Upregulation of activin signaling in experimental colitis

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Ulcerative colitis, a form of inflammatory bowel disease (IBD), is a chronic tissue-destructive disease. It is believed that both colonic epithelial cells and the underlying lamina propria cells are critical components during the development of IBD, in which cellular inflammation and inflammatory cytokines ultimately lead to damage of the colonic mucosa. The pathogenesis of IBD remains elusive although extensive evidence has demonstrated that, in predisposed individuals, a complex interplay between genetic, environmental, and immunological factors causes an abnormal immune response to otherwise innocuous enteric microbial flora (9). Once the balance is broken, a T helper (Th1) cell-mediated immune response arises, in which cytokines such as interferon-γ, tumor necrosis factor (TNF-α), interleukin-1 (IL-1), and IL-6 collaborate to participate disease (30). Considering this destructive sequence, acceleration of the restoration of the epithelial lining could limit the exposure to bacterial products and resolve intestinal inflammation, thus improving the clinical status of patients with IBD.

The architectural features of the gastrointestinal (GI) tract are maintained by a rapid cellular turnover through continuous cell replication of multipotential stem cells within the crypts, with progeny cells moving in vertical columns toward the villus apex. These progeny cells, which differentiate into absorptive enterocytes, mucus-secreting goblet cells, hormone-secreting enteroendocrine cells, and antimicrobial peptide-secreting Paneth cells, form a potent tissue polarity (31). This continuous renewal of the intestinal epithelium is critical for the regeneration of tissue damaged by inflammation, such as in IBD. Mucosal repair involves both the rapid migration of crypt epithelial cells into the injured area of the mucosa, and the replacement of the mucosa by cell replication (34). Recent studies have sought to clarify the mechanisms involved in promoting intestinal mucosal wound repair. Growth factors such as EGF, hepatocyte growth factor, and transforming growth factor (TGF)-α have been shown to play important roles in these processes (10, 37). However, despite our increased understanding of mucosal homeostasis, therapeutic modalities facilitating colonic mucosal repair have not yet been fully established. It is therefore important to study the factors that regulate the proliferation of epithelial cells in the intestine and define conditions that may optimize repair during IBD.

Activins, members of the TGF-β superfamily, regulate a wide range of fundamental physiological phenomena, including embryonic development, cell differentiation, and apoptosis in various tissues (21). Activins are synthesized as either a single homo- or heterodimer of two highly related β-subunits (βA and βB), resulting in three commonly recognized activins, activin A (βA βA), activin B (βB βB), and activin AB (βA βB) (18). Activins bind directly to activin type IIA or type IIB receptors and signal through type I (type IA or type IB) receptors that, once activated, phosphorylate the downstream transcriptional coactivators, Smad2 or Smad3, to facilitate signal transduction. Several studies have demonstrated the expression of activins, activin receptors, and the activin antagonist, follistatin, in GI tissues (14, 35). Activin A has been reported to inhibit cell proliferation of the rat intestinal cell line, IEC-6 cells (14), as well as to play a role in destructive inflammatory responses (6). Activin A is released early in the cascade of circulatory cytokines during systemic inflammatory episodes, roughly coincident with TNF-α and before IL-6 and follistatin are elicited (29). Moreover, clinical data have shown that the activin A protein is elevated in IBD (11) and inflammatory arthropathies (40), and circulating concentrations of follistatin are elevated in patients with sepsis (22). Interestingly, monocyte secretion of activin A is enhanced upon contact with antigen-specific T cells (1) and following antigen sensitization in the
lung (13). Expression of activin receptors has also been reported in the gut of patients with IBD but not in healthy controls (6). However, the mechanism by which activins contribute to the development and progression of pathological processes is still largely unknown. Follistatin is an antagonist of activins, binding to them and blocking their interaction with their receptors (23). The presence and participation of follistatin in IBD development has not been fully elucidated.

In this study, we utilized the dextran sulfate sodium (DSS)-induced murine colitis model as well as a genetic model of colitis, mdr1a−/− mice, to investigate the hypothesis that activin signaling plays an important role in the pathogenesis of colitis. DSS-induced colitis is a well-established experimental model that mimics many clinical symptoms of human IBD, including diarrhea, bloody feces, weight loss, mucosal ulceration, and shortening of the large intestine (8, 28). The mdr1a−/− mouse, which lacks expression of the mdr1a glycoprotein, develops a spontaneous colitis at 5–8 wk of age when conventionally housed (32). The experiments presented here evaluated the potential role and mechanism of activin signaling in the pathogenesis of colitis.

