J. Toxicol. Pub. Health
Vol. 20, No. 4, pp. 293-298 (2004)

Official Journal of
Korean Society of Toxicology

Available online at http://www.toxmut.or.kr

Gliotoxin Protects Trinitrobenzene Sulfonic Acid-Induced Colonic Damage through Induction of Heme Oxygenase-1

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Received October 11, 2004; Accepted November 15, 2004

ABSTRACT. Background: Crohn's disease is characterized by a chronic relapsing inflammation of the bowel. Gliotoxin has been known to play strong immunosuppressive properties, while mechanisms for its anti-inflammatory actions are not completely understood. Here, we investigated the effects of gliotoxin in trinitrobenzene sulfonic acid (TNBS) induced mouse colitis, an animal model of Crohn's disease. **Results**: Gliotoxin dramatically improved clinical and histopathological symptoms in accompanied with reduced expression of TNF-α, IL-1β, and ICAM-1 protein levels in TNBS induced colitis. Interestingly Gliotoxin induced Heme oxygenase-1 (HO-1) and the HO-1 inducer cobalt protoporphyrin IX (CoPPIX) completely mimicked the protective effects of gliotoxin in TNBS induced colitis mice. In contrast, the HO-1 inhibitor zinc protoporphyrin IX (ZnPPIX) could reverse the anti-inflammatory effects of gliotoxin and CoPPIX. **Conclusions:** Gliotoxin is a potential therapeutic agent targeting for the treatment of Crohn's disease by inducing HO-1.

Keywords: Gliotoxin, HO-1, TNBS colitis, Crohn's disease.

INTRODUCTION

The major causes for imbalance of mucosal homeostasis in the pathogenesis of Crohn's disease are changes in cytokine production by macrophages and lymphocytes (Strober *et al.*, 1998). TNBS-induced colitis is clinically and histologically similar to Crohn's disease, and the course of colonic injury has been well characterized. In TNBS induced colitis, increases in mucosal pro-inflammatory cytokines, such as TNF-α and IL-1β, play important roles in sustaining the inflammatory response (Elson *et al.*, 1995; Fiocchi, 1998). In addition, the levels of both TNF-α and IL-1β were elevated in colonic tissues of the patients with inflammatory bowel disease (IBD) (Rogler and Andus, 1998).

Gliotoxin, a member of the epipolythiodioxopiperazine family, is a toxic metabolite of *Aspergillus fumigatus*. However this fungal metabolite produces potent immunosuppressive effects both *in vitro* and *in vivo* (Waring *et al.*, 1988; Sutton *et al.*, 1994; Pahl *et al.*, 1996; Ward *et al.*, 1999). Specifically, gliotoxin affects the functions of several types of immune cells including lymphocytes, neutrophils, and macrophages (Waring *et al.*, 1988; Sutton *et al.*, 1994; Pahl *et al.*, 1996; Ward *et al.*, 1999). Gliotoxin at low concentrations inhibits activation of NFκB in T and B cell lines by suppressing degradation of IκB (Pahl *et al.*, 1996).

Heme oxygenase-1 (HO-1) is the rate-limiting enzyme for heme catabolism; it acts by opening the tetrapyrrole ring structure of heme to produce the bile pigment, biliverdin, free iron, and carbon monoxide (CO). HO-1 is strongly and rapidly induced during oxidative stress and pathological conditions such as inflammation (Maines, 1988; Keyse and Tyrrell, 1989). A recently published communication reported that the protective effect of glutamine against intestinal ischemic injury was related to induction of HO-1 (Tamaki *et al.*, 1999). Another study reported that administration of tin mesoporphyrin (SnMP), a HO-1 inhibitor, on TNBS-treated mice, potentiated colonic damage and reduction of HO-1 activity (Wang *et al.*, 2001). However the mechanism for HO-1 activity in defending against inflammatory injury is

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largely unclear. This work was carried out to identify the protective mechanism of gliotoxin in TNBS induced colitis through investigation of relationships between gliotoxin and HO-1 expression.

MATERIALS AND METHODS

Materials

Unless otherwise stated, all chemicals were purchased from Sigma (St. Louis, MO). TNF-α were from R & D Systems (Minneapolis, MN). HO-1, ICAM-1, TNF-α, IL-1β antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Zinc protoporphyrin IX (ZnPPIX) and cobalt protoporphyrin IX (CoPPIX) were from Porphyrin Products (Logan, UT).

Cell culture

A human colonic epithelial cell line (HT-29) was obtained from the American Type Tissue Culture Collection (St. Louis, MO) and cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 2% sodium bicarbonate, and 10 mM HEPES.

Mice and induction of colitis

The Animal Studies Ethics Committee of Wonkwang University, South Korea, approved all experiments. Mice weighing 20~25 g were obtained from Dae-Han Bio Animal Center Company (Daejun, Korea). Mice were housed under specific pathogen-free conditions, and supplied with sterile food and water. Experiments were conducted with 6-week-old male BALB/c mice. Colitis was induced by rectal administration of two doses (separated by a 7-day interval) of 5% TNBS in 40% ethanol using a vinyl catheter positioned 5 cm from the anus (10 mice per group). Mice were anesthetized with diethyl ether during instillation, and after instillation, mice were kept vertical for 60 sec. To study the effects of gliotoxin on TNBS-induced colitis, mice were treated intra-rectally with gliotoxin dissolved in sterile PBS at day 1 before TNBS administration and days 3, and 6 after TNBS administration. The control group received an identical procedure except that physiological saline was used in place of the gliotoxin solution. Groups of mice were sacrificed at days 1 and 9 after the first TNBS administration.

Measuring myeloperoxidase (MPO) activity

Samples of colon were obtained from inflamed portions of lesions after samples were taken for histological examination. Samples were immediately washed in cold PBS, pH 7.4, and blotted to remove any blood or other contaminants. Samples were weighed on an analytical balance, then the distal segment of colon (150~300 mg) was suspended in 1 ml of ice-cold 0.5% hexadecyltrimethyl ammonium bromide in 50 mM phosphate buffer, pH 6.0, and homogenized three times for 30 sec each, using a tissue homogenizer (OMNI GLH-2017; Yamato Scientific Co., Ltd., Tokyo, Japan). The probe was rinsed twice with 1.0 ml of buffer. After three freeze-thaw cycles, the homogenate was centrifuged for 15 min at 12,000 g. The level of MPO activity in the tissue was measured spectrophotometrically; 0.1 ml of supernatant was combined with 2.4 ml of 60 mM phosphate buffer, pH 6.0, containing 0.2 mg/ml O-dianisidine hydrochloride. Samples were preincubated for 10 min at 25°C, 0.5 ml of 0.003% H_2O_2 was added, and the samples were incubated for an additional 10 min. Reactions were stopped by addition of 0.5 ml of 0.1% sodium azide, and absorbance was measured at 460 nm with a spectrophotometer (DU-640; Beckman Instruments, Inc., Fullerton, CA). One unit of MPO activity was defined as the activity required to convert 1 μmol of H_2O_2 to water in 1 min at 25°C, activity was divided by the colonic wet weight. The MPO assay was performed in a blind fashion using coded tubes.

Western blot analysis

When the colon was harvested, homogenates were made with a tissue homogenizer in lysis buffer (150 mM NaCl, 1% Triton X-100, 10 mM Tris pH 7.4, 5 mM EDTA pH 8.0, 10 mM PMSF). Tissue was lysed for 30 min on ice, followed by two centrifugations (10 min, 14,000 g, 4°C). Homogenates were stored at -70°C until use. Cells in culture were lysed in the same lysis buffer.