Clotrimazole Ameliorates Intestinal Inflammation and Abnormal Angiogenesis by Inhibiting Interleukin-8 Expression through a Nuclear Factor-κB-Dependent Manner

Dinesh Thapa, Jong Suk Lee, Su-Young Park, Yun-Hee Bae, Soo-Kyung Bae, Jun Bum Kwon, Kyoung-Jin Kim, Mi-Kyoung Kwak, Young-Joon Park, Han Gon Choi, and Jung-Ae Kim

College of Pharmacy, Yewungnam University, Gyeongsan, South Korea (D.T., J.S.L., S.-Y.P., J.B.K., K.-J.K., M.-K.K., H.G.C., J.-A.K.); School of Medicine, Pusan National University, Pusan, South Korea (Y.-H.B., S.-K.B.); and Research Center, Samil Pharmaceutical Co. Ltd., Ansan, South Korea (Y.-J.P.)

Received June 16, 2008; accepted August 25, 2008

ABSTRACT

Increased interleukin (IL)-8 plays an important role not only in activation and recruitment of neutrophils but also in inducing exaggerated angiogenesis at the inflamed site. In the present study, we investigated the fact that clotrimazole (CLT) inhibits intestinal inflammation, and the inhibitory action is mediated through suppression of IL-8 expression. In the trinitrobenezene sulfonic acid (TNBS)-induced rat colitis model, CLT dose-dependently protected from the TNBS-induced weight loss, colon ulceration, and myeloperoxidase activity increase. In the lesion site, CLT also suppressed the TNBS-induced angiogenesis, IL-8 expression, and nuclear factor (NF)-κB activation. In a cellular model of colitis using tumor necrosis factor (TNF)-α-stimulated HT29 colon epithelial cells, treatment with CLT significantly suppressed TNF-α-mediated IL-8 induction and NF-κB transcriptional activity revealed by a luciferase reporter gene assay. Furthermore, cotreatment with CLT and pyrrolidine dithiocarbamate, a NF-κB inhibitor, synergistically reduced the NF-κB transcriptional activity as well as IL-8 expression. In an in vitro angiogenesis assay, CLT suppressed IL-8-induced proliferation, tube formation, and invasion of human umbilical vein endothelial cells. The in vivo angiogenesis assay using chick chorioallantoic membrane also showed that CLT significantly inhibited the IL-8-induced formation of new blood vessels. Taken together, these results suggest that CLT may prevent the progression of intestinal inflammation by not only down-regulating IL-8 expression but also inhibiting the action of IL-8 in both colon epithelial and vascular endothelial cells during pathogenesis of intestinal inflammation.

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the gastrointestinal tract. Patients with IBD suffer from rectal bleeding, severe diarrhea, abdominal pain, and weight loss. Although the etiology of IBD has not been fully elucidated, many studies have suggested that multiple factors, genetic, immunologic, and environmental factors, all contribute to the pathogenesis of IBD (Fiocchi, 1998). The common pathological changes associated with IBD include disruption of epithelial barrier, infiltration of dysregulated immune cells, and production of proinflammatory mediators including chemokines.

Chemokines are essential for activating and recruiting inflammatory cells. Interleukin (IL)-8, a multifunctional member of the chemotactic CXC cytokines, has been reported to be up-regulated in the colon of patients with IBD (Banks et al., 2003; Subramanian et al., 2008). Furthermore, it has been shown that the severity of inflammation in the colon is congruent with the IL-8 expression (Mazzucchelli et al., 1994;...
Daig et al., 1996). In normal tissues, IL-8 expression is low or undetectable, whereas upon stimulation by various stimuli including tumor necrosis factor (TNF)-α, IL-8 expression can be induced up to 100-fold (Baggerlini et al., 1994). In contrast to the constitutively expressed chemokines that direct baseline leukocyte trafficking, the inducible chemokines such as IL-8 further attracts leukocytes to local sites and aggravates inflammation (Laing and Secombes, 2004).

Angiogenesis, the process of new capillary formation from pre-existing vessels, is thought to be intrinsic to inflammation. Inflammatory mediators either directly or indirectly promote angiogenesis, which, in turn, contributes to inflammatory pathology by allowing further recruitment of inflammatory cells (Jackson et al., 1997). Recent studies showing that excessive angiogenesis in IBD is particularly prominent in areas of active inflammation indicates that angiogenesis is an integral component of IBD pathogenesis (Saito et al., 2003; Danese et al., 2007). In addition to its proangiogenic action, IL-8 also acts as an angiogenic factor by directly enhancing endothelial cell survival, proliferation, and metalloproteinase production (Heidemann et al., 2003; Li et al., 2003). Thus, the development of strategies to block IL-8 production or IL-8-induced actions may hold a promise in treating chronic inflammatory diseases like IBD.

