples from the active lesions of patients who have UC and CD (Fig. 1*B*), as compared with samples from the normal and inactive IBD mucosa. IL-24 mRNA expression was not detected in samples from infectious and ischemic colitis.

To characterize the cellular origin of IL-24 in the inflamed mucosa, the samples were double immunostained with anti-α-SMA, a marker for myofibroblasts, and anti-human IL-24 Abs (Fig. 2). In the normal mucosa, α-SMA (Fig. 2, red fluorescence) was clearly immunostained in the subepithelial regions, but IL-24 was not detected. In contrast, IL-24 protein was clearly detected, mainly in the subepithelial regions in the active mucosa of patients who have UC and CD (Fig. 2, green

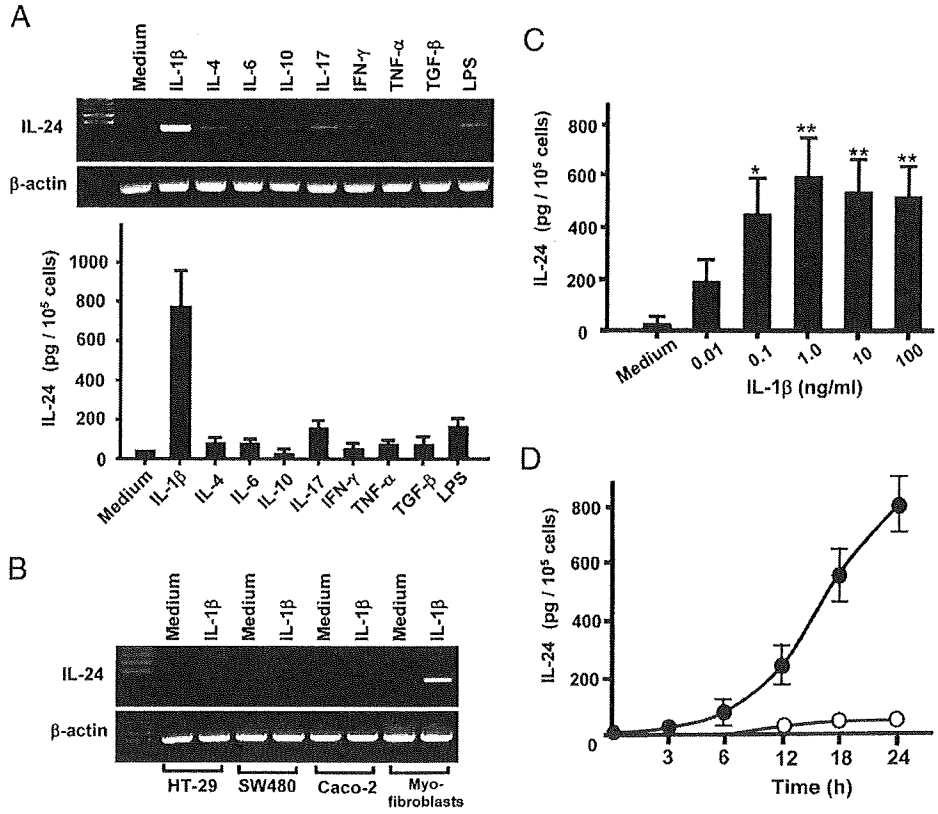

FIGURE 3. IL-24 expression in human colonic SEMFs. *A*, IL-24 mRNA expression in colonic SEMFs (*top blot*). The cells were stimulated with cytokines (100 ng/ml) for 12 h, and then the IL-24 mRNA expression was analyzed by RT-PCR. IL-24 protein secretion in colonic SEMFs. The cells were stimulated with cytokines (100 ng/ml) for 24 h, and then the IL-24 mRNA expression was analyzed by ELISA (*bottom*). *B*, IL-24 mRNA expression in colonic epithelial cell lines (HT-29, SW480, and Caco-2). The cells were stimulated with cytokines (100 ng/ml) for 12 h, and then the IL-24 mRNA expression was analyzed by RT-PCR. *C*, Dose-dependent effects of IL-1β on IL-24 secretion. Colonic SEMFs were incubated for 24 h with increasing concentrations of IL-1β. The levels of IL-24 secreted were then determined by ELISA. Data are expressed as mean ± SD (number of samples (n) = 5). *, $p < 0.05$; **, $p < 0.01$, for significant difference from the values for medium alone. *D*, Kinetics of IL-24 secretion. Colonic SEMFs were stimulated with IL-1β (10 ng/ml) for the predetermined times, and then the IL-24 levels were determined by ELISA. Data are expressed as mean ± SD (n = 5).