MATERIALS AND METHODS

Mice. C57BL/6J mice (6–8 wk old) and mdr1a−/− mice (5–8 wk old) on an FVB background were used in this study. C57BL/6J and mdr1a−/− mice were maintained in the specific pathogen-free facilities at the Scripps Research Institute (TSRI) and the University of California, San Diego (UCSD), respectively. All studies were approved by the Institutional Animal Care and Use Committee at UCSD.

Reagents and antibodies. Human recombinant activin A (rh-activin A) and follistatin (rh-follistatin, prepared in a carrier protein-free buffer, 0.05 M sodium acetate, pH 5.0) were kindly provided by Dr. Yuzuru Eto (Pharmaceutical Research Laboratories, Ajinomoto, Japan). The rabbit anti-ββB antibody was a generous gift from Dr. Wylie Vale (the Clayton Foundation Laboratories for Peptide Biology, the Salk Institute for Biological Studies, La Jolla, CA). This antibody specifically recognizes inhibin-βB (3). The mouse monoclonal antibody for the βA-subunit of activins and antibodies for the activin type IIA, type IIB, and type IA receptors, as well as anti-human follistatin, were purchased from R&D systems (Minneapolis, MN); anti-phosphorylated Smad2 antibody was purchased from Cell Signaling Technology (Beverly, MA). 3,3′-Diaminobenzidine tetrahydrochloride was purchased from Sigma-Aldrich (St. Louis, MO). Rat anti-bromodeoxyuridine (BrdU) antibody was purchased from Accurate Chemical and Scientific (San Diego, CA).

Induction of colitis. C57BL/6J mice were given DSS (MW, 40,000–50,000; USB, Cleveland, OH) at a concentration of 5% (w/vol) in their drinking water. The animals were treated with DSS for 5 days, and the control group received plain water without DSS. On day 10, mice were injected intraperitoneally with BrdU (100 μg/g body wt) and were then euthanized 1.5 h later. Total colonic tissues were removed, rinsed with PBS, and opened longitudinally, and the length of the colon was measured. Colonic tissues from 8-wk-old mdr1a−/− mice displaying clinical signs of colitis were harvested and subjected to immunostaining as described below.

Immunohistochemical staining and Western blotting. For immunohistochemical analysis, the colon tissues were fixed with Bouin’s Fixative and were embedded in paraffin. Sections (4-μm thick) were cut and stained as previously described (44). Images were obtained using a Zeiss Axioskop (Oberkochen, Germany). For double staining, Cy3-conjugated anti-rat, anti-mouse and anti-rabbit antibodies from Jackson Research Laboratories (West Grove, PA) and Alexa Fluor 488 donkey anti-goat, anti-rabbit FITC-conjugated antibodies from Molecular Probes (Eugene, OR) were used. Sections were viewed using a Bio-Rad MRC 1024 scanning confocal microscope (Richmond, CA) mounted on a Zeiss Axiovert TV-100 with a ×40 objective. Negative controls were performed without the primary antibodies. For Western blotting, tissues or cells were washed with PBS and lysed with ice-cold RIPA buffer and subjected to immunoblotting. Densitometric analysis was carried out using Image J software.

RT-PCR. Total RNA was extracted using the TRIzol Reagent (Invitrogen, Carlsbad, CA). RNA samples were then treated with deoxyribonuclease to remove contamination of genomic DNA. First-strand cDNA was synthesized by using Moloney murine leukemia virus reverse transcriptase (Invitrogen). The oligonucleotide primers used in this paper are summarized in Supplemental Table S1. Supplemental material for this article is available online on the American Journal of Physiology Gastrointestinal and Liver Physiology website.