Clotrimazole (CLT) is an antifungal imidazole derivative that has been in clinical use for more than 20 years. Recent studies have shown that CLT inhibits proliferation of human cancer cells (Khalid et al., 2005) and vascular endothelial cells, leading to inhibition of growth factor-stimulated angiogenesis (Takahashi et al., 1998) and, thus, tumor growth (Belo et al., 2004). However, it has not been reported whether CLT modulates inflammatory angiogenesis. In this study, we investigated the fact that CLT suppresses intestinal inflammation by down-regulating proinflammatory cytokine IL-8 expression and blocking the epithelial and endothelial responses to IL-8.

**Materials and Methods**

**Materials.** The recombinant human IL-8 (also called CXCL8) was purchased from R&D Systems (Minneapolis, MN). An endothelial growth medium-2 bullet kit and endothelial growth medium-2 SingleQuotes [hydrocortisone, human basic fibroblast growth factor, vascular endothelial growth factor (VEGF), R3-IGF-1, ascorbic acid, human epidermal growth factor, heparin, gentamicin, and fetal bovine serum (FBS)] were purchased from Cambrex (San Diego, CA). HEPEs, trypsin/EDTA, and trypsin neutralizing solution were purchased from Lonza Walkersville, Inc. (Walkersville, MD). The Matrigel was purchased from BD Biosciences (Bedford, MA). Trinitrobenzene sulfonic acid (TNBS), pyrrolidine dithiocarbamate (PDTC), 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyl tetrazolium bromide (MTT), sodium pyruvate, dimethyl sulfoxide, protease inhibitor cocktail, and SDS were obtained from Sigma-Aldrich (St. Louis, MO). 5-Aminosalicylic acid (5-ASA) was purchased from Sigma-Aldrich, where as clotrimazole (1-[o-chloro-o,a-diphenylbenzyl]-imidazole) was supplied from Samil Pharmaceutical Co. Ltd. (Ansan, Korea) and Korea United Pharm, Inc. (Seoul, Korea).

**TNBS-Induced Experimental Colitis.** Sprague-Dawley rats (7–8 weeks old) were purchased from Samtaco bio Korea (Osan, Korea). Rats were fasted (able to drink ad libitum) for 24 h before induction of colitis. They were then anesthetized lightly using diethyl ether. Using a polyethylene catheter fitted onto a 1-ml syringe, rats received injections slowly with 0.8 ml of 5% TNBS in 50% (v/v) ethanol into the lumen of the colon (8 cm proximal to the anus through the rectum), and they were kept in a vertical position for 60 s before being returned to their cages. Rats in the control group were handled similarly, but 50% (v/v) ethanol alone was administered instead. To investigate the effect of the drugs, rats were administrated CLT (10–50 mg/kg/day) intraperitoneally for 5 days after the TNBS administration. In our experiments, 5-ASA, an active metabolite of sulfasalazine that is a commonly used drug for IBD, was used as a positive control.

All rats were sacrificed on day 7 after the TNBS administration. The macroscopic ulceration and severity of colitis was evaluated by two independent investigators who were blinded to the treatment. The colon tissues from 5 to 7 cm proximal to the rectum were cut out and used for histologic examination as well as myeloperoxidase (MPO) activity measurement. Animal experiments were performed according to the institutional guidelines of the Institute of Laboratory Animal Resources (1996) and Yeungnam University for the care and use of laboratory animals.

**MPO Activity Measurement.** MPO serves as a marker for tissue neutrophil infiltration. To measure MPO activity, we used a MOP assay kit (ASA-001; CytoStore, Calgary, Alberta, Canada). The 1-cm segment of dissected colon tissues were washed in cold phosphate-buffered saline (PBS) (pH 7.4), weighed, suspended in 0.5 ml of ice-cold sample buffer per 50 mg of tissue, and homogenized for 30 s using a tissue homogenizer at 4°C (Biospec Products Inc., Basel, Switzerland). The homogenized sample was mixed with an additional sample buffer to give a final concentration of 50 mg/ml and centrifuged at 10,000 rpm for 5 min twice. The level of MPO activity in the tissue was measured spectrophotometrically. Each 20 μl of sample solution was mixed with 200 μl of development reagent (12.5 μl of H2O2 containing chromogen powder in phosphate buffer (2.5 ml)), and the absorbance at 450 nm was then measured immediately at 1-min intervals using a spectrophotometer (VersaMax; Molecular Devices, Sunnyvale, CA). One unit of MPO activity was defined by Bradley et al. (1982) as the activity required for converting 1 μM H2O2 at 25°C for 7 min and expressed as units per gram of tissue.

**Western Blot Analysis.** Cytoplasmic or nuclear extracts were collected using an NE-PER (Pierce, Rockford, IL) kit per the manufacturer’s instructions. In brief, colon tissue homogenate and collected HT29 cells were resuspended in 50 μl of cytoplasmic extraction reagent 1 containing protease inhibitor cocktail, then homogenized by vigorous vortexing for 15 s, and left on ice (4°C). After 10 min, 2.3 μl of cytoplasmic extraction reagent II was added and vortexed on the highest setting for 5 s. The homogenates were then centrifuged at 14,000 rpm for 5 min. Supernatant (cytoplasmic fraction) was transferred to a fresh tube and stored at −70°C. To lyse the pellet containing nuclear fraction, 30-μl aliquots of nuclear extraction reagent and protease inhibitor cocktail were added. The pellets were vortexed for 1 min, left on ice (4°C) for 40 min, and centrifuged for 10 min (4°C, 14,000 rpm) to collect the supernatant (nuclear fraction).

