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Effect of Atorvastatin, a HMG-CoA Reductase Inhibitor, in Experimental Colitis in Mice

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Background : The statins, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, are approved for cholesterol reduction, and may also be beneficial in the treatment of inflammatory disease. In this study, atorvastatin was tested in experimental colitis, a disease model of inflammatory bowel disease. **Methods :** To induce colitis, dextran sodium sulfate (DSS) or trinitrobenzene sulfonic acid (TNBS) were administrated to C57BL/6 or BALB/c mice. Mice were monitored daily for loss of body weight and survival for indicated days. Colon length and histology were examined after sacrifice. **Results :** The administration of DSS induced marked colonic inflammation and shortening, and resulted in a loss of body weight. DSS-induced colitis was not affected by atorvastatin treatment, but in contrast, the administration of atorvastatin relieved TNBS-induced colitis with a resultant rapid recovery of weight loss and a reduction in colonic length shortening. Histologically, inflammatory cell infiltration in the colonic wall, mucosal ulceration and crypt disruption were also suppressed in atorvastatin treated mice. **Conclusions :** These results suggest that atorvastatin preserves intestinal integrity in colitis, probably via the modulation of Th cell-mediated immune response, in a manner independent of innate immunity.

Key Words : Atorvastatin; Inflammatory bowel disease; Experimental colitis; Th cell mediated immunity; Immunity, Natural

Inflammatory bowel diseases (IBDs), such as Crohn's disease and ulcerative colitis, are idiopathic chronic diseases that are being diagnosed with increasing frequency in the Western and in Korea.[1,2] IBD is characterized by tissue edema, increased gut epithelial permeability, and extensive leukocyte infiltration of the gut. General morbidity and weight loss in individuals with IBD can be attributed to leukocyte sequestration in the gut in this condition.[3,4] The current literature suggests that multiple immune, genetic, and environmental factors influence both the initiation and progression of colitis.[3,4] However, initiating events remain unknown and thus studies in human IBD are necessarily retrospective and inferential. This has led to the development of animal models of IBD primarily to identify early events in the disease process and to test new treatments.[5,6] One of these models is based on the local exposure of colonic mucosa to the contact-sensitizing agent trinitrobenzene sulfonic acid (TNBS).[7] The colonic administration of a single dose of TNBS in 50% ethanol induces a chronic distal colitis in mice. Another IBD model is induced using dextran sulfate sodium, a heparin-like polysaccharide containing up to three sulfate groups per glucose molecule. Administered orally, this has been successfully used to induce both acute and chronic colitis in mice.[8] These models have been proven useful in investigations into the fundamental mechanisms underlying the pathophysiology of IBD, and for the screening of potential therapeutic interventions.

3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, known as 'statins', block mevalonate synthesis and are potent cholesterol-lowering drugs. Statins are extensively used in medical practice, and large clinical trials have demonstrated that this class of lipid-lowering drugs greatly reduces cardiovascular-related morbidity and mortality in patients with or without coronary disease.[9-11] Recent *in vitro* and *in vivo* findings indicate that statins,

in addition to their lipid-lowering effects, have anti-inflammatory properties, which suggests that they are able to regulate molecules important for immunomodulation. Lovastatin was found to inhibit the production of TNF-α and inducible nitric oxide synthetase by microglia and astrocytes.[12] Statins also prevented IFN-γ-inducible major histocompatibility complex (MHC) class II expression on non-professional antigen-presenting cells (APC), thus suggesting that they may inhibit antigen presentation to pro-inflammatory Th cells.[13] In addition, Youssef *et al*. demonstrated that atorvastatin treatment could either inhibit or reverse chronic and relapsing experimental autoimmune encephalomyelitis, the archetypal model for multiple sclerosis *in vivo*.[14] These observations suggest that statins may be beneficial in multiple sclerosis and other autoimmune diseases. Here we examine the immunomodulatory effects of atorvastatin in acute and chronic experimental colitis induced by either dextran sulfate sodium or trinitrobenzene sulfonic acid *in vivo*.