Cell culture and measurement of DNA synthesis. The mouse crypt cell line, m-Icc12, was kindly provided by Dr. Alain Vandewalle (Inserm U 478, Institute Federatif de Recherche, Faculte de Medicine Xavier Bichat, 75870 Paris Cedex 18, France). These cells were derived from transgenic mice with targeted expression of SV40 large T antigen in intestinal cells, and they retain the main features and differentiated functions of intestinal crypt cells, expressing cytokeratins, villin, aminopeptidase N, dipeptidyl-peptidase IV, and glucoamylase (2). The cells were maintained in DMEM high glucose with 10% FBS. All cultures were kept at 37°C under humidified conditions of 95% air and 5% CO2. To measure their proliferation, m-Icc12 cells were plated in 96-well dishes, and their growth was arrested by serum starvation for 24 h. Stimulation was performed using serum-free media in the presence or absence of recombinant human activin A or recombinant human follistatin. After a 44-h incubation, 10 μCi/ml thymidine was added to the cultures, and the incubation was continued for an additional 4 h. Thymidine incorporation was quantitated on a 1205 Betaplate liquid scintillation counter (Perkin Elmer, Shelton, CT).

Measurement of viability of m-Icc12 cells. To analyze the role of activin A in the survival of m-Icc12 cells, we used the Live/Dead Viability/Cytotoxicity Kit from Molecular Probes. M-Icc12 cells were plated in a four-well cover glass chamber from Nalge Nunc International (Naperville, IL). After growth arrest for 24 h, the cells were treated with or without activin A (4 nM) in 0.5% FCS-containing medium for 48 h, after which the cells were washed with PBS and stained with the Live/Dead Viability Kit. The cells were viewed using a Bio-Rad MRC 1024 scanning confocal microscope with a ×40 objective.

MTT and cell death assays. FET cells were plated at a density of 10,000–20,000 cells/well in 48-well plates with four wells per treatment (control vs. activin A, 2 nM). The compound 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (MP Biomedicals, Aurora, OH) was added to a final concentration of 0.04 mg/ml to the medium, and cells were incubated for an additional 3 h. The reaction product was lysed with SDS, and the absorbency was detected at 570 nm using a Beckman-Coulter DU640B spectrophotometer (Beckman-Coulter, Fullerton, CA). For cell death assay, FET cells were grown to near confluency in 10-cm dishes (~2.4 × 10⁶) and treated with activin A (2 nM) for 24 h. Following trypan blue staining and counting, viable and dead cells were counted using a hemocytometer.

In situ detection of apoptosis. FET cells were grown to near confluency on glass microscope slides (~2.4 × 10⁶) and were treated with activin A (2 nM) for 24 h. Cells were then fixed in 1% paraformaldehyde and washed with PBS, and equilibration buffer was added (Chemicon ApopTag Plus Fluorescein Kit) followed by addition of TdT enzyme directly to the specimen. Following PBS washes, the slides were incubated with antidigoxigenin fluorescein antibody and counterstained with DAPI, and the percentage of apoptotic cells in a given area was determined via fluorescence microscopy.

Measurement of plasma activin A concentration. Blood samples were collected from tail vein (on day 3 following DSS treatment) and (on day 6 at euthanasia under anesthesia) into heparinized capil-
lary tubes. Activin A levels in the plasma were measured by using a Quantikine Human/Mouse/Rat Activin A immunoassay kit (R&D systems).

**In vivo administration of rh-follistatin.** Rh-follistatin protein was dissolved in 1% mouse serum in PBS; the total volume used for each injection was 150 μL. Control mice were injected at the same time with 150 μL of 1% mouse serum in PBS. As previously shown, administration of rh-follistatin at 0.3 μg/mouse is sufficient to induce its biological effect (42). On the basis of this previous finding, 1 μg/mouse follistatin was injected intraperitoneally every day for 6 days starting from the time DSS was first included (DSS treatment lasted for 5 days) in the drinking water, and the mice were then euthanized. In a separate set of experiments, 1 μg/mouse follistatin was injected intraperitoneally beginning on day 5 of DSS treatment. DSS treatment was continued for 2 additional days (7 days of DSS treatment in total), while follistatin injections were continued for 5 additional days (5 days of follistatin treatment in total). Mice were euthanized at the end of the follistatin treatment period. The second protocol was performed to determine whether follistatin could accelerate repair of epithelial cells.