The soluble protein concentrations in cytoplasmic or nuclear lysates were determined by a BCA protein assay kit (Pierce). The equal
amount of proteins (15 μg regardless of type of lysate) was separated on SDS-polyacrylamide gel electrophoresis and transferred to a Hybond ECL nitrocellulose membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK) at 200 mA for 1 h. The membranes were blocked in 5% skim milk in Tris-buffered saline (TBS)-Tween 20 (TBST) (20 nM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Tween 20) at room temperature for 1 h. The membrane was incubated with antibodies specific to nuclear factor (NF)κB (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), IκBα (Santa Cruz Biotechnology, Inc.), phospho-IκBα (Cell Signaling Technology, Beverly, MA), cyclooxygenase (COX)-1, COX-2 (Cayman Chemical Co., Ann Arbor, MI), pSTAT3/Tyr705, and STAT3 (Cell Signaling Technology Inc., Danvers, MA) in skim milk-TBS at 4°C overnight. The membrane was then washed three times with TBST and incubated with horseradish peroxidase-conjugated secondary antibody in skim milk-TBS for 1 h at room temperature. The immunoreactive proteins were visualized with an ECL kit (GE Healthcare).

**Histology and Immunohistochemical Examination.** For histological examination, colon tissues from 6 to 7 cm proximal to the rectum were cut out and fixed in 4% paraformaldehyde/PBS. The paraffin-embedded tissue sections (3-μm thickness) were stained with hematoxylin and eosin (H&E) using standard techniques. The histological lesions on the prepared cross-trimmed H&E stained histological samples were evaluated by a pathologist, who was blinded to the experimental groups, according to previously established criteria (Peran et al., 2005) as shown in Table 1. For immunohistochemistry, colon tissues were prepared for frozen section in 7-μm thickness and incubated with rabbit anti-von Willebrand factor (vWF) (Millipore Bioscience Research Reagents, Temecula, CA) overnight at 4°C. Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) was used as a secondary antibody at 1:500 dilutions and incubated for 1 h at room temperature. After mounting with the media containing 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) for nuclear counterstaining, tissue slides were visualized under a fluorescent microscope (Olympus, Tokyo, Japan).

**Reverse Transcription-Polymerase Chain Reaction Analysis.** Total RNA from rat colon tissues and HT29 cells was extracted by using a RNeasy kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer’s instruction and reverse transcribed using a Reverse Transcription Kit (QIAGEN GmbH). Two microliters of cDNA from rat colon and HT29 cells were used for conventional polymerase chain reaction (PCR) and real-time PCR, respectively.

The predicted size of PCR products for rat IL-8 (primer sets sense 5'-ACGCTGGCTTCTGACAACACTAGT-3' and antisense 5'CT-CCTCTGCTCAGACGAAAGG-3') was 496 base pairs. For equal loading normalization, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (sense 5'-ACCACAGTCCATGCCATCAC-3' and antisense 5'-TCCACCACCTGTTGCTGTA-3') was used. PCR products were separated on 1.8% agarose gels and visualized by UV transillumination. Real-time PCR was carried out with a PCR kit (Quantitect SYBR Green; QIAGEN GmbH) using a Rotor-Gene 6000 (Corbett Life Science, Mortlake, Australia) in the condition of 95°C for 15 min followed by 45 cycles of denaturation at 95°C for 5 s, annealing at 55°C for 10 s, and extension at 72°C for 20 s using primer sets specific for human IL-8 (sense 5'-ACCCCCAATTTATCATCAAAG-3' and antisense 5'-TCAAAACTTTCTCCACAAACC-3'), and GAPDH (provided by QIAGEN). The real-time PCR product was normalized

---

**Fig. 1.** CLT improves the clinical and morphological features of TNBS-induced colitis in rats. Colitis was induced by rectal administration of TNBS. The control group received 50% ethanol as a vehicle. A, the body weight was recorded daily from day 1 to day 6. The macroscopic appearance (B), the mean values of the colon length distal to cecum (C), and the wet weight of colon (distal 5–6-cm portion) (D) were analyzed. The data represent the mean ± S.E.M. for 5 rats per group. *p < 0.05, compared with the vehicle-treated control group. #, p < 0.05, compared with the TNBS-treated group.

---
Fig. 2. CLT suppresses the TNBS-induced inflammatory response and pathogenic angiogenesis. A, the MPO activity of colon tissue was measured by using the MPO assay kit and expressed as mean ± S.E.M. for 5 rats per group. B, the expression level of COX-1, COX-2, and NF-κB was detected by Western blotting. The Western blot is a representative of three independent experiments. In the bar graph, relative expression of COX-2 and NF-κB
using GAPDH as an internal control. The fold change over control level was determined according to the comparative cycle threshold method.