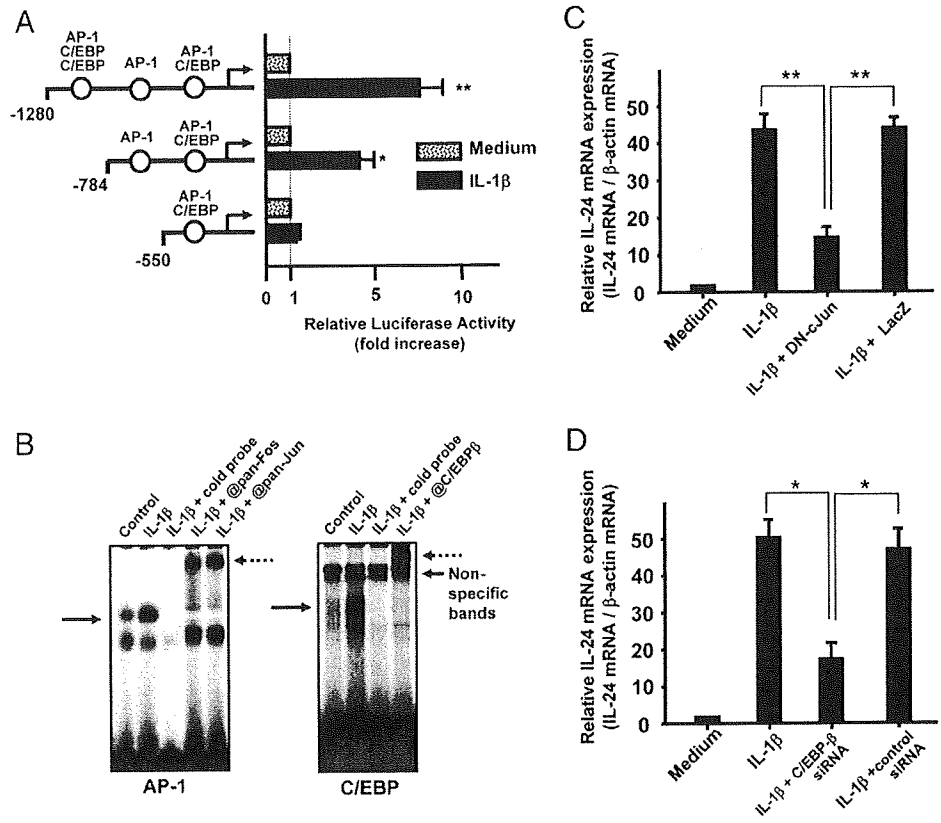

FIGURE 4. Effects of IL-1β on IL-24 promoter activities. Human IL-24 promoter DNA and β-galactosidase reporter vectors were cotransfected to the colonic SEMFs, and were incubated for 24 h. Next, the cells were incubated with IL-1β (10 ng/ml) for 6 h. *A*, The luciferase activities were measured by the Luciferase Assay System kit (Promega), and were expressed as relative activities normalized to β-galactosidase activity. Data are expressed as mean $\pm$ SD (number of samples (n) = 5). *, $p < 0.05$; **, $p < 0.01$, a significant difference from the values for medium alone. *B*, EMSA analysis for AP-1 and C/EBP DNA-binding activity. Colonic SEMFs were incubated with medium alone or IL-1β (10 ng/ml) for 1.5 h, and then nuclear extracts were prepared. Specific DNA-protein bound complexes (dashed arrow), and nonspecific binding (dot arrow) are shown. *C*, Effects of a recombinant adenovirus expressing a dominant negative mutant of c-Jun (Ad-DN-c-Jun) and β-galactosidase cDNA (Ad-LacZ). Forty-eight hours after infection with the adenovirus, colonic SEMFs were stimulated with IL-1β (10 ng/ml) for 12 h. The IL-24 mRNA expression was then determined by real-time PCR. The data were normalized vs β-actin for human IL-24. Data are expressed as mean $\pm$ SD (n = 5). **, $p < 0.01$. *D*, Effects of C/EBP-β-specific siRNA. Colonic SEMFs were transfected with C/EBP-β siRNA or control siRNA, and then stimulated by IL-1β (10 ng/ml) for 12 h. The IL-24 mRNA expression was then determined by real-time PCR. The data were normalized vs β-actin for human IL-24. Data are expressed as mean $\pm$ SD (n = 5). *, $p < 0.05$.

fluorescence). The α-SMA/IL-24 double immunopositive cells were detected (Fig. 2, yellow), and the IL-24 immunopositive cells coincided with part of the α-SMA immunopositive cells (Fig. 2, *right column*). These observations indicated that α-SMA immunopositive SEMFs are a major source of IL-24 in the inflamed mucosa of IBD patients.

Regulation of IL-24 expression in colonic SEMFs

Based on the in vivo expression of IL-24 in the inflamed IBD mucosa, we examined IL-24 expression in isolated human colonic SEMFs. Colonic SEMFs were stimulated with various cytokines and LPS for 12 h, and the IL-24 mRNA expression was determined by real-time PCR (Fig. 3*A*, *top blot*). Very weak IL-24 mRNA expression was detected in unstimulated colonic SEMFs, and IL-1β stimulation significantly enhanced this IL-24 mRNA expression. IL-17 or LPS weakly enhanced IL-24 mRNA expression, but these stimulations were much weaker than those induced by IL-1β. Similar effects on IL-24 protein secretion were also observed (Fig. 3*A*, *bottom*). In contrast, in the colonic epithelial cell lines (HT-29, SW480, and Caco-2), IL-1β failed to induce IL-24 mRNA expression (Fig. 3*B*).

