Cyclosporine regulates intestinal epithelial apoptosis via TGF-β-related signaling

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Cyclosporine regulates intestinal epithelial apoptosis via TGF-β-related signaling. Am J Physiol Gastrointest Liver Physiol 297: G514–G519, 2009. First published July 16, 2009; doi:10.1152/ajpgi.90608.2008.—Cyclosporine is a potent immunomodulator and has a beneficial effect in the treatment of ulcerative colitis (UC). We analyzed the mechanism of the effects of cyclosporine on the regulation of epithelial apoptosis via TGF-β-related signaling, because the balance between the apoptosis and regeneration of epithelial cells seems to be a key factor to maintain the intestinal homeostasis. For this purpose, colitis was induced by treatment of 4% dextran sulfate sodium (DSS), and the effect of treatment with cyclosporine and anti-TGF-β antibody was assessed. Treatment with cyclosporine ameliorated body weight loss, mucosal destruction, and epithelial apoptosis in DSS-induced colitis. Cyclosporine was shown to upregulate the expression of TGF-β in the colonic tissue, enhance the expression of p-Smad2 and cFLIP in epithelial cells, and inhibit caspase-8 activity but not caspase-1 or -9. Upregulation of cFLIP in the colonic epithelial cells, amelioration of body weight loss, and mucosal destruction by cyclosporine were attenuated by anti-TGF-β antibody treatment. These results indicated that cyclosporine could have a protective role against epithelial apoptosis associated with upregulation of TGF-β-related signaling.

caspase-8; dextran sulfate sodium; Smad2; cFLIP; ulcerative colitis

CROHN’S DISEASE (CD) and ulcerative colitis (UC), the major forms of inflammatory bowel disease (IBD), are chronic, relapsing, immunologically mediated disorders. Although the etiology and pathogenesis of IBD are not fully defined yet, a growing body of work suggests that defects of immune regulation, diminished barrier integrity, and alteration in pattern recognition receptors expressed in epithelial cells exacerbate the pathogenesis of IBD (13, 32).

Intestinal epithelial cells play an important role in mucosal immune responses in the homeostatic conditions, and also participate in the pathogenesis of IBD (25). Toll-like receptors on the epithelial cells (6), intestinal defensins (29), and the balance between the apoptosis and regeneration of epithelial cells (24) seem to be key factors to maintain the intestinal homeostasis. The frequency of epithelial apoptosis is considerably increased in both UC (26) and CD (31), which is thought to contribute to the impairment of intestinal barrier function. Moreover, many studies clarified that increased apoptosis of intestinal epithelial cells is closely associated with the onset of mucosal destruction, such as deletion of epithelial NEMO (17), T-bet deficiency in innate immune system (8), and loss of TGF-β-related signaling pathway (3, 20). In this regard, the regulation of epithelial apoptosis might be a target of therapeutic reagents.

Cyclosporine is an immunosuppressive macrolide that inhibits the production of interleukin 2 by activated T lymphocytes through a calcineurin-dependent pathway (21). In addition, cyclosporine induces the synthesis of TGF-β in various cell types via autocrine mechanisms (1, 10, 23, 28), and TGF-β also regulates the synthesis of other inflammatory cytokines (19). The efficacy of intravenous cyclosporine in the treatment of severe UC has been confirmed by several clinical trials (11, 27). In fact, the treatment with cyclosporine for severe colitis has been shown to reach immediate clinical improvements and remission induction. However, the precise mechanisms how cyclosporine ameliorates intestinal destruction are not well defined yet.

Therefore, we hypothesized that the effect of cyclosporine would be associated with the regulation of intestinal epithelial apoptosis via TGF-β-related signaling. The present study demonstrated that cyclosporine ameliorated mucosal destruction through reduction of epithelial apoptosis in dextran sulfate sodium (DSS)-induced colitis. Cyclosporine was shown to upregulate the expression of TGF-β in the colonic tissue, enhance expression of cellular Fas-associated death domain-like IL-1-converting enzyme-like inhibitory protein (cFLIP) in epithelial cells, and inhibit caspase-8 activity. These results indicated that cyclosporine could have a protective role against epithelial apoptosis associated with upregulation of TGF-β-related signaling.