**IL-8 Enzyme-Linked Immunosorbent Assay.** The secreted IL-8 level in culture supernatant was quantified by an enzyme-linked immunosorbent assay ELISA kit (R&D Systems) according to the manufacturer’s protocol. The measurements were made in three independent experiments with duplicates.

**Cell Culture.** Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza Walkersville, Inc. HUVECs were maintained in cell culture flasks coated with 0.2% gelatin and cultured with EBM-2 containing hydrocortisone, human basic fibroblast growth factor, VEGF, R3-IGF-1, ascorbic acid, human epidermal growth factor, heparin, gentamicin, and 2% FBS. Confluent cultures of HUVECs were serially passaged, and between passages 2 and 6 were used.

The HT29 human colon cancer cell line and U937 human leukemia cell line were purchased from American Type Culture Collection (Manassas, VA), and the cells were grown in RPMI 1640 (Invitrogen) medium supplemented with 10% FBS, 1% penicillin/streptomycin, 1 mM sodium pyruvate, and 2 mM glutamine. Culture medium was replaced every other day.

**Transient Transfection and Luciferase Assay.** NF-κB transactivation was studied using luciferase reporter plasmids from Stratagene (La Jolla, CA) according to the manufacturer’s instruction. In brief, vector DNA (pNF-κB-Luciferase plasmid) was allowed to form complexes with GeneJammer (Stratagene) at a 0.003% (v/v) final concentration. The DNA-GeneJammer mixture was added to 40 to 50% confluent HT29 cells, which were already washed once in an antibiotic-free complete medium. The transfection was continued at 37°C for 20 h, and the medium was then changed to normal growth medium. After the cells were allowed to grow for another 24 h, the transfected cells were pretreated with CLT or PDTC for 1 h followed by TNF-α for 3 h. The cell lysates were used for luciferase assay using a dual luciferase reporter assay system (Promega, Madison, WI), and the emitted light was measured with a luminometer (Turner BioSystems, Sunnyvale, CA).

**Monocyte Adhesion Assay.** The monocyte-epithelial cell adhesion was evaluated using U937 monocyte cells and HT29 colonic epithelial cells as described previously (Kobayashi et al., 2006). U937 cells were preloaded with BCECF/AM (10 μM) for 1 h at 37°C. HT29 cells in 48-well plates were pretreated with CLT or 5-ASA for 1 h and then stimulated with IL-8 for 3 h. The cells were then coincubated with BCECF/AM-preloaded U937 cells (5 × 10⁵ cells/well) for 40 min at 37°C. The plates were washed twice with PBS to remove nonadhering U937 cells. A set of cells representing each group was taken and imaged by fluorescence microscopy connected to a digital camera (TE2000-U; Nikon, Tokyo, Japan). The cells in the other sets were lysed with 0.1% Triton X-100 in 0.1 M Tris, and the BCECF fluorescence was analyzed by using a Fluostar Optima microplate reader (BMG Labtech GmbH, Offenburg, Germany) using excitation at 485 nm and emission at 520 nm.

**HUVEC Proliferation Assay.** HUVECs plated at a density of 5 × 10⁴ cells/well were incubated in serum-reduced (1% FBS) EBM-2 for 24 h and then cotreated with VEGF (20 ng/ml) in the absence or presence of CLT for 48 h. After incubation, the viable cell number was measured by MTT assay. The cells were incubated with 10 μl of MTT solution (5 mg/ml) added to the medium for 4 h at 37°C. Then, the culture medium was removed, and 200 μl of dimethyl sulfoxide was added to each well to dissolve formazan. Absorbance was measured at 540 nm using a microplate reader (VersaMax; Molecular Devices). The viable cell number was expressed as a percentage of the control culture.

**Tube Formation Assay.** The tube formation assays were performed on 48-well plates coated with 100 μl of Matrigel per well and polymerized at 37°C for 30 min. HUVECs were suspended in EBM-2 medium containing 1% FBS, plated on Matrigel at a density of 1 × 10⁴ cells per well, and then treated with test compounds. After 14 h, four fields of each culture well were randomly selected and photographed with a microscope attached to a CCD camera (TE2000-U; Nikon).

**Matrigel-Invasion Assay.** In vitro invasion assay was performed using Transwell inserts (Corning Inc., Corning, NY), which consisted of a 24-well companion plate with 12 cell culture inserts containing 8-μm pore-size filters. In brief, the upper and lower parts of Transwell inserts were coated with 10 μl of Matrigel (0.5 mg/ml) and 40 μl of type I collagen (0.5 mg/ml), respectively. HUVECs (5 × 10⁴ cells/100 μl) were added to each insert (upper chamber), and the chemotaxtractant (1% FBS) was placed in each well. The cells were incubated for 24 h at 37°C. After incubation, the upper surface of the Transwell was wiped out with a cotton swab to remove noninvading cells. The cells that had invaded to the lower surface of the membrane were fixed with methanol and stained with H&E. Invasion was expressed as the number of invaded cells per microscopic field by counting the cells in randomly chosen microscopic fields. At least five microscopic fields were counted per group, and three independent experiments were performed.