These effects were confirmed at the protein level. Colonic SEMFs were stimulated with various concentrations of IL-1β for 24 h, and then IL-24 secretion was determined by ELISA. As shown in Fig. 3*C*, IL-1β dose-dependently induced IL-24 secretion. Furthermore, the colonic SEMFs were incubated with IL-1β (10 ng/ml), and then IL-24 protein secretion was sequentially analyzed by ELISA. This IL-24 secretion was observed in a time-dependent manner (Fig. 3*D*).

IL-1β induces IL-24 promoter activity

Multiple recognition sites for the AP-1 and C/EBP transcription factors are present in the promoter region of the *il24* gene (36). The IL-24(−1280) plasmid contains three AP-1 and three C/EBP sites, and IL-24(−784) plasmid has two AP-1 sites and one C/EBP site. IL-24(−550) contains one AP-1 and one C/EBP site. As shown in Fig. 4*A*, IL-1β significantly increased the relative luciferase activity in SEMFs transfected with the IL-24(−1280) and IL-24(−784) plasmids, but the effects of IL-1β were not detected in cells transfected with the IL-24(−550) plasmid. These results suggest a major role for AP-1 and C/EBP binding between bp −550 to 1280 for the effects of IL-1β.

Next, we performed EMSA to determine whether IL-1β stimulation actually results in the formation of active AP-1 DNA or C/EBP DNA binding complexes. As shown in Fig. 4*B*, stimulation with IL-1β (10 ng/ml) induced the formation of AP-1 DNA and

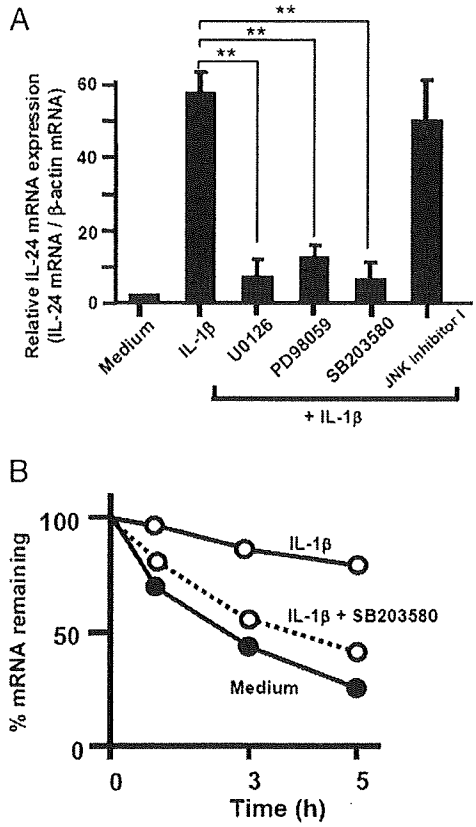

FIGURE 5. Effects of MAPK inhibitors on IL-24 mRNA expression in colonic SEMFs. The cells were pretreated with 10 μM MAPK inhibitors (SB203580, PD098059, or U02016) and 3 μM JNK inhibitor (JNK Inhibitor I) for 15 min, and then stimulated with IL-1β (10 ng/ml) for 12 h. *A*, The IL-24 mRNA expression was then determined by real-time PCR. Data are expressed as mean ± SD (number of samples (n) = 5). **, $p < 0.01$, a significant difference from the values for IL-1β stimulation. *B*, Stability studies on IL-24 mRNA induced by IL-1β. Colonic SEMFs were stimulated with or without I IL-1β (10 ng/ml) for 12 h, washed, and further incubated with actinomycin D (5 μM) for various time periods. The total RNA was sequentially extracted, and the IL-24 mRNA expression was then determined by real-time PCR. To assess the role of p38 MAPK activation, the cells were treated with SB203580 (10 μM) for 15 min before the addition of actinomycin D. The IL-24 mRNA expression was expressed as the percentage mRNA remaining relative to the corresponding levels before the addition of actinomycin D.

C/EBP DNA binding complexes, and these bindings were blocked by cold probe or supershifted by Abs against pan-Fos, pan-Jun, and C/EBP-β.

Furthermore, we evaluated the effects of a recombinant adenovirus expressing a dominant negative mutant of c-Jun (Ad-DN-c-Jun) and C/EBP-β-specific siRNA. As shown in Fig. 4C, Ad-DN-c-Jun significantly decreased the IL-1β-induced IL-24 mRNA expression. Similarly, in the C/EBP-β siRNA-transfected cells, IL-24 expression induced by IL-1β was significantly reduced (Fig. 4D). These findings indicate that the IL-1β-induced IL-24 mRNA expression actually mediated by AP-1 and C/EBP-β activation.