MATERIAL AND METHODS

Animals. Six- to 8-wk-old C57BL/6 female mice were purchased from Clea Japan. All mice were kept in a specific pathogen-free environment. This study was carried out in accordance with the Guidelines for Animal Experimentation of Hirosaki University.

Induction of colitis. Colitis was induced in animals by treatment 4% DSS dissolved in drinking distilled water (molecular weight 5,000; Wako Pure Chemical, Osaka, Japan), as described previously (18).

Treatment with cyclosporine and anti-TGF-β antibody. Cyclosporine was dissolved in pharmaceutical grade olive oil with sonication before use (16), and 20 mg/kg of cyclosporine were daily injected intraperitoneally. This treatment was started 1 day before DSS treatment. When DSS treatment was started, mice were injected intraperitoneally with 10 mg/ml of monoclonal antibody against TGF-β1,2 (2 mg/mouse, 1D11.16.8, mouse IgG1, American Type Culture Collection). Isotype-matched IgG was injected as a control (20).

Histological assessment of colon sections. Paraffin-embedded sections from colonic tissues were cut and stained with hematoxylin and eosin (H&E). Histological severity was graded on a scale of 0 to 3 (0, normal; 1, slight; 2, moderate; 3, severe) as cell infiltration into lamina...
propria, appearance of erosions, decrease of crypts and glands, and height of epithelium, as previously described (20).

Analysis of intestinal epithelial cell apoptosis. Paraffin sections of colonic tissue were analyzed with the ApoTAQ kit (Oncor-Appligene, Heidelberg, Germany), according to the manufacturer’s instruction. Terminal deoxynucleotidyl transferase-mediated UTP-biotin nick-end labeling (TUNEL)-positive images were detected by microscopy.

Isolation of IECs. Intestinal epithelial cell (IECs) were freshly purified as previously described (14). In brief, mice were anesthetized by inhalation of isoflurane and the abdomen was opened. The thoracic cavity was opened and perfused through the left ventricle with 10 ml of 30 mmol/l EDTA in Hanks’ balanced salt solution (HBSS). At the end of perfusion, entire colon excluding cecum was removed, inverted, and placed in a cold tube with 2 ml of cold HBSS. The tube was shaken by a minibeater, and the tissue remnants were discarded. The crypts in the supernatant were collected and washed three times with HBSS.

Western blotting and caspase activity. Freshly isolated IECs or colonic tissues were lysed in lysis buffer containing 100 mM HEPES, 10% sucrose, 0.1% 3-[3-cholanidopropyl]dimethyl-ammonio] -1-propanesulfonate, 10 mM dithiothreitol, 1 mM EDTA, protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany), at pH 6.8, followed by a 30-min incubation on ice. Lysis was completed by two 10-s sonications. Protein contents were determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA). Cytosolic extracts (20 μg) were fractionated by 15% sodium dodecyl sulfate-polyacylamide gel electrophoresis and electrotransferred to Immobilon p15 membranes (Millipore, Bedford, MA). Protein expression was detected by Western blot analysis with anti-FLIPL primary antibody (antibody dilution; 1:5,000, Upstate Biotechnology, Lake Placid, NY), anti-Smad2 primary antibody (1:2,000, Cell Signaling

Fig. 1. Cyclosporine ameliorated the weight loss in dextran sulfate sodium (DSS)-induced colitis. Graphical representation of the average percent of weight loss for cyclosporine-treated mice and vehicle-treated mice compared with the weight at the start of DSS treatment. The data shown are means ± SE for 6 mice per group. **P < 0.05 and *P < 0.01 vs. vehicle-treated mice.