MATERIALS AND METHODS

Mice

C57BL/6 and BALB/c mice were purchased from Daehan Biolink (Chungbuk, Korea). All mice were housed in a specific pathogen-free facility at Hallym University. All experimental animals were cared for, maintained, and terminated by CO_2 inhalation in accordance with Hallym University Guidelines.

Dextran sodium sulfate-induced colitis

Colitis was induced in C57BL/6 mice (n=6) by adding 2.5% dextran sodium sulfate (DSS) (M.W. 36,000-50,000; MP Biomedicals, Irvine, CA) to drinking water for 12 days. DSS+atorvastatin (n=6) group animals were administrated 2.5% DSS and 4% atorvastatin (Pfizer Inc.) (prescription formulation) in distilled drinking water 1 day after DSS administration. Control mice (n=3) received drinking water only. Animals were monitored daily for loss of body weight and for survival over 12 day period. Mice were sacrificed at 7 days after starting DSS administration, when clinical symptoms such as diarrhea, hematochezia, and weight loss were obvious, and colon length was recorded.

Trinitrobenzene sulfonic acid-induced colitis

Colitis was induced in BALB/c mice as previous described.[7] In brief, mice were sensitized by the epicutaneous application of TNBS solution onto shaved abdomens. Seven days later mice were lightly anesthetized, and a 3.5 F catheter was inserted into the colon such that its tip was 4 cm proximally to the anus. To induce colitis, 1.5-2.0 mg of TNBS (Sigma Chemical Co, St Louis, MO) in 100 μL of 50% ethanol was administrated to mice (n=13) via the catheter into the lumen. The TNBS+atorvastatin (n=13) group were administrated 4% atorvastatin in drinking water 1 day after TNBS treatment. Control mice (n=3) received 50% ethanol alone. Animals were monitored daily for loss of body weight and survival for 7 days. Four days after TNBS administration, when weight loss was peaked in the non-atorvastatin treated group, mice were sacrificed and colon lengths were recorded.

Histopathological analysis

Colons were removed, fixed in 10% neutral formalin, embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin. Lesion severity was compared between groups by light microscopic examination.

RESULTS

Effects of atorvastatin on DSS-induced colitis

The administration of DSS dissolved in water to mice induces acute colitis, which occurs during the DSS administration, and chronic colitis, which occurs a little time after the DSS administration.[15] In this study, we investigated the effect of atorvastatin on the course of DSS-induced acute colitis. The average weight of the C57BL/6 mice before the initiation of this study was 20.7 $\pm$0.8 g. Body weights were monitored for 7 days after DSS administration, and histopathologic features were evaluated in these day 7 tissue samples. Clinically, symptoms of acute colitis, such as a progressive loss of body weight, hematochezia, and diarrhea were noted; loose stool was detected after 4 days, and diarrhea and hematochezia were noted after 5 days of 2.5% DSS administration. Following the induction of colitis, body weights significantly reduced, resulting in a 32.72$\pm$0.02% decreases in the DSS group versus the control group (Fig. 1A). To investigate the effect of atorvastatin, the atorvastatin group received both 4% atorvastatin and 2.5% DSS in drinking water from 1 day after DSS administration, and changes in body weight was compared with the group that received only DSS. Both groups lost body weight to similar extents (Fig. 1A); their survival rates were also