Role of MAPK activation in IL-24 induction

Previously, we demonstrated that IL-1β induces MAPK activation in colonic SEMFs (41). To investigate the role of MAPKs in the IL-1β-induced IL-24 mRNA expression in SEMFs, we evaluated the effects of p42/44 MAPK inhibitors (PD98059 and U0216) (42, 43), a p38 MAPK inhibitor (SB203580) (44), and a JNK inhibitor (JNK Inhibitor I) (45). As shown in Fig. 5*A*, p42/44 and p38 MAPK inhibitors significantly reduced the IL-β-induced IL-24 mRNA expression, but JNK inhibitor had no effect.

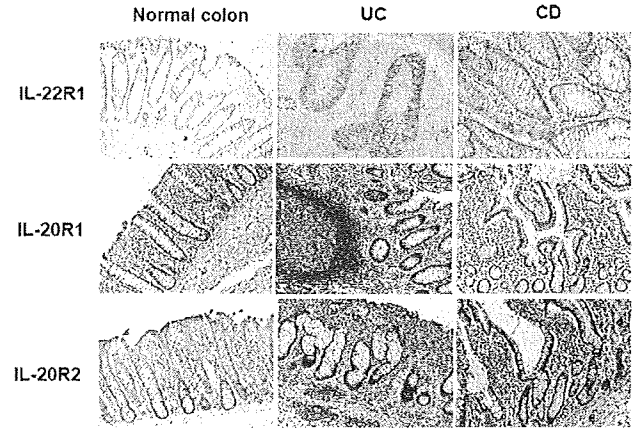

FIGURE 6. Immunohistochemical analyses of IL-22R1, IL-20R1, and IL-20R2 expression in normal and active IBD mucosa.

Effects of IL-1β on IL-24 mRNA stability

Next, we evaluated the possibility that the IL-1β-induced IL-24 mRNA expression was dependent on increased mRNA stability. Colonic SEMFs were stimulated for 12 h in medium alone or with IL-1β, and then treated with actinomycin D for various time periods to block further RNA transcription (Fig. 5*B*). In those cells treated with medium alone, IL-24 mRNA expression decreased by 70% at 5 h after the addition of actinomycin D. Treatment with IL-β actually prolonged the rate of DAF mRNA degradation; 80% of the IL-24 mRNA still remained after 5 h. SB203580, a p38 MAPK inhibitor, abolished these effects of IL-1β. These findings suggest that some of the effects of IL-1β may be mediated by an induction of IL-24 mRNA stabilization via p38 MAPK activation.

IL-24 receptor expression in colonic epithelial cell lines and SEMFs

IL-24 signaling has been reported as being mediated through both IL-20R1/IL-20R2 and IL-22R1/IL-20R2 (7–9), and expression of IL-20R1 and IL-22R1 is restricted in nonimmune cells. However, in vivo expression in human colonic mucosa remains unclear. As shown in Fig. 6, immunohistochemical studies showed an expression of IL-22R1, IL-20R1, and IL-20R2 in epithelial cells from normal colonic mucosa. Epithelial expression of IL-22R1, IL-20R1, and IL-20R2 was also detected in IBD mucosa. Furthermore, IL-20R1, IL-22R1, and IL-20R2 mRNA was detected in the three colonic epithelial cell lines and in colonic SEMFs (Fig. 7*A*).

IL-24 induces JAK/STAT and MAPK activation in colonic epithelial cell line

To define the biological activities of SEMF-derived IL-24 in the inflamed mucosa, we investigated how IL-24 modulates the functions of colonic epithelial cell lines. First, we evaluated the effects of IL-24 on the activation of JAK1/2, STAT 1/3, p42/44 (ERK1/2), p38, and JNK in HT-29 cells. As shown in Fig. 7, IL-24 induced the phosphorylation of JAK-1 and STAT-3 as early as 5 min after stimulation. However, we could not detect any STAT-1 phosphorylation (Fig. 6*B*) or JAK-2 activation (data not shown). Thus, JAK1/STAT-3 activation is a major pathway for IL-24 signaling in HT-29 cells. Like JAK1/STAT-3 phosphorylation, IL-24 also induced the phosphorylation of p42/p44, p38 MAPKs, and JNK in HT-29 cells (Fig. 7).