Fig. 2. Cyclosporine ameliorated mucosal injury via epithelial apoptosis. Colonic sections of vehicle-treated mice and cyclosporine-treated mice were stained with hematoxylin and eosin on day 5. A: histology of the colons in vehicle-treated mice and cyclosporine-treated mice at ×40 and ×200 magnification. B: histological scores of colitis in vehicle-treated mice (open bar) or cyclosporine-treated mice (solid bar) were calculated. Data shown are means ± SE for 6 mice per group. *P < 0.01 vs. vehicle-treated mice. C: terminal deoxynucleotidyl transferase-mediated UTP-biotin nick-end labeling (TUNEL)-labeled apoptotic epithelial cells were detected in the colonic sections of vehicle-treated mice or cyclosporine-treated mice on day 4 after DSS treatment (original magnification, ×40 and ×200). D: apoptotic index was calculated as the number of TUNEL-positive cells per 100 consecutive crypts. TUNEL-labeled apoptotic epithelial cells were increased in the colonic sections of vehicle-treated mice (open bar) compared with cyclosporine-treated mice (solid bar) on day 4 after DSS treatment. Data shown are means ± SE for 6 mice per group. *P < 0.01 vs. vehicle-treated mice.
Technology. Beverly, MA), anti-phospho-Smad2 primary antibody (1:2,000, Cell Signaling Technology), anti-β-actin antibody (1:5,000, Cell Signaling Technology), and horseradish peroxidase-conjugated secondary antibody (1:5,000–10,000, GE Healthcare, Chalfont St Giles, UK). The visualization of the blots was carried out by use of chemiluminescent substrate (ECL detection kit). Caspase-1, -8, -9 activities in purified IECs were measured by using the colorimetric protease assay kit (Bio-Rad Research Products, Mountain View, CA), as described previously (20).

Cytokine-specific ELISA. Proteins were extracted from the colonic tissues. Protein contents were determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA). TGF-β concentrations of these fractions were measured by enzyme-linked immunosorbent assay (ELISA) kit (BioSource International, Camarillo, CA).

Statistical analysis. Data were expressed as mean ± SE and the Student’s t-test or Mann-Whitney U-test were used to determine the significance of the differences of the body weight loss, numbers of TUNEL-positive cells, histological scores and the level of cFLIP expression between the control and experimental groups. P values of <0.05 or 0.01 were considered significant.

RESULTS

Cyclosporine inhibited mucosal destructions in DSS-induced colitis. To confirm the therapeutic efficacy of cyclosporine in DSS-induced colitis, we assessed clinical signs and changes in body weight of mice. After DSS treatment, mice treated with vehicle showed rectal bleeding with diarrhea at day 5. In contrast, these clinical signs were not observed in cyclosporine-treated mice by day 7 after initiation of DSS treatment. The body weight decreased to 91.4 ± 6.5% on day 6 and to 84.3 ± 6.8% on day 7 in mice treated with DSS plus vehicle. In contrast, body weight fell to 95.9 ± 3.2% on day 6 (P < 0.05, vs. vehicle-treated group) and to 94.1 ± 3.5% on day 7 (P < 0.01, vs. vehicle-treated group) when mice were treated with DSS plus cyclosporine (Fig. 1).

Cyclosporine ameliorated DSS-mediated mucosal injury and epithelial apoptosis. Histological changes in DSS-treated mice were examined by H&E staining to evaluate the effectiveness of cyclosporine against mucosal destruction. Histological scores were calculated based on the scoring system previously described (20). On day 5, severe mucin depletion and epithelial destruction with marked infiltration of mononuclear cell were observed in mice treated with DSS plus vehicle. On the other hand, epithelial destruction and severe crypt loss were not observed in mice treated with DSS plus cyclosporine (Fig. 2A). There was significant difference in histological scores between these two groups (Fig. 2B).

To evaluate the effect of cyclosporine on epithelial apoptosis, we assessed apoptotic epithelial cells in colonic sections from cyclosporine-treated mice or vehicle-treated mice on day 4 after DSS treatment. TUNEL-positive cells in vehicle-treated mice were significantly increased on day 4, compared with those in cyclosporine-treated mice (Fig. 2C). We calculated the number of epithelial cells that expressed TUNEL-positive signals per 100 consecutive crypts as an apoptotic index. Apoptotic index in cyclosporine-treated mice was significantly reduced compared with that in vehicle-treated mice (Fig. 2D). These results suggested that cyclosporine might have a protective effect against mucosal destruction via epithelial apoptosis in DSS-induced colitis.