**Chick Chorioallantoic Membrane Assay.** In vivo neovascularization was examined by using chick chorioallantoic membrane (CAM). In brief, 10-day-old embryos were purchased from Baek-ja farm (Cheongsong, Korea) and were incubated at 37°C with 5% relative humidity. A small hole was punctured in the shell concealing the air sac using a hypodermic needle. A second hole was punctured in the shell on the broadside of the egg directly over avascular portion of the embryonic membrane, as observed during candling. A false air sac was created beneath the second hole by the application of negative pressure to the first hole, causing the CAM to be separated from the shell. A window, approximately 1 cm², was cut in the shell over the dropped CAM with the use of a small crafts grinding wheel (Dremel, Racine WI). Sterile filter disks (Whatman No. 1 Filter Paper; Whatman, Maidstone, UK) were soaked in 3 mg/ml cortisone acetate in a solution of 90% ethanol and water and subsequently air dried under sterile conditions. IL-8 was used to stimulate vessel growth on the CAMs of 10-day-old chick embryos. Sterile filter disks absorbed with IL-8 (10 ng/disk) dissolved in PBS containing 0.1% bovine serum albumin were placed on the growing CAMs. Test compounds or a vehicle was then added directly to CAMs topically. After 72 h, CAM tissue directly beneath the disk was resected from the embryo and was harvested under light microscopy (Leica, Wetzel, Germany). The number of vessel branch points contained in a circular region equal to the area of filter disk was counted for each section.

**Data Analysis.** The data are expressed as mean ± S.E.M. of three independent experiments. Statistical analysis was done with a Student’s t test to calculate differences between groups. For histological assessment, a multiple comparison test for different dose groups were conducted using Levene and one-way analysis of variance tests. Statistical analysis was performed using SPSS for Windows (Release 14K; SPSS Inc., Chicago, IL). P values less than 0.05 were considered statistically significant.

---

Inhibition of Intestinal Inflammation via IL-8 Suppression

357

normalized to the actin level was expressed as mean ± S.E.M. C, the colon section of each experimental group was stained with H&E (first column) or incubated with primary rabbit anti-vWF antibody overnight at 4°C. Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG was used as a secondary antibody (middle column). 4′,6-Diamidino-2-phenylindole was used for nuclear counterstaining (third column) (magnification, 200×). D, total RNA was extracted from the colon tissue, and the level of IL-8 mRNA was determined by reverse transcription-PCR. The equal loading of RNA was confirmed using GAPDH as a reference. E, the phospho-STAT-3 and STAT-3 expressions were detected by Western blotting. In the bar graph, relative expression of phospho-STAT-3 normalized to STAT-3 level was expressed as mean ± S.E.M., *p < 0.05, compared with the vehicle-treated control. ¶, p < 0.05, compared with TNBS-treated group.
**Results**

**CLT Ameliorates TNBS-Induced Rat Colitis.** The rats treated with TNBS developed significant signs of colitis, bloody diarrhea, and wasting conditions with sluggish and weak movement. In addition, TNBS induced stunted body weight in contrast to the weight gain in vehicle-treated control groups (Fig. 1A). The colon length distal to cecum was shortened by TNBS (Fig. 1, B and C), whereas the weight of colon tissue was increased by TNBS (Fig. 1D). However, the rats given CLT (10–50 mg/kg) or 5-ASA (100 mg/kg), an active metabolite of sulfasalazine that is a commonly used drug for IBD, for 5 days significantly recovered the body and colon weight (Fig. 1, A and D). To confirm the TNBS-induced colitis, we measured colonic MPO activity, which serves as a marker for tissue neutrophil infiltration, and COX-2 induction. In addition, because many studies have elucidated that NF-κB is actively involved in the regulation of inflammatory genes (Baeuerle and Henkel, 1994; Blackwell and Christman, 1997; Tak and Firestein, 2001), we examined the activation of inflammatory transcription factor NF-κB. TNBS-induced increase in MPO activity (Fig. 2A) and COX-2 and nuclear NF-κB was significantly reduced by CLT (Fig. 2B). The cytosolic IκB-α level was decreased by TNBS, which was blocked by CLT administration. Such inhibitory effects of CLT at 10 mg/kg dose were comparable with 100 mg/kg 5-ASA, and a high dose of CLT (50 mg/kg) almost completely suppressed the MPO activity.