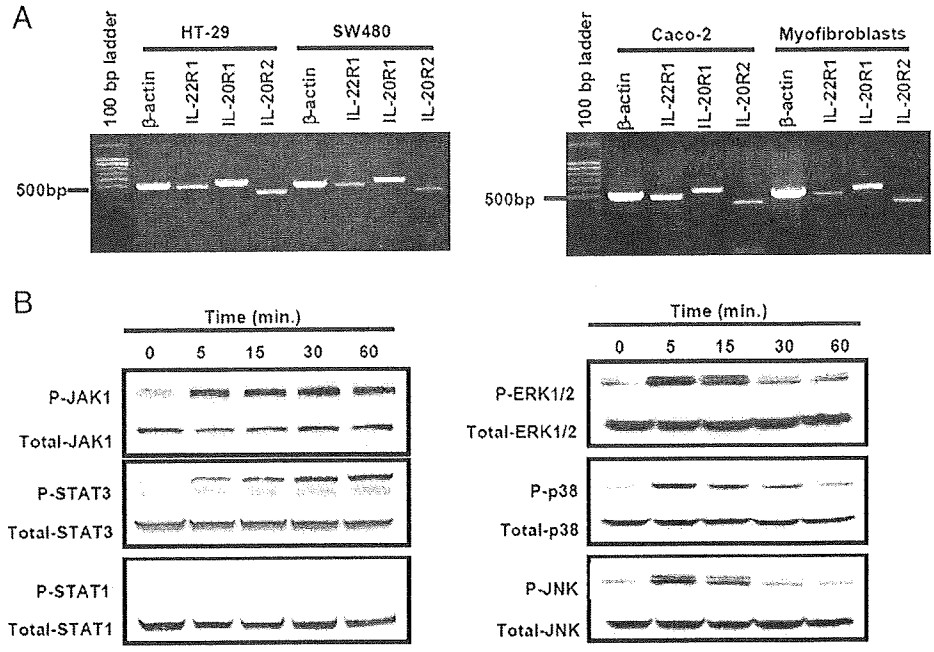

FIGURE 7. mRNA in the three colonic epithelial cell lines and in colonic SEMF. *A*, RT-PCR analyses for the mRNA expressions of IL-20R1, IL-20R2, and IL-22R1 in colonic epithelial cells (HT-29, SW480, and Caco-2) and SEMFs. *B*, IL-24 induced JAK/STAT and MAPK activation in HT-29 colonic epithelial cells. The cells were stimulated with IL-24 (100 ng/ml), and the activation of JAK/STAT and MAPK were then evaluated by Western blotting. Abs directed against phosphorylated (P) and total JAK/STAT and MAPKs were used.

Effects of IL-24 on proliferation of colonic epithelial cells

Based on finding of IL-24 receptor expression in our system, we investigated the effects of IL-24 on proliferation of colonic epithelial cell lines. As shown in Fig. 8*A*, IL-24 had no effects on [^{3}H]thymidine incorporation in HT-29 cells. Similar results were also observed in Caco-2 and SW480 cells (data not shown).

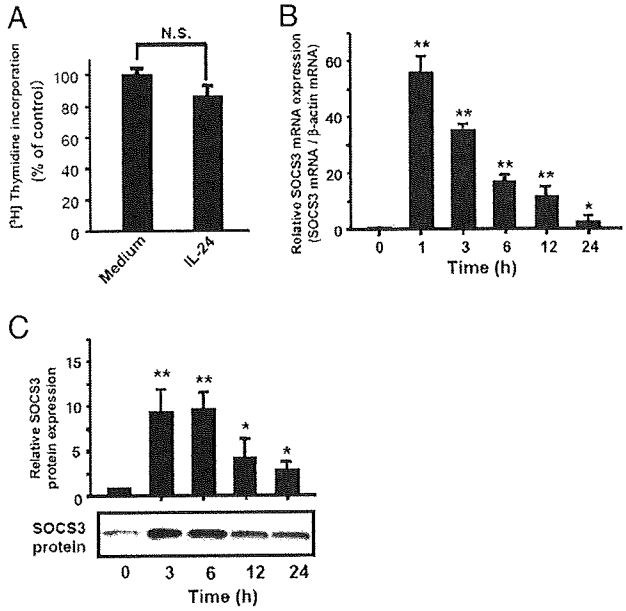

FIGURE 8. Effects of IL-24 on cell proliferation and SOCS3 expression. *A*, Effects of IL-24 on [^{3}H]thymidine incorporation in HT-29 cells. Cells were stimulated with IL-24 (100 ng/ml) for 24 h, and the [^{3}H]thymidine incorporation was determined. Data are expressed as mean ± SD (number of samples (n) = 5). *B*, IL-24-induced SOCS3 expression in HT-29 cells. The cells were stimulated with IL-24 (100 ng/ml), and the SOCS3 mRNA expression was sequentially determined by real-time PCR. Data are expressed as mean ± SD (n = 5). *C*, IL-24-induced SOCS3 protein secretion in HT-29 cells. The cells were stimulated with IL-24 (100 ng/ml) for 24 h, and the SOCS3 protein secretion was determined by Western blotting and image analyzer. Data are expressed as mean ± SD (n = 5). *, $p < 0.05$; **, $p < 0.01$, a significant difference from values for culture start.