Upregulation of TGF-β associated with cyclosporine attenuation of DSS-induced mucosal destruction. We assessed the expression of TGF-β in the colon from vehicle- or cyclosporine-treated mice on days 0 to 4 after DSS treatment. The levels of TGF-β expression in mice treated with DSS plus cyclosporine were significantly higher than that in vehicle-treated mice on days 1 and 2 after DSS treatment (Fig. 3A). These results suggested that the protective effect of cyclosporine was closely related to upregulation of TGF-β in the colon.

To clarify the role of TGF-β, we investigated whether blockade of TGF-β would have an impact on the protective effect of cyclosporine in DSS-induced colitis. The positive effects of cyclosporine on DSS-induced loss of body weight were attenuated in mice treated with anti-TGF-β antibody (P < 0.05, Fig. 3B).
To evaluate the mechanism of TGF-β-mediated effects, we blocked TGF-β with antibody and assessed epithelial cell apoptosis in colonic sections from either cyclosporine-treated mice or their vehicle-treated controls on day 4 after DSS treatment. Histological scores were calculated based on the scoring system previously described (20). On day 5, with blockade of TGF-β, severe mucin depletion and epithelial destruction with marked infiltration of mononuclear cell were observed in both of vehicle-treated and cyclosporine-treated mice (Fig. 4A). There was no significant difference in histological scores between these two groups (Fig. 4B). TUNEL-positive cells in cyclosporine-treated mice were not significantly reduced on day 4, compared with those in vehicle-treated mice (Fig. 4C). In addition, the apoptotic index in cyclosporine-treated mice was not significantly reduced compared with that in vehicle-treated mice (Fig. 4D). These results suggested that cyclosporine might have its protective effect in DSS-induced colitis via TGF-β-mediated antiapoptotic activity in mucosal epithelial cells.

To confirm the activation of TGF-β-associated cascade in intestinal epithelial cells, we further investigated that the expression of Smad protein in purified epithelial cells. Smad2 phosphorylation in intestinal epithelial cells from cyclosporine-treated mice was remarkably increased compared with that from vehicle-treated mice on day 2 after DSS treatment (Fig. 5). These results supported that TGF-β upregulation might underlie the protective effect of cyclosporine treatment against epithelial destruction through apoptosis.

Cyclosporine regulates epithelial apoptosis through the regulation of cFLIP. To investigate the regulatory molecules in apoptosis of IECs, we assessed the expression of cFLIP and activities of caspase-1, -8, and -9 in isolated IECs. cFLIP is one of the antiapoptotic factors that inhibits death-receptor-mediated apoptosis by caspase-8 and -10 activation. The expression of cFLIP in IECs from cyclosporine-treated mice was significantly upregulated compared with that from vehicle-treated mice on day 2 after DSS treatment (Fig. 6, A and B). However, upregulation of cFLIP might underlie the protective effect of cyclosporine treatment against epithelial destruction through apoptosis.

Fig. 4. Effect of cyclosporine was associated with TGF-β activity. A: histology of the colons in vehicle-treated mice and cyclosporine-treated mice with blockade of TGF-β at ×40 and ×200 magnification. B: histological scores of colitis in vehicle-treated mice (open bar) or cyclosporine-treated mice (solid bar) with blockade of TGF-β were calculated. Data shown are means ± SE for 6 mice per group. C: TUNEL-labeled apoptotic epithelial cells were detected in the colonic sections of vehicle-treated mice or cyclosporine-treated mice with blockade of TGF-β on day 4 after DSS treatment (original magnification, ×40 and ×200). D: apoptotic index was calculated as the number of TUNEL-positive cells per 100 consecutive crypts. TUNEL-labeled apoptotic epithelial cells in the colonic sections of vehicle-treated mice (open bar) compared with cyclosporine-treated mice (solid bar) with blockade of TGF-β on day 4 after DSS treatment were calculated. Data shown are means ± SE for 6 mice per group.