Next, we investigated whether the protective effects of CLT in TNBS-induced colitis is associated with the inhibition of pathogenic angiogenesis and IL-8 reduction by examining histological changes. In histomorphometrical examinations, as shown in Table 2, TNBS induced a significant increase of thickness of mucosa, submucosa, and total colonic walls and increased the number of infiltrated inflammatory cells in both the mucosa and submucosa. However, such histopathological changes were dramatically suppressed by the treatment with 5-ASA and CLT. In addition, the quantitation of microscopic scores of the colon tissues revealed that TNBS induced ulceration, mucous cell depletion, inflammatory cell infiltration, and edematous changes, whereas treatment with CLT or 5-ASA reduced the TNBS-induced changes, which were summarized in Table 3. The lesion site in TNBS-treated rat colon showed that the swollen submucosal layer contained an increased number of blood vessels, which was detected by using anti-vWF antibody (Fig. 2C). The mucosa and submucosal blood vessels in response to the stimulation with TNBS was recovered by the administration of CLT. In addition, IL-8 tissue level was highly increased in the TNBS-treated inflamed colon, and such an abnormal increase was significantly suppressed by the administration of CLT and 5-ASA (Fig. 2D). Because STAT3, a transcriptional regulator of several cytokines including IL-8 (Yeh et al., 2004), is reported to be up-regulated in IBD, we also examined any changes in STAT3. As depicted in Fig. 2E, STAT3 was highly phosphorylated in the TNBS-treated inflamed colon, and such abnormal phosphorylation was significantly suppressed by the administration of CLT and 5-ASA.

**CLT Inhibits TNF-α-Induced IL-8 Expression via Suppression of NF-κB Activation in HT29 Cells.** To examine the mechanism by which CLT regulates IL-8 expression in colon epithelial cells, we used an in vitro model of colitis. Treatment of HT29 colon epithelial cells with TNF-α induced a dramatic increase in IL-8 mRNA expression measured by real-time PCR (Fig. 3A) and protein secretion detected by ELISA (Fig. 3B). However, CLT significantly suppressed the TNF-α-induced IL-8 expression in a concentration-dependent manner. Furthermore, the effective concentration of CLT was almost 1000 times lower than 5-ASA. Because IL-8 expression has been reported to be related to the activation of NF-κB, we also examined whether the inhibitory effect of CLT on the expression of IL-8 is mediated through the regulation of NF-κB activation. As in TNBS-induced rat colitis, TNF-α increased NF-κB nuclear translocation and IκB-α phosphorylation along with IκB-α decrease in HT29 cells (Fig. 3C). Furthermore, TNF-α stimulation (10 ng/ml) led to 7-fold increase in NF-κB transcriptional activity in the cells transfected with NF-κB-Luc plasmid (Fig. 3D). However, treatment of the cells with CLT concentration-dependently inhibited the TNF-α-induced transcriptional activity of NF-κB. Such effects of CLT (5 μM) are similar to those of PDTC (50 μM), a NF-κB inhibitor. Moreover, the combined treatment with CLT (5 μM) and PDTC (50 μM) synergistically suppressed the NF-κB activation. Likewise, cotreatment with PDTC and CLT also synergistically inhibited the TNF-α-induced IL-8 secretion (Fig. 3E).

**Inhibitory Effects of CLT on IL-8-Induced Monocyte Adhesion to Colon Epithelial Cells.** Because IL-8 is known as a chemokine playing an important role in the pathogenesis of IBD (Mazzucchelli et al., 1994), we examined whether CLT inhibits IL-8-induced monocyte adhesion to colon epithelial cells, which is an initial event of colon inflammation. As shown in Fig. 4, CLT significantly reduced the IL-8-induced U937 cell adhesion to HT29 cells. It is noteworthy that such inhibitory action of CLT was effective at concentrations between 1 and 10 μM, which was comparable with the effect of 20 mM 5-ASA.

**Table 2**

Effects of CLT treatment on the colon morphological changes in TNBS-induced rat colitis

<table>
<thead>
<tr>
<th>Histomorphometry Groups</th>
<th>Thicknesses (Cross-Trimmed Sections)</th>
<th>μm</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mucosa</td>
<td>Submucosa</td>
<td>Total Colonic Walls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>254.30 ± 54.09</td>
<td>193.14 ± 14.86</td>
<td>661.18 ± 65.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNBS</td>
<td>481.97 ± 45.21*</td>
<td>522.37 ± 88.35*</td>
<td>1110.56 ± 96.92*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNBS + 5-ASA</td>
<td>302.28 ± 16.09#</td>
<td>261.00 ± 57.73#</td>
<td>801.38 ± 91.29#</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNBS + CLT10</td>
<td>232.42 ± 42.09##</td>
<td>242.97 ± 52.91##</td>
<td>830.02 ± 66.51##</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNBS + CLT50</td>
<td>290.95 ± 40.46###</td>
<td>199.52 ± 42.36###</td>
<td>688.43 ± 86.99###</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p < 0.01 and # p < 0.05 compared with the vehicle-treated control group; ## p < 0.01 compared with the TNBS-treated group.
CLT Inhibits the IL-8-Induced Angiogenesis in Vitro and in Vivo. IL-8 is also known to act as a proangiogenic factor in the inflamed site. Because we found that CLT suppressed the TNBS-induced abnormal angiogenesis in a rat colitis model, we also examined the possibility that CLT inhibits IL-8-induced neovascularization by analyzing each step of angiogenesis, endothelial proliferation, tube formation, and migration, using HUVECs. Treatment with CLT concentration-dependently inhibited the IL-8-induced endothelial cell proliferation (Fig. 5A). When HUVECs were placed on a Matrigel-coated plate in the presence of IL-8 (10 ng/ml), they rapidly aligned with one another and formed tube-like structures within 14 h, and CLT prevented the IL-8-stimulated tube formation in a concentration-dependent manner (Fig. 5B). In an invasion assay using a Matrigel-coated Transwell insert, treatment of the cells with IL-8 (10 ng/ml) increased the number of invaded cells into the lower compartment through the Matrigel by more than 3-fold compared with the control in which only low levels of FBS (1%) were present as a chemoattractant (Fig. 5C). However, CLT significantly suppressed the IL-8-induced invasion of the cells. To further confirm the antiangiogenic effects of CLT, we performed CAM assay, which is an in vivo model of angiogenesis. IL-8 increased the number of newly formed blood vessels, which was assessed as the number of vessel branch points. Such IL-8-induced neovascularization was significantly suppressed by the treatment with CLT in a dose-dependent manner (Fig. 6).