Effects of IL-24 on SOCS3 expression in HT-29 cells

It has not been reported whether IL-24 can induce SOCS3 in any cell types. As shown in Fig. 8, *B* and *C*, in HT-29 cells IL-24 rapidly induced SOCS3 mRNA expression, followed by SOCS3 protein expression. In contrast, we could not detect any mRNA expression of proinflammatory cytokines such as IL-8 and IL-6 (data not shown), although IL-24 has been reported to induce IL-6 and TNF-α in monocytes (5).

IL-24 stimulates mucin gene expression in colonic epithelial cells

Recently, IL-22 has been reported to stimulate mucin synthesis (46). To look for similar functions, we evaluated the effects of IL-24 on mucin gene expression in HT-29 cells. IL-24 (100 ng/ml) induced significant increase in MUC1 (8.1-fold), MUC3 (4.5-fold), and MUC4 (6.8-fold) mRNA expression (Fig. 9*A*). These were sufficient effects, but weaker than those induced by IL-22 (Fig. 9*B*). To clarify the role of STAT-3 activation, we used STAT-3 specific siRNA. As shown in Fig. 9*C*, STAT-3-specific siRNA effectively reduced total and phosphorylated STAT-3 expression in HT-29 cells. Stimulation with IL-24 induced a significant increase in the mRNA expression of MUC1, MUC3, and MUC4 in HT-29 cells (Fig. 9*D*), and this increased expression was significantly abolished by STAT-3-specific siRNA. Similar observations were detected in SW480 cells (data not shown).

Discussion

Recent studies have focused on the functions of IL-24 as an intracellular cytotoxic factor against cancer cells (15–17). In particular, when IL-24 is administered via an adenoviral vector, it selectively and efficiently kills cancer cells derived from various tissues (14, 16, 17, 47, 48). In contrast, the biological functions as a cytokine still remain unclear. In this report, we demonstrated some novel findings concerning the expression and function of IL-24: 1) IL-24 expression is increased in the inflamed mucosa from IBD patients, and colonic SEMFs are a major local source; 2)