Fig. 5. TGF-β-associated cascade in intestinal epithelial cells were activated by cyclosporine treatment. The expression of Smad protein in purified epithelial cells was analyzed by Western blotting. Smad2 phosphorylation in intestinal epithelial cells (IECs) from cyclosporine-treated mice was remarkably increased compared with that from vehicle-treated mice on day 2 after DSS treatment. Data shown are representative of 2 independent experiments giving similar results.
cFLIP in IECs by cyclosporine treatment was not observed with concurrent anti-TGF-β antibody treatment (Fig. 6, A and B). In addition, the activity of caspase-8 in IECs from cyclosporine-treated mice was significantly reduced compared with that from vehicle-treated mice (Fig. 6C). There was no significant difference in caspase-1 and -9 activities of IECs between vehicle and cyclosporine treatment.

**DISCUSSION**

Cyclosporine is a potent immunomodulator and beneficial therapeutics for treatment of UC. Cyclosporine treatment of continuous intravenous infusion showed the high response rates among patients with severe UC refractory to cortico-steroids (11, 27). However, the precise mechanism of improvement has not been fully defined. Here we showed that cyclosporine had a protective effect for IECs from the destruction via apoptosis in DSS-induced colitis.

TGF-β has known to play an important role to protect from intestinal epithelial destruction (3, 7, 20), and cyclosporine induces the synthesis of TGF-β in various cell types via autocrine mechanisms (1, 10, 23, 28). In the present study, we showed that the treatment with cyclosporine upregulated the expression of TGF-β in the colon on days 1 and 2 after DSS administration. TGF-β-associated signaling, including the phosphorylation of Smad2, in intestinal epithelial cells was significantly upregulated in cyclosporine-treated mice, compared with control mice. In addition, blockade of TGF-β significantly diminished the protective effect of cyclosporine. Together, these results indicated that cyclosporine ameliorated DSS-induced mucosal destruction via a mechanism involving TGF-β signaling in intestinal epithelial cells.

Intestinal epithelial barrier function comprised with intestinal epithelial cells is important in the dynamic frontline defense response to luminal stimuli (32). Modulation of epithelial cell growth and apoptosis and differentiation function as repair mechanisms to maintain barrier integrity (5, 22, 32). Previous studies showed that FasL-mediated epithelial apoptosis might lead to a breakdown of the epithelial barrier function in UC (24, 26). Moreover, it was reported that increased epithelial apoptosis in the deletion of epithelial NEMO (17) or the deletion of TGF-β-related signal pathway (3, 7, 20) is involved in the pathogenesis of mucosal destruction. As cyclosporine has been shown to improve DSS-colitis model (2, 12, 15), precise mechanisms of epithelial destruction has not been well defined. In the present study, cyclosporine treatment inhibited the increased epithelial apoptosis and ameliorated mucosal destruction in DSS-induced colitis associated with TGF-β. These findings indicated that one of the beneficial effects of cyclosporine seemed to regulate epithelial apoptosis.

Caspases are cysteine proteases that are activated through death receptors like FasL and TNF-α receptor. FLIP has been described as a natural inhibitor of Fas and TNF-α-mediated apoptosis (9). Recent studies showed that upregulation of cFLIP in intestinal epithelial cells increased the resistance to apoptosis (4, 30). Our present data showed that cyclosporine reduced epithelial apoptosis in DSS-induced colitis to attenuate the balance between cFLIP and caspase-8 activity. Furthermore, blockade of TGF-β diminished the upregulation of cFLIP in intestinal epithelial cells by cyclosporine treatment, indicating that increased cFLIP expression in epithelial cells was associated with the upregulation of TGF-β in the colon.

In conclusion, our present study indicates that cyclosporine could attenuate the balance between cFLIP and caspase-8 activity in intestinal epithelial cells via TGF-β signaling pathway. Treatment with cyclosporine ameliorated DSS-induced mucosal destruction through the increase of resistance to epithelial apoptosis. Our findings about the potential role of cyclosporine in the epithelial apoptosis regulation would provide a new clue to understand the mucosal homeostasis and pathophysiology of UC.
REFERENCES