Symptoms of colitis such as weight loss, histopathology, and the existence of epithelial restoration (observed as the appearance of proliferating and/or migrating epithelial cells over the disrupted mucosal layer) were evaluated in each experimental group. A histological score for each hematoxylin and eosin-stained colonic was determined according to the published criteria to evaluate the severity of inflammation (15). The score ranges from 0–14 (total score), which repre-

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**Fig. 1.** Expression of activins in normal and colitic colon. Sections of distal part of the colon were stained with βA-antibodies (A and B) or βB-antibodies (C–F), detected by 3,3′-diaminobenzidine tetrahydrochloride (DAB), and counterstained with hematoxylin. B and D: mice were treated with 5% dextran sodium sulfate (DSS) for 5 days and were euthanized at day 10. A and C were the age-matched controls. F: genetic colitis model of mdr1a−/− mice and its age-matched control (E). m, muscle; magnification: ×20 objective.
sents the sum of scores from 0–3 for severity and extent of inflammation, and 0–4 for crypt damage and the percentage of the colon involved. Epithelial cell proliferation was determined by counting BrdU-positive cells per crypt in both follistatin-treated and DSS alone colitic mice. At least 15 crypts were counted on each slide, and four slides were counted for each mouse. To investigate the extent of apoptosis occurring in epithelial cells during colitis, TUNEL staining was performed using the Roche In Situ Cell Death Detection POD kit (Roche, Indianapolis, IN).

**Statistical analysis.** Statistical analysis was carried out using the Student’s *t*-test for two-tailed unpaired data. The results from this study were expressed as means ± SD. Differences with *P* < 0.05 were considered to be significant.

**RESULTS**

Expression of activins and their receptors is upregulated during colitis. To investigate the role of activins in the development of colitis, we first evaluated the expression of activins, activin receptors, and the activin antagonist, follistatin in the colons of control and colitic mice. In control mice, activin-βA immunoreactivity was not detected in the colon, and activin-βB immunoreactivity was only found occasionally located in intraepithelial cells. However, in DSS-treated mice, we found that activins (both βA- and βB-subunits) were extensively expressed in the lamina propria of colitic colon tissues (Fig. 1, A–D). The expression of activins also significantly increased in the lamina propria of colonic tissue from mdrla−/− model, compared with tissues from age-matched wild-type mice (Fig. 1, E and F). These data suggest that the upregulation of activins is a generalized phenomenon during murine colitis and may be independent of the initiating stimulus.

Next, we studied the expression of activin receptors. We found that activin type IIA receptors were expressed by intestinal epithelial cells in both normal and DSS-induced or mdrla−/− colitic tissues; however, their expression was strikingly increased in colitic tissues compared with normal tissues. In addition, activin type IIA receptors were also expressed in

![Fig. 2. Expression of activin receptors in DSS-induced colitis. Mice were treated with 5% DSS for 5 days and were euthanized at day 10. Sections of distal part of the colon from DSS-treated and control mice were stained with anti-type IIA (A and B), type IIB (C and D), and type IA (E and F) antibodies, detected by DAB, and counterstained with hematoxylin; magnification: ×20 objective.](http://ajpgi.physiology.org/.../ajpgi.org)
the inflamed lamina propria of colitic tissues but not in the lamina propria of normal colon tissues (Fig. 2, A and B). The expression of activin type IIB receptors was also increased in colitic tissues, predominately in the inflamed lamina propria areas. In contrast, in normal control mice, only a few cells located in the intestinal epithelium stained positively for activin type IIB receptors (Fig. 2, C and D). In addition, the expression of the type I receptor was also significantly increased in colitic tissues (Fig. 2, E and F). Upregulation of activin receptors was also observed in the mdr1a−/− mice in a pattern similar to that of DSS-mice (Fig. 3, A–D). To confirm the results of our immunohistochemical findings, we isolated RNA from control and colitic colons and performed RT-PCR studies. We found that the expression of activins and their receptors were upregulated in colitis, paralleling the results of our immunohistochemical observations (Fig. 3E). Of note, although we were not able to detect follistatin in either normal or colitic tissues by immunohistochemistry, using RT-PCR analysis, we were able to show that follistatin was expressed at similar levels in both normal and colitic colons (Fig. 3E). These data suggested that enhanced expression of activins during colitis may not be properly balanced by their antagonist, and therefore the homeostasis of activin is likely disrupted during colitis development. To further investigate whether plasma activin level changes during colitis, C57BL/6J mice were treated with or without 5% DSS in drinking water, and the plasma were collected on day 3 and day 6 following treatment and activin A levels measured. Our results showed that plasma activin A level significantly increased on day 3 compared with that of the control mice; however, no significant differences were found on day 6, indicating that activin was produced during acute phase of colitis (Fig. 3F).