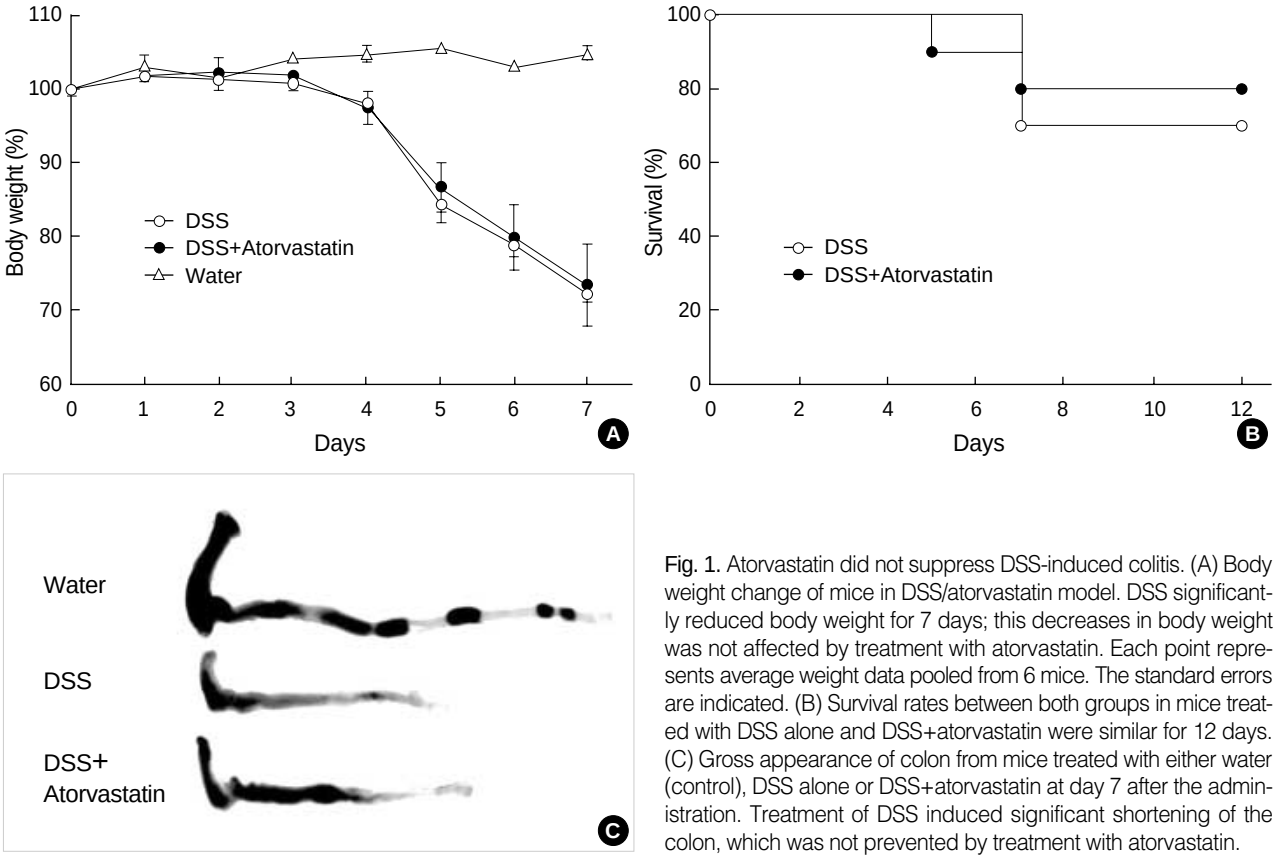

Fig. 1. Atorvastatin did not suppress DSS-induced colitis. (A) Body weight change of mice in DSS/atorvastatin model. DSS significantly reduced body weight for 7 days; this decreases in body weight was not affected by treatment with atorvastatin. Each point represents average weight data pooled from 6 mice. The standard errors are indicated. (B) Survival rates between both groups in mice treated with DSS alone and DSS+atorvastatin were similar for 12 days. (C) Gross appearance of colon from mice treated with either water (control), DSS alone or DSS+atorvastatin at day 7 after the administration. Treatment of DSS induced significant shortening of the colon, which was not prevented by treatment with atorvastatin.

similar (Fig. 1B). The mean colon length in the DSS group (45 ±0 mm) was markedly shorter than that in the control group (80±0 mm), but this was not affected by atorvastatin treatment (Fig. 1C).

Histopathologic examination of the colons at after 7 days of DSS administration showed massive mixed inflammatory cell infiltration in mucosa and submucosa with epithelial denudation, destruction of crypt architecture, ulceration, and muscular thickening (Fig. 2B). Neutrophils comprised the major proportion of these infiltrating inflammatory cells (Fig. 2C). Pathologic changes in colons induced by 7-days of DSS administration were similar in atorvastatin-treated and untreated mice (Fig. 2D), implying that administration of atorvastatin failed to protect the development of DSS-induced colitis.