Discussion

CLT, an antymycotic agent, has been reported as a potent growth inhibitor of tumor (Khalid et al., 2005) and vascular endothelial cells (Takahashi et al., 1998). In the present study, we demonstrated that CLT also suppresses intestinal inflammation by inhibiting proinflammatory cytokine expression and pathogenic angiogenesis.

In TNBS-induced colitis, which is a well-established model of intestinal inflammation with some resemblance to human IBD, the histopathological lesions of the colon are generally documented as ulceration, mucus cell depletion, inflammatory cell infiltration, and edematous changes. In the present study, the inhibition of the histopathological changes (Tables 2 and 3) against TNBS-induced colitis by CLT was considered as direct evidence that CLT ameliorated or treated the TNBS-induced colitis. In addition, similar or more favorable inhibitory effects were detected in the CLT (50 mg/kg)-treated group compared with the 5-ASA (100 mg/kg) group, indicating that CLT may be a better drug for IBD treatment.

Several lines of evidence have implicated that the pathogenesis of chronic inflammatory disease, including IBD, involves angiogenesis (Pousa et al., 2008). Based on these notions, the antiangiogenic approach is suggested as a new therapeutic intervention for chronic inflammatory diseases (Danese et al., 2007). Inflammatory cells release proinflammatory cytokines and chemokines, which activate vascular endothelial cells directly or indirectly to make new blood vessels at the inflammatory sites. On the other hand, pathogenic angiogenesis during chronic inflammation may aggravate the inflammatory response by inducing an enhanced supply of nutrients and oxygen, abnormal and dysfunctional vessel architecture, and enhanced recruitment of inflamma-
Fig. 3. Inhibitory effect of CLT on TNF-α-induced IL-8 expression is mediated through suppression of NF-κB activation in HT29 cells. HT29 cells were pretreated with various concentrations of CLT or 20 mM of 5-ASA for 1 h and then stimulated with TNF-α (10 ng/ml). A, total RNA was extracted from the cells 3 h after TNF-α stimulation, and IL-8 mRNA expression was quantified by real-time PCR. B and E, IL-8 protein secretion in culture supernatant was quantified by using an ELISA kit. C, proteins were extracted from the cells stimulated with TNF-α (10 ng/ml) for 15 min. Nuclear
Inhibitory effects of CLT on the IL-8-induced adhesion of U937 cells to HT29 cells. HT29 cells were pretreated with various concentrations of CLT and 20 mM of 5-ASA for 1 h and then stimulated by 10 ng/ml TNF-α. After 3 h, HT29 cells were cocultured with U937 cells, which were already labeled with BCECF-AM (10 μg/ml). The adhesion of BCECF fluorescence-labeled U937 cells to HT29 colon epithelial cells was captured by using a fluorescence microscope (A) and quantitated by using a fluorescence-detecting microplate reader (Fluostar Optima; BMG Labtech GmbH) using excitation at 485 nm and emission at 520 nm (B). Data are means ± S.E.M. from three independent experiments. *, p < 0.05, compared with the vehicle-treated control group. ‡, p < 0.05, compared with the vehicle-treated control group. #, p < 0.05, compared with the IL-8-treated group.