To identify whether downstream signaling events induced by activin occur during colitis, we investigated the expression of Smad2/3, the intracellular targets of activins. By immunohistochemistry, we found expression of Smad2/3 in the epithelial cells of both normal and DSS-induced or mdr1a−/− colitic tissues, with expression significantly upregulated during colitis. In fact, nuclear localization of Smad2/3 was predominantly observed in colitic tissue, whereas normal tissues exhibited cytoplasmic localization. Nuclear translocation of Smad2/3 indicates the phosphorylation of Smad2/3 and therefore the activated activin signaling, suggesting that activin signaling during colitis could be mediated by the phosphorylation of Smad2/3 (data not shown).

Fig. 3. A–D: sections from mdr1a−/− and their age-matched control were stained with anti-type IIA (A and B) and anti-type IIB (C and D) antibodies, detected by DAB, and counterstained with hematoxylin; magnification: ×20 objective. E: expression of mRNA for activin, activin receptors, and follistatin in the colon of control and DSS-induced colitis. Total RNA was extracted from control and colitis colons, and first-strand cDNA was synthesized from 1 μg RNA using Moloney murine leukemia virus reverse transcriptase. PCR was performed using a Peltier thermal cycler. M, marker; RT, samples without reverse transcriptase. F: plasma activin A levels following DSS treatment. C57BL/6J mice were treated with or without 5% DSS in drinking water for 5 days, and the blood was collected on day 3 and day 6. Results are from duplicate assays for every sample from 4 mice in each group. **P < 0.01.
Cellular specificity of activin expression. The mature activin protein is a homodimer of βA (activin A), βB (activin B), or a heterodimer of βA and βB (activin AB) subunits. βA- and βB-subunits are broadly distributed in different types of cells and tissues and exert diverse effects (39). We investigated whether activin-βA and -βB are coexpressed by the same cells during colitis by double staining with anti-βA and anti-βB antibodies. Our results demonstrated that most of the βA- and βB-immunoreactivity colocalized within the same cells (Fig. 4A). The same result was also found in the mdr1a−/− mice (data not shown), suggesting that, during colitis, βA- and βB-subunits were produced in the same cells. Interestingly, we also found that expression of activin and activin type IIB receptor colocalized within the inflamed areas (Fig. 4B). Together, these data suggest that activins may serve as both autocrine and paracrine factors during colitis to exert their effect on inflammatory cells and intestinal epithelial cells.

In the next set of experiments, we sought to determine the cellular source of activins during colitis. We performed colocalization studies for activin and various cell type-specific markers selected on the basis of their relevance to the development of colitis. We first asked whether activin was expressed in macrophages by double staining tissue sections with anti-activin and anti-F4/80 antibodies. Using DSS-mice, we found ∼40% of the activin-positive cell population costained for F4/80, suggesting that the induced activins were partially derived from infiltrating macrophages (Fig. 5A). We also found that activin type IIB receptor was colocalized with F4/80 staining (Fig. 5B). Similar results were also observed in mdr1a−/− mice (data not shown). It is possible that activins are derived from a subpopulation of macrophages that are undergoing activation. Because cyclooxygenase (COX)-2 is an inducible enzyme that is abundant in activated macrophages at sites of inflammation, we studied the relationship between COX-2 and activin or the activin receptor by double immunostaining experiments. We found that both activin and the activin receptor were colocalized with COX-2 (Fig. 6, A and B). We therefore hypothesize that activin is induced selectively in activated macrophages. Of note, we did not observe any double staining between activins and epithelial cell marker, E-cadherin, suggesting that activins are not directly elicited from this cell compartment (Fig. 6, C and D). Interestingly, activin type IIA receptors were found to be colocalized with E-cadherin in the epithelium but not in the inflammatory cells (Fig. 6E). Although the possibility that epithelial cells might undergo a phenotypic change to a more mesenchymal phenotype during inflammation may limit our staining results, these data indicated that the activins secreted from macrophages might exert a paracrine effect on intestinal epithelial cells. Because activins have been reported to be involved in the fibrosis that accompanies inflammatory diseases (29), we therefore investigated whether the increased activins were produced by fibroblasts using an α-smooth muscle actin (a marker of myofibroblasts) antibody for double staining, but did not...
not observe any colocalization, indicating that activins were not produced by fibroblasts during the development of colitis (Supplemental Fig. S1). Our results thus far have identified activated macrophages as a significant cellular source of activins during colitis. Furthermore, the observations that macrophages also express increased levels of activin receptors indicate that activins may act in an autocrine manner on macrophages. A recent study demonstrated that activin A has potent autocrine effects on the capacity of human dendritic cells to stimulate immune responses (33); it is therefore possible that the increased activins during colitis settings may be also partially derived from dendritic cells. Further studies are needed to address this hypothesis in future studies.