Effects of atorvastatin on TNBS-induced colitis

To evaluate the effects of atorvastatin on experimental colitis induced by TNBS, BALB/c mice were sensitized with TNBS solution epicutaneously on shaved abdomens, and this was followed by the administration of TNBS per rectum 7 days later. The average weight of BALB/c mice before the initiation of this study was 24.2±2.2 g. Body weights were monitored during 7 days after the TNBS administration per rectum. BALB/c mice treated as described above developed a colitis that was marked by a gradual weight loss that peaked 4 days after induction (Fig. 3A) and a shortening of colonic length (Fig. 3C). Microscopic examination of colon extracted from these mice 4 days after TNBS challenge per rectum showed histologic evidence of severe colitis. Compared to control mice, which received PBS/ethanol enemas and showed histologically normal colons (Fig. 4A), TNBS treated mice developed multifocal lymphocytic colitis of moderate intensity (Fig. 4B), which was accompanied by mucosal inflammation characterized by a multifocal or locally extensive inflammatory reaction composed of lymphocytes, and macrophages (Fig. 4C). The epithelium ranged in appearance from relatively normal, through acutely damaged, to regenerative.

In these TNBS treated mice, the oral administration of atorvastatin 1 day after the injection of TNBS per rectums led to a more rapid recovery in weight loss (Fig. 3A). Colon length in controls was 62±4 mm, and the colon length in the TNBS group (37±3 mm) was shorter than that in the TNBS+atorvastatin group (49±4 mm) (Fig. 3C), showing that atorvastatin reduced the colon shortening associated with the induction of TNBS col-