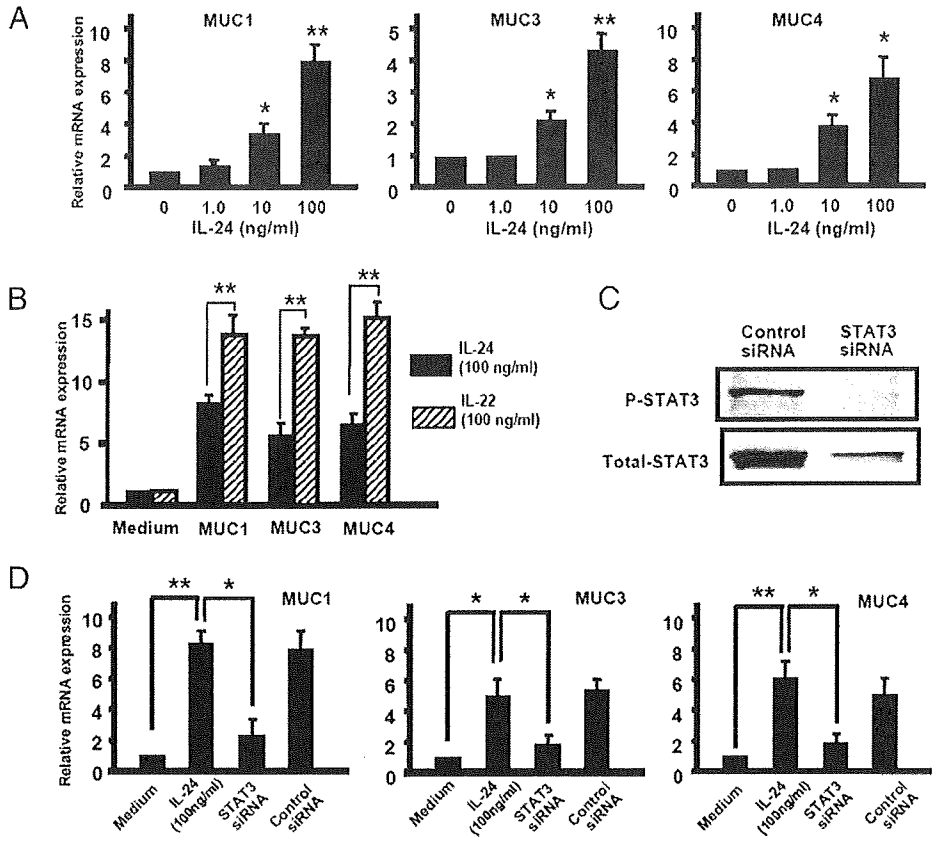

FIGURE 9. IL-24 induces mucin gene expression in colonic epithelial cells through STAT-3-mediated pathways. *A*, HT-29 cells were stimulated with increasing concentrations of IL-24 for 48 h, and the mucin gene expression was then determined by real-time PCR. *B*, Comparison of the effects of IL-24 and IL-22. HT-29 cells were stimulated with 100 ng/ml of IL-24 or IL-22 for 48 h, and the mucin gene expression was evaluated by real-time PCR. *C*, STAT-3 siRNA effectively reduced total and phosphorylated STAT-3 protein expression in IL-24-stimulated HT-29 cells. The cells were transfected with STAT-3 siRNA or control siRNA, and after 24 h they were stimulated by IL-24 (100 ng/ml) for 15 min. STAT-3 expression was evaluated by Western blotting. *D*, HT-29 cells were stimulated with IL-24 (100 ng/ml) for 48 h, and the mucin gene expression was then determined by real-time PCR. Data were normalized vs β-actin for human IL-24. Data are expressed as mean ± SD (number of samples (n) = 5). *, $p < 0.05$; **, $p < 0.01$.

Among various cytokines, IL-1β is the sole cytokine that can induce IL-24 mRNA expression in colonic SEMFs. This was mediated by both transcriptional and posttranscriptional mechanisms; 3) IL-24-activated JAK1/STAT-3 signaling pathways and induced SOCS3 expression in colonic epithelial cells; and 4) IL-24 stimulated MUC gene expression via STAT-3 activation.

There are few reports concerning the in vivo expression of IL-24 under normal and pathological conditions. Soo et al. (19) found that IL-24 expression was greatly increased at the edge of cutaneous wounds in an animal model. A recent study by Kunz et al. (12) showed that IL-24 was expressed by keratinocytes from patients with psoriasis. However, there are no reports concerning IL-24 expression in the gut. In the present study, we demonstrated that IL-24 mRNA expression was significantly increased in the inflamed mucosa of patients with IBD. Furthermore, these IL-24-expressing cells coincided with α-SMA immunopositive cells located in the subepithelial regions, suggesting α-SMA immunopositive SEMFs as the local source of IL-24 in the inflamed mucosa of patients with IBD. Because IL-24 expression was not detected in the normal mucosa and infectious/ischemic colitis, a specific role for IL-24 in IBD is suspected.

The molecular mechanisms responsible for IL-24 expression have not fully been identified in any cell types. To investigate the regulatory mechanisms involved in IL-24 expression in the IBD mucosa, we used colonic SEMFs isolated from the normal human colonic mucosa (23). Among the various cytokines, IL-1β exerted remarkable effects on IL-24 induction. IL-17 and LPS weakly stimulated IL-24 expression, but these were negligible as compared with IL-1β. However, IL-1β could not induce IL-24 expression in any colonic epithelial cell lines, and this was compatible with the immunohistochemical observation that IL-24 was not detected in colonic epithelial cells.

Previously, Madireddi et al. (36) demonstrated a role for the transcription factors AP-1 and C/EBP in the basal promoter activity of the *il24* gene during melanoma cell differentiation. However, the role of these transcription factors in the cytokine-induced promoter activity of the *il24* gene has not been investigated. In the present study, we set out to determine the relevant transcriptional processes in IL-1β-induced IL-24 mRNA expression. From the results of the *il24* promoter luciferase assays, IL-1β significantly increased *il24* gene promoter activity in SEMFs transfected with the reporter plasmids containing the AP-1 and C/EBP binding sequences. The EMSA demonstrated that IL-1β actually induced AP-1 and C/EBP DNA-binding activity in colonic SEMFs. The supershift assay indicated that the C/EBP DNA-binding activity was mediated by C/EBP-β molecules. Furthermore, the expression of dominant negative c-Jun and the knockdown of C/EBP-β by siRNA significantly reduced the IL-1β-induced IL-24 mRNA expression in SEMFs. Based on these observations, we concluded that the AP-1- and C/EBP-β-mediated transcriptional activation of the *il24* gene contributes to an IL-1β-induced up-regulation of IL-24 mRNA expression in colonic SEMFs.

The stabilization of their mRNAs contributes to the strong and rapid induction of genes during the inflammatory response (49). The *il24* gene has three AU rich elements in its 3′ untranslated region (36), which is implicated as key determinants in regulating transcript stability (50). The p38 MAPK also plays a role in the induction of mRNA stabilization by inflammatory stimuli (49, 50). In this study, we showed that IL-1β induced a marked increase in IL-24 mRNA stability, indicating that posttranscriptional mechanisms mediated the IL-1β-induced up-regulation of the *il24* gene. SB203580 abolished this IL-1β-induced IL-24 mRNA stabilization, suggesting that the p38 MAPK may play a role in IL-1β-induced *il24* transcript stabilization.