Activin inhibits the proliferation and reduces the survival of mouse crypt cells and colon cancer cells. Activins are widely known to inhibit proliferation and induce apoptosis of various types of epithelial cells. On the basis of our findings that activin expression is upregulated in colitis and that activin receptors are extensively expressed in colonic epithelial cells, particularly in the field of inflammation, we hypothesized that activins are involved in the inflammatory process and could inhibit the proliferation of intestinal epithelial cells. We tested this hypothesis using a mouse crypt cell line, m-ICc12 cells. As shown in Fig. 7A, activin A inhibited the proliferation of m-ICc12 cells in a dose-dependent manner; the effect was significant at 2 nM ($P = 0.048$). At 10 nM activin A, the incorporation of thymidine was inhibited by roughly 55%, compared with control samples ($P = 0.001$).

To investigate the effect of activin A on the survival of m-ICc12 cells, we used the Live/Dead Viability/Cytotoxicity Kit from Molecular Probe (West Grove, PA) and measured the viability of these cells in the presence and absence of activin A. As shown in Fig. 7B, following activin A-treatment, the proportion of dead cells (indicated by the red nuclei) was significantly increased relative to the live cells (indicated by the green color). The average percentage of dead cells following activin A-treatment was 23.7%, whereas it was only 1.6% ($P < 0.001$) in controls (Fig. 7C). To determine whether Smad2 acts as a downstream mediator of activin signal transduction, we performed Western blotting with an antibody to phosphorylated Smad2. We found that activin A (4 nM) stimulated the phosphorylation of Smad2 in m-ICc12 cells within 30 min, and the peak activation was found at 60 min following treatment (Fig. 7D). Furthermore, follistatin reversed activin A-induced growth inhibition of m-ICc12 cells (Fig. 7E). Interestingly, follistatin alone slightly promoted the growth of m-ICc12 cells, suggesting that the growth of these cells may be normally limited by endogenous activins. These results strongly support our hypothesis that activins induced during colitis may be involved in the inhibition of the repair of the colonic epithelium.

To further investigate the effects of activin A on colonic epithelial cell viability and apoptosis, we used colon cell line FET
cells, which were derived from a well-differentiated early-stage colon carcinoma and are nontumorogenic in athymic mice (19). In FET colon cancer cells, activin A treatment resulted in a decrease in cellular viability as evidenced by decreased metabolic activity, increased cell death, and apoptosis (Fig. 7). Together, these results suggested that activin A reduces the survival and induces apoptosis of colonic epithelial cells.

**In vivo blockade of activins by administration of follistatin accelerates the growth of epithelial cells in colonic crypts.** To ask whether in vivo administration of follistatin could inhibit
immune infiltration during colitis and ameliorate the symptoms of colitis induced by DSS, we administered recombinant follistatin (1 μg/mouse) for 6 days starting at the initiation of DSS treatment. Mice were then euthanized at day 7. Mice weight and cage conditions were checked every day. Although we did not find significant body weight differences between these two groups, from day 3 following the initiation of DSS treatment, we found reduced rectal bleeding in the DSS/follistatin-treated group compared with the DSS alone group. At euthanasia, we found that there was less blood in the colons of the DSS/follistatin-treated group and the integrity of the intestinal epithelium was preserved with a lower histological score in the DSS/follistatin-treated group compared with DSS alone group (Fig. 8 and Table 1). To determine whether administration of follistatin could promote the regeneration of colon crypt cells, we counted BrdU-positive cells in the crypt. Approximately 17% of crypt epithelial cells were BrdU positive in the DSS/follistatin-treated group, compared with 12% in the DSS-treated group (Fig. 9, A–D, n = 21 from 3 different mice in each group, P = 0.0015), suggesting that follistatin treatment accelerates epithelial proliferation/repair during colitis. It should be noted that follistatin administration in control mice did not enhance the proliferation of colonic epithelial cells, suggesting that follistatin may exert its function only when activin signaling is activated (data not shown).