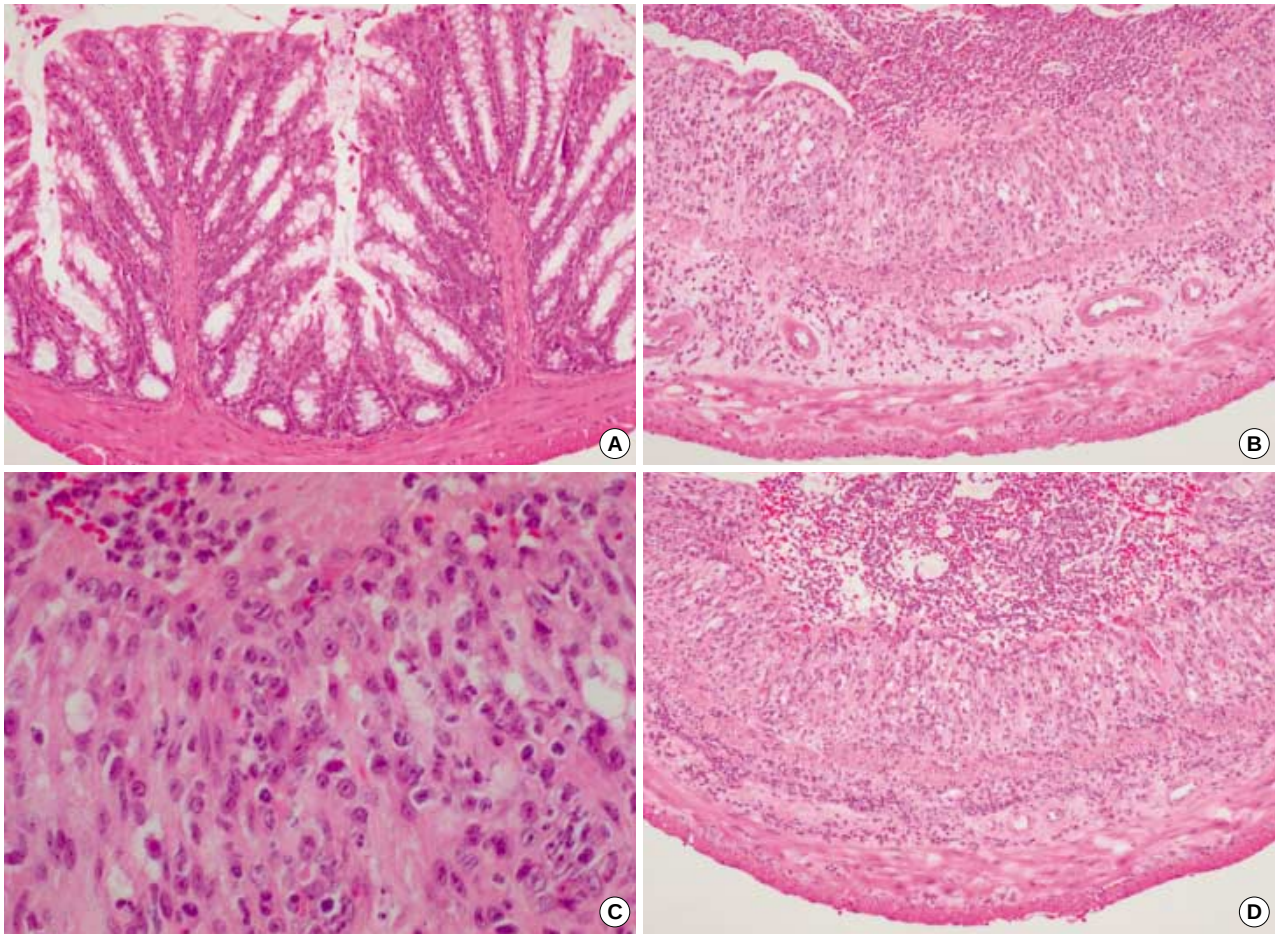

Fig. 2. Histology of colonic samples taken from mice receiving either water (control), DSS, DSS + atorvastatin on day 7. Compared with that of control animals (A), colons of DSS-treated mice (B, C) shows complete destruction of epithelial architecture, with nearly complete loss of crypts, loss of epithelial integrity, and intense cellular inflammation composed by mainly leukocytes (C) in all layers. Treatment with atorvastatin (D) does not attenuated morphological damage and colonic inflammation shown in these mice is comparable to that of DSS-treated mice.

itis. Survival rate was also slightly improved in the group treated with atorvastatin, although this was not statistically significant (Fig. 3B). Histologic findings showed significantly less inflammatory cells in the colons of atorvastatin treated mice. In most cases, atorvastatin treatment abrogated the TNBS-induced inflammation and restored a normal histologic appearance of the colon. Mild lymphoid hypertrophy and mild inflammatory cell infiltration in mucosa and submucosa were noted, but severe mucosal inflammation including crypt disruption or ulceration was no longer detected (Fig. 4D). Compared with DSS-induced colitis (Fig. 1, 2), atorvastatin evidently suppressed the inflammation induced by TNBS-induced colitis.

DISCUSSION

This study shows that oral statin treatment suppresses TNBS induced colitis but not DSS induced colitis *in vivo*. The administration of DSS dissolved in water to mice caused hematochezia, body weight loss, shortening of colon, mucosal ulcers, and neutrophil infiltration.[15] Acute colitis, which occurred during the administration of DSS, and chronic colitis, which occurred a little time after the administration of DSS, were seen in this model. Acute colitis was considered to be induced by innate immunity, but not acquired immunity.[6] It is known that DSS induces colitis in mice, irrespective of strain. Here, C57BL/6 mice were used in colitis induction with DSS, which strain was also used to compare experimental colitis in another transgenic mice in C57BL/6 background (unpublished data). As shown in Fig. 1 and 2, atorvastatin did not relieve acute colonic inflammation induced by DSS, and this resulted in disease progression. In the TNBS colitis model, colonic inflammation can be induced by either a single sensitizing dose[16] or by re-challenge after initial sensitization.[7] However, several studies show that the induction of coli-