In vivo expression of IL-24R remains unclear. As shown in Fig. 6, we observed that IL-20R1, IL-22R1, and IL-22R2 are expressed by colonic epithelial cells in vivo, and they were also detected in colonic epithelial cell lines. These suggest that colonic epithelial cells are targets of IL-24 in the mucosa. So, we used human colonic epithelial cell lines (HT-29, Caco-2, and SW480) to elucidate the biological activity of IL-24. IL-24 induced a rapid activation of JAK-1 and STAT-3 molecules, and induced SOCS3 expression in these cell lines, although IL-24 did not modulate proliferation of these cells. To our knowledge, this is the first study reporting that IL-24 activates the JAK1/STAT-3/SOCS3 cascade. SOCS3 is a negative feedback modulator of STAT-3 activation by inhibiting JAK downstream of the cytokine signal (51). Thus, the biological activity of IL-24 may be controlled by a SOCS3-mediated negative feedback mechanism in these cells. Furthermore, it has been reported that the STAT-3-mediated activation of the innate immune response contributes to a suppression of colitis (52–54), whereas the STAT-3-mediated activation of the acquired immune response plays a pathogenic role in colitis by enhancing the survival of pathogenic T cells (2, 52–56). IL-24 specifically targets innate immune pathways (8), and epithelial cells are major expression sites for IL-24 receptors (IL-22R1, IL-20R1, and IL-20R2) in human colonic mucosa. These suggest that the IL-24-induced selective activation of STAT-3 in colonic epithelial cells, but not in acquired immune cells, may contribute to the suppression of mucosal inflammation.

Recently, Sugimoto and Mizoguchi et al. (46) showed that IL-22 contributes to the improvement of colitis-associated mucus layer destruction by enhancing the production of membrane-bound mucins, such as MUC-1 and MUC3. Membrane-bound mucins form a static external barrier at the epithelial surface (46, 57), and play a role in a reduction of colitis (58–61). In the present study, we found that IL-24 can stimulate the expression of membrane-bound MUC genes (MUC-1 (8.1-fold increase), MUC-3 (4.5-fold), and MUC-4 (6.8-fold)) through the STAT-3 pathway in intestinal epithelial cells. These were sufficient effects, but weaker than those induced by IL-22. In contrast to a marked protective role of IL-22 in epithelial-barrier function (46, 62), several studies showed proinflammatory properties for IL-22 (40, 62, 63). For example, IL-22 (100 ng/ml) induced TNF-α and IL-8 mRNA expression in colonic epithelial HT-29 cells (62). There are paradoxical reports regarding the role of IL-22 in IBD mucosa (64). However, in our preliminary study IL-24 did not induce such proinflammatory responses in HT-29 cells (data not shown). Lack of evidence for proinflammatory properties of IL-24 suggests that IL-24 mainly play a protective role in the pathophysiology of IBD via a stimulation of STAT-3 activation in cells mediating the innate immune response (54).

In conclusion, we demonstrated that IL-24 expression is enhanced in the inflamed mucosa of active IBD patients. Our data suggest that IL-24 targets epithelial cells and plays anti-inflammatory and protective roles in intestinal mucosa. Recently, a replication-incompetent adenovirus expressing IL-24 has undergone evaluation in a phase I clinical trial for solid tumors, and has demonstrated safety (65). So, based on further characterization of biological activities of IL-24, this cytokine has the potential of clinical application to regulate the inflammatory pathways in IBD.

Disclosures

The authors have no financial conflict of interest.

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An Open-Label Prospective Randomized Multicenter Study Shows Very Rapid Remission of Ulcerative Colitis by Intensive Granulocyte and Monocyte Adsorptive Apheresis as Compared With Routine Weekly Treatment

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OBJECTIVES: **Granulocyte and monocyte adsorptive apheresis (GMA) has shown efficacy in patients with active ulcerative colitis (UC). However, with routine weekly treatment, it may take several weeks to achieve remission, and to date, the efficacy of a more frequent treatment schedule remains unknown. The aim of this study was to assess the clinical efficacy and safety of intensive GMA treatment in patients with active UC.**

METHODS: **This was an open-label, prospective, randomized multicenter study to compare an intensive, two GMA sessions per week, with the routine, one GMA session per week. A total of 163 patients with mild-to-moderately active UC were randomly assigned to routine weekly treatment or intensive treatment. The maximum number of sessions of GMA permitted was 10. However, when patients achieved remission, GMA was discontinued. Remission rate at the end of the study, time to remission, and adverse events were assessed in both groups.**

RESULTS: **Of the 163 patients, 149 were available for efficacy analysis as per protocol, 76 were in weekly GMA, and 73 were in intensive GMA. At the end of the study period, clinical remission was achieved in 41 of 76 patients (54.0%) in weekly GMA and in 52 of 73 patients (71.2%) in intensive GMA ($P=0.029$). The mean time to remission was 28.1±16.9 days in the weekly GMA treatment group and 14.9±9.5 days in the intensive GMA group ($P<0.0001$). Intensive GMA was well tolerated without GMA-related serious adverse side effects.**

CONCLUSIONS: **Intensive GMA in patients with active UC seems to be more efficacious than weekly treatment, and significantly reduced the patients' morbidity time without increasing the incidence of side effects.**

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