Activins have been reported to induce apoptosis in a variety of tissues and cells (24, 43). We therefore asked whether follistatin administration could reduce apoptosis and promote cell survival. We performed TUNEL staining and quantitated cells that were both TUNEL positive and fit the morphological criteria for apoptosis. The percentage of apoptotic epithelial cells in the crypts of DSS-treated mice was 8.2 ± 4.3% (n = 17 from 3 different mice). However, in DSS/follistatin mice, the corresponding percentage was reduced to 4.7 ± 2.1% (n = 17 from 3 different mice, P = 0.006) (Fig. 9E). This result indicated that follistatin enhances crypt cell survival in DSS-treated mice and suggested that activins regulate the turnover of crypt epithelial cells during DSS-induced colitis.
To determine whether follistatin can accelerate the repair of the damaged intestinal epithelium, we administered follistatin at a later stage of DSS treatment (beginning day 5 after the initiation of DSS treatment) and continued for the next 5 days with DSS treatment terminated at day 7. We observed a similar body weight loss in the presence and absence of follistatin at day 6 after DSS initiation. However, rectal bleeding in the follistatin-treated group was less than that in the DSS alone group. Importantly, mice in the follistatin-treated group appeared healthier compared with that of the DSS alone group. Following euthanasia on day 10, we found that the length of the colon was longer in follistatin-treated group compared with that of the DSS alone group (Table 2). Furthermore, the follistatin-treated group demonstrated a reduced histological score and an increased BrdU labeling compared with DSS alone group (Fig. 10 and Table 2). Moreover, weight loss was significantly reduced in the mice that had received DSS plus follistatin compared with DSS alone (Table 2). Together, these results demonstrate that follistatin clearly can both reduce the development of DSS-induced colitis and speed the resolution and regeneration of the colonic epithelium.

**DISCUSSION**

The epithelium of the GI tract rapidly renews and regenerates itself in response to certain inflammatory stimuli. This mucosal repair and regeneration process maintains the

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Table 1. Parameters in DSS and follistatin-treated mice (first set of experiment)

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<thead>
<tr>
<th>Parameters Assessed</th>
<th>DSS Alone (n = 5)</th>
<th>DSS + Follistatin (n = 5)</th>
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<td>Colon length, cm</td>
<td>3.95 ± 0.3</td>
<td>4.6 ± 0.45 (P = 0.017)</td>
</tr>
<tr>
<td>Histological score</td>
<td>7 ± 2.8</td>
<td>5.2 ± 1.9 (P = 0.0016)</td>
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Values are mean ± SD. Dextran sodium sulfate (DSS) (5%) mice were treated for 5 days, and follistatin (1 μg/mouse per day) was administered for 6 days starting from the time of DSS treatment. Mice were euthanized at day 7. P values are calculated by comparing the DSS alone with that of DSS + follistatin.
epithelial integrity necessary for gut homeostasis. However, the growth factors regulating this continuous proliferation have not been fully defined. In the present study, we report that expression of activin and their receptors were significantly increased during the development of colitis. In addition, the upregulated activin signaling correlated with immune cell infiltrations. Interestingly, in vivo administration of follistatin ameliorated the severity of colitis and promoted the proliferation of colonic crypt cells.

Activins have been recognized as multifunctional cytokines expressed by many cell types, including epithelial cells, macrophages, and fibroblasts (5). In this study, we found that a subset of activin-positive cells colocalized with macrophage-specific markers, indicating that macrophages represent a significant cellular source of activins during colitis. Although we did not observe significant changes of activin expression in colonic epithelial cells by immunohistochemistry, our in vitro studies using m-ICc12 cells suggested that activins are likely produced by these cells, as follistatin treatment augmented the basal growth of these cells (Fig. 7E). Other studies also suggest that activins are expressed in intestinal epithelial cells (14, 35). We therefore suggest that activin signaling has two distinct effects during the development of colitis. First, it may stimulate the inflammation during colitis as we have found that administration of follistatin ameliorates the severity of colitis. Second, the increased activins inhibit the growth of intestinal epithelial cells, and repress their mitosis and survival in vivo, which was evidenced by the fact that administration of follistatin reduced the apoptotic rate among colonic crypt cells. Such a notion is further supported by our in vitro experiments showing that activin A-inhibited cell growth and viability of intestinal crypt cells were significantly reversed by simultaneous addition of follistatin. The present study is consistent with a previous study demonstrating that neutralization of activins by follistatin promotes epithelial cell division and tissue repair (7). Importantly, our experimental system involved not only the administration of follistatin during the development of colitis but also during its resolution. Our results showed that follistatin administration at either of these times is effective in reducing the severity of colitis.