IL-8 alone + 5-ASA (20 mM) B

+ CLT (1 μM) + CLT (5 μM) + CLT (10 μM)

Fig. 4. Inhibitory effects of CLT on the IL-8-induced adhesion of U937 cells to HT29 cells. HT29 cells were pretreated with various concentrations of CLT and 20 mM of 5-ASA for 1 h and then stimulated by 10 ng/ml TNF-α. After 3 h, HT29 cells were cocultured with U937 cells, which were already labeled with BCECF-AM (10 μg/ml). The adhesion of BCECF fluorescence-labeled U937 cells to HT29 colon epithelial cells was captured by using a fluorescence microscope (A) and quantitated by using a fluorescence-detecting microplate reader (Fluostar Optima; BMG Labtech GmbH) using excitation at 485 nm and emission at 520 nm (B). Data are means ± S.E.M. from three independent experiments. *, p < 0.05, compared with the vehicle-treated control group. ‡, p < 0.05, compared with the vehicle-treated control group. #, p < 0.05, compared with the IL-8-treated group.
The inhibitory effect of CLT on IL-8 expression was well correlated with the inhibitory actions of CLT on NF-κB transcriptional activity. Furthermore, the NF-κB-inhibiting activity of CLT was comparable with that of PDTC, a NF-κB inhibitor, in TNF-α-treated HT29 cells (Fig. 3, D and E), representing the fact that the molecular target of CLT action is NF-κB. These findings were further supported by the synergistic effect of CLT in combination with PDTC on the suppression of IL-8 increase.

It is well known that TNF-α preferentially induce activation of NF-κB, whereas many other cytokines activate STAT family. Phosphorylated STAT proteins by kinases such as...
Janus kinases in response to cytokine or growth factor receptor activation migrate into the nucleus and regulate the transcription of cytokine genes. STAT3 activation has been observed in many colitis models and in the colonic mucosa of patients with active IBD (Mitsuyama et al., 2007). In the present study, CLT was able to suppress TNBS-induced phosphorylation of STAT3 in the rat colon tissue. Thus, inhibition of STAT3 activation seems likely to be involved in amelioration of TNBS-induced colitis by CLT. Furthermore, recent reports suggest that STAT-3 is involved in the synthesis of IL-8, a proinflammatory and proangiogenic factor (Yeh et al., 2004). Our results showing that CLT suppressed both STAT-3 phosphorylation and IL-8 expression by TNBS suggest that the regulatory effect of CLT on the IL-8 level is associated with suppression of STAT-3 phosphorylation.

In addition to the well characterized role of IL-8 in the chemoattraction and recruitment of leukocytes into the intestinal mucosa, IL-8 is also known to exert effects on vascular endothelial cells to promote in vitro tube formation (Heidemann et al., 2003) and pathological angiogenesis (Koch et al., 1992). It is noteworthy that such angiogenic action of IL-8 is well documented in tumor-associated angiogenesis and tumor progression (Chen et al., 2003). In recent studies, IL-8 has also been reported to induce inflammatory angiogenesis by regulating endothelial cell proliferation, survival, migration, and matrix metalloproteinase-2 production (Koch et al., 1992; Li et al., 2005). Likewise, our results showed that IL-8 induced endothelial cell proliferation, tube formation, and migration. It is noteworthy that CLT suppressed the IL-8-induced angiogenesis in vitro as well as in vivo (Figs. 5 and 6). Like TNF-α that is an inducer of NF-κB and also a NF-κB-dependent gene product, it has been suggested that IL-8 expression is NF-κB-dependent, and its action not only in inflammation but also in angiogenesis is dependent on NF-κB activity (Manna and Ramesh, 2005). Based on these reports and our results of the inhibitory action of CLT in NF-κB translocation and transcriptional activity, the action of CLT in IL-8-induced angiogenesis is also believed to be mediated through suppression of NF-κB.

Although we clearly demonstrated that CLT down-regulates the IL-8 expression and inflammatory angiogenesis in the colon tissue of rats, we cannot eliminate the possibility that other cytokines may be the prime target of CLT action. TNBS-induced colitis is a model of T-helper 1-type colitis in which many other important cytokines such as TNF-α, IFN-γ, IL-2, or IL-6 are increased. However, although we did not analyze them in this study, suppression of NF-κB activation by CLT is consistently observed both in vivo and in vitro, indicating the possible regulation of CLT on the NF-κB-dependent cytokines including TNF-α and IL-8. In addition, we also noted the inhibitory effect of CLT on STAT-3 phosphorylation in TNBS-induced colitis. These results indicate that CLT ameliorates TNBS-induced colitis via NF-κB and STAT-3 pathways.

Finally, we found that the inhibitory action of CLT in NF-κB activation, inflammation, and abnormal angiogenesis was achieved at much lower concentrations than 5-ASA in both in vivo TNBS-treated rat colitis and in vitro HT29 cells or HUVECs. These results suggest that CLT may be a more effective therapeutic agent for IBD than a conventional drug, 5-ASA. In summary, our results provide strong evidence that CLT is able to ameliorate intestinal inflammation by inhibiting abnormal angiogenesis and blocking the production and action of IL-8 via inhibition of NF-κB activation.

**Acknowledgments**

We thank Prof. Sae Kwang Ku (Department of Anatomy and Histology, Daegu Hany University, South Korea) for expert support in histological evaluation of colon tissues. We appreciate Samil Pharmaceutical Co. Ltd. (Ansan, Korea) and United Pharm. Inc. (Seoul, Korea) for providing CLT.

**References**