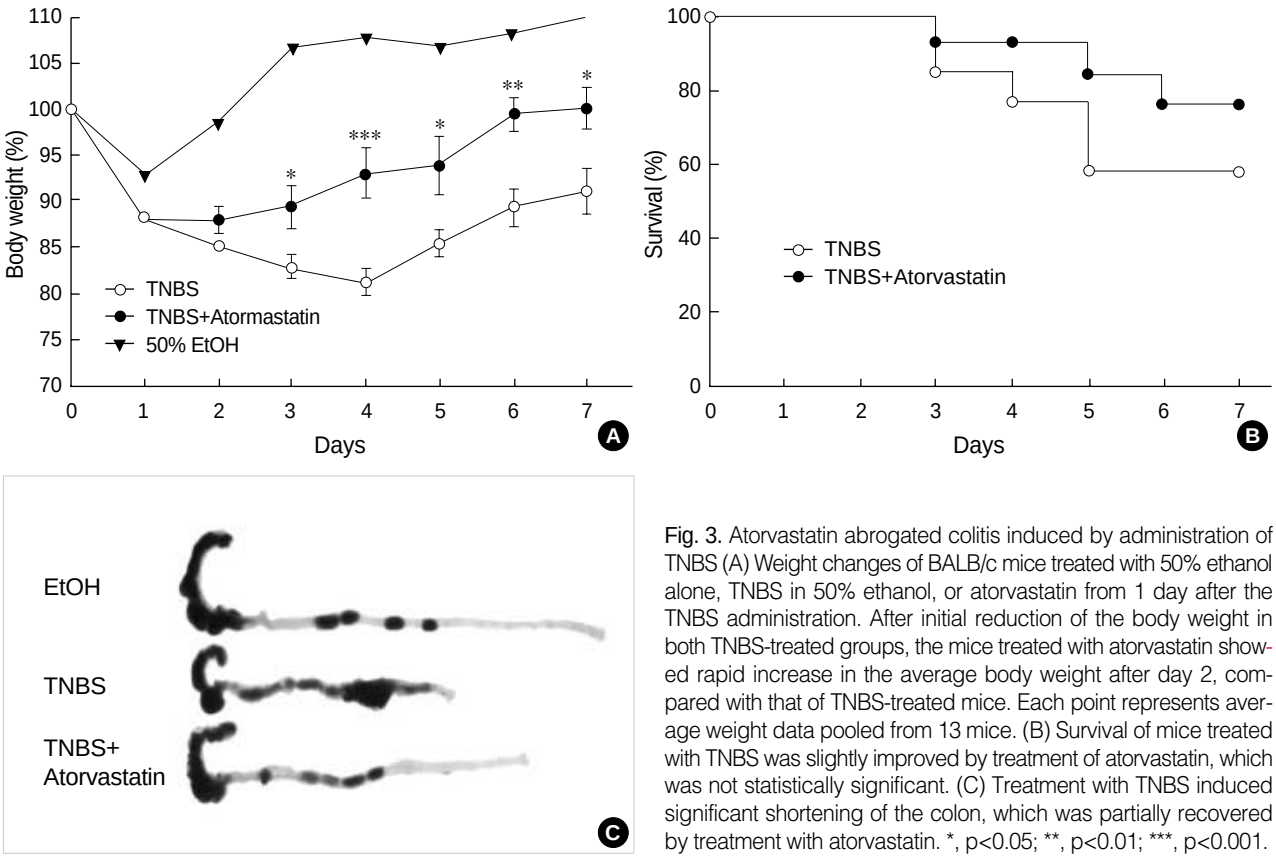

Fig. 3. Atorvastatin abrogated colitis induced by administration of TNBS (A) Weight changes of BALB/c mice treated with 50% ethanol alone, TNBS in 50% ethanol, or atorvastatin from 1 day after the TNBS administration. After initial reduction of the body weight in both TNBS-treated groups, the mice treated with atorvastatin showed rapid increase in the average body weight after day 2, compared with that of TNBS-treated mice. Each point represents average weight data pooled from 13 mice. (B) Survival of mice treated with TNBS was slightly improved by treatment of atorvastatin, which was not statistically significant. (C) Treatment with TNBS induced significant shortening of the colon, which was partially recovered by treatment with atorvastatin. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