Activins signal through their type I and type II receptors. Interestingly, we also found that both type I and type II activin receptors are increased during colitis. Expression of activin receptors has also been reported in the gut of patients with IBD but not in healthy controls (6), suggesting that activin signaling is activated during colitis. Activins are implicated in both pro- and anti-inflammatory responses, depending on the tissue settings. For example, in bone marrow-derived macrophages, activin A stimulates the production of the proinflammatory cytokines TNF-α and IL-1β

Table 2. Parameters in DSS and follistatin-treated mice (second set of experiment)

<table>
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<tr>
<th>Parameters Assessed</th>
<th>DSS Alone (n = 3)</th>
<th>DSS + Follistatin (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon length, cm</td>
<td>2.9±0.26</td>
<td>3.7±0.1 (*P = 0.008)</td>
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<tr>
<td>Body weight, %</td>
<td>57±3.6</td>
<td>67±4 (*P = 0.03)</td>
</tr>
<tr>
<td>Histological score</td>
<td>12.55±1.4</td>
<td>7.67±2.5 (*P = 0.0004)</td>
</tr>
<tr>
<td>BrdU-positive cells</td>
<td>2±0.64</td>
<td>8±3.9 (*P = 0.005)</td>
</tr>
</tbody>
</table>

Values are mean ± SD. DSS (5%) mice were treated for 7 days, and follistatin (1 μg/mouse per day) was administered on day 5 following the initiation of experiment for an additional 5 days. Mice were euthanized at day 10. *P values are calculated by comparing the DSS alone with that of DSS + follistatin. Body weight percentage is ratio of the original body weight. BrdU, bromodeoxyuridine.
as well as the upregulation of COX-2 (25). Activin A is also reported to suppress the level of the anti-inflammatory cytokine IL-10 in a human prostatic epithelial cell line (38). In contrast, activin A can antagonize IL-6 (4, 40) and inhibits the production of other proinflammatory cytokines, such as IL-1β (27). In the present study, we found that both activin and activin receptors colocalized with COX-2, which is undetectable in most normal tissues but is abundantly expressed by activated macrophages at sites of inflammation (16). This suggested that the increased activins might have a close relationship with COX-2 during colitis although this mechanism needs further investigation in future studies.

Considering the mechanism responsible for the increased activins during colitis, a common trigger must exist as increased activins were observed in both DSS-induced and mdr1a−/− models of colitis. A recent elegant study indicated that systemic injection of LPS, the major component of the Gram-negative bacterial cell wall, rapidly increases the circulation levels of activin (12). Previous studies also demonstrated that treatment with LPS increases activin production in monocytes and mouse peritoneal macrophages (26, 41). As the luminal microflora plays a pivotal role in the development of colitis, we thus hypothesize that activins induced in the colitis are possibly direct stimuli from the pathogen-derived inflammatory signal, or an indirect effect from other cytokines; further studies are necessary to clarify this hypothesis.

Follistatin is a specific activin-binding protein and blocks the action of activins in most biological systems by preventing the binding of activins to their type II receptors (23). In vitro neutralization of activins by follistatin has been shown to promote the regeneration of liver (17), kidney (20), and pancreas (42). In the present study, intraperitoneal injection of follistatin during colitis significantly improved body weight loss and colonic injury in the DSS-induced model. In addition, follistatin treatment also promoted BrdU incorporation and reduced the rate of apoptosis of colonic epithelial cells. As both BrdU incorporation and apoptosis are the indicators of repair for colonic epithelium during tissue injuries, we therefore suggest that activins induced in the experimental colitis may accelerate the breakdown of the epithelium by inhibiting the proliferation and inducing the apoptosis of epithelial cells in the colon. Interestingly, a recent study has shown that follistatin strongly reduces macrophage trafficking in the epidermis (36). Given that the induction of colitis in DSS-treated mice has been associated with the influx of macrophages into the intestinal mucosa, it is possible that administration of follistatin inhibits the macrophage accumulation during colitis and thereby ameliorates the symptoms of colitis. Therefore, inhibition of activin signaling may favor the survival of colonic cryptal cells and thus provide a potential molecular target for therapeutic interventions against colitis.

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