tis with a single dose of TNBS produces inconsistent results, and thus have raised the question as to whether other non-immunologic mechanisms are involved.[7,16] Thus, in the present study, mice were sensitized by the epicutaneous application of TNBS solution, followed by the administration of TNBS per rectum 7 days later as described in Materials and Methods. The administration of TNBS per rectum leads to colonic inflammation in susceptible (BALB/c or SJL/J) but not in the resistant (C57BL/6J) strain. The strain used also influences disease manifestation; TNBS-mediated colitis has a Th1 character in the SJL/J mouse strain, whereas it shows a mixed Th1/Th2 character in the BALB/c background.[17] It is known that an initial Th1 response is followed by a later Th2 response in BALB/c mouse treated with TNBS. In the present study we used the BALB/c mouse, and found that colonic inflammation was successfully induced by TNBS treatment (Fig. 3, 4). The infiltration of inflammatory cells and epithelioid histiocytes was seen in all layers of the intestine of this model. In addition, lymphocytic infiltration and lymphoid tissue hypertrophy were frequently noted in TNBS treated mice. These findings suggest that the colitis seen in TNBS treated BALB/c mice might be induced by acquired immunity, i.e., a T-helper response. Unlike DSS induced colitis, TNBS induced colonic inflammation was prevented by atorvastatin treatment in BALB/c mice. Taken together with these current results, it is suggested that orally administrated atorvastatin modulates immune response induced by acquired immunity, especially by CD4 T cells and has little effect on inflammation caused by innate immunity.

However, Sasaki *et al*. reported that intraperitoneally injected pravastatin relieved DSS-induced colitis by preventing leukocyte infiltration and gut injury, probably by increasing eNOS (constitutive NO synthetase) expression and activity.[18] This result is not consistent with the findings of the present study. The effects and the potencies of statins varied widely depending on the form of statins, for example, atorvastatin is known to be the most powerful MHC class II repressor,[13] whereas the role of atorvastatin in the modulation of NOS has not been reported. Therefore, the different statins are likely to have different effects on experimental colitis.

Statins may have multiple targets with respect to immune modulation.[12,13,19,20] Certain statins bind LFA-1 and inhibit its interaction with ICAM-1 in T-cell adhesion/co-stimulation.[19] Other recognized effects of statins are mediated via the inhibition of the mevalonate pathway. Mevalonate is a substrate in cholesterol biosynthesis, but it also participates in the post-translation-

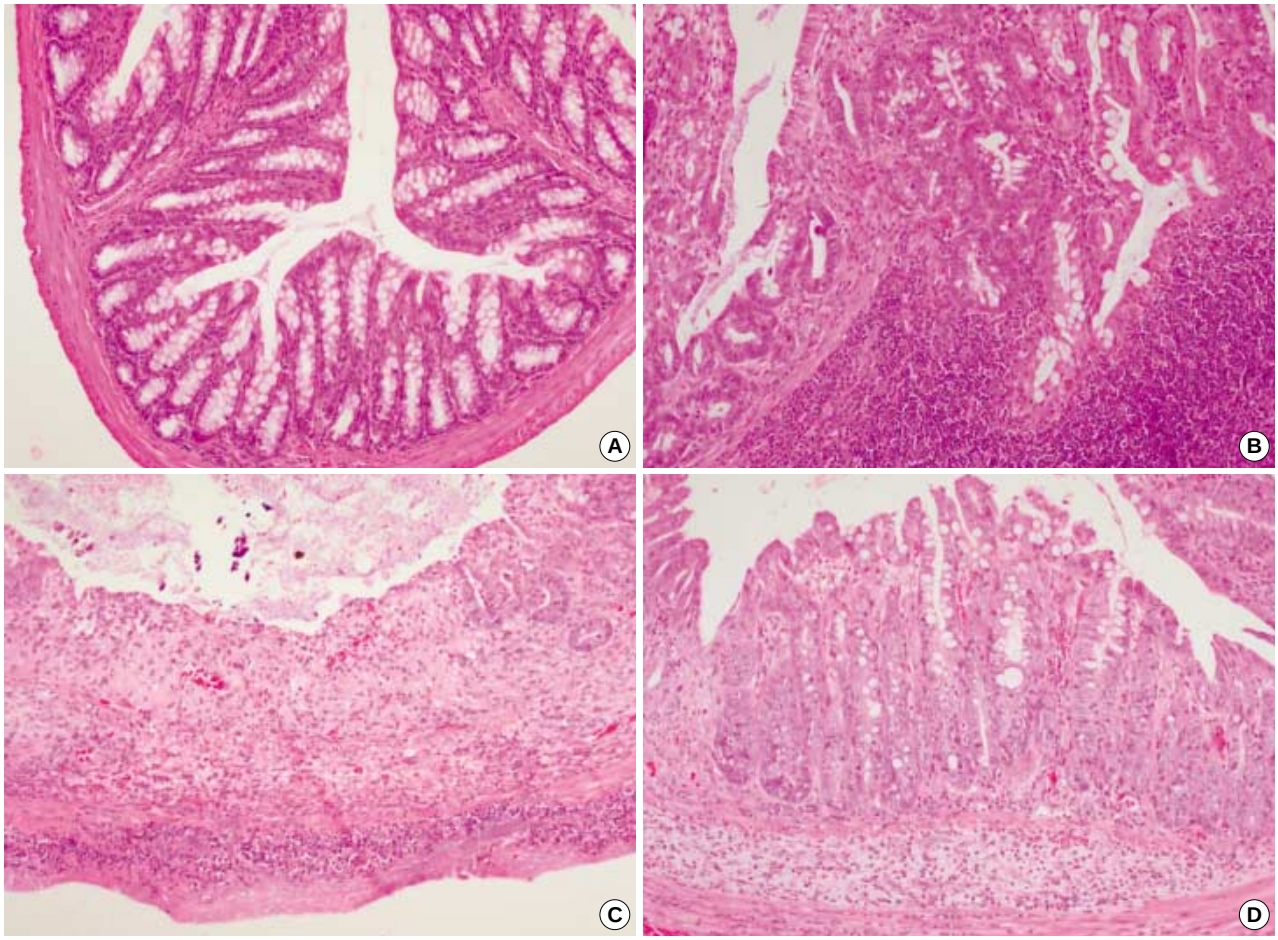

Fig. 4. Histology of colonic samples taken from mice receiving 50% ethanol (control), TNBS, or TNBS+atorvastatin. Compared with that of control mice (A), colon from TNBS-treated mice on day 4 after the challenge of TNBS per rectum shows lymphocytic infiltration in mucosa (B) loss of epithelial lining, and severe transmural inflammatory cell infiltration (C). Treatment of atorvastatin 1 day after TNBS challenge (D) strikingly reduces the inflammatory activity of the colon showing mild cellular infiltrate, whereas epithelial architecture is preserved in these mice.

al modification of proteins involved in cell division and maturation.[21] Statins were found to inhibit the production of proinflammatory cytokines and chemokines, and these effects were reversed by mevalonate.[12,20] More recently, Youssef *et al*. demonstrated that the inhibitions in expression of IFN-γ-inducible CIITA, MHC class II, and costimulatory molecules on APC induced by atorvastatin are mediated via the inhibition of the mevalonate pathway.[14] Thus atorvastatin may more effectively suppress Th-mediated immune response than innate immune reactions, as is shown by current study.

We have demonstrated that atorvastatin treatment suppressed TNBS-induced colitis in BALB/c mice, another Th mediated autoimmune disease model. As statins have modes of action that differ from currently approved IBD treatment modalities, they may be useful in combination therapy. Our results also provide a rationale for testing atorvastatin in other organ-specific Th-mediated autoimmune disease, including diabetes and rheumatoid arthritis.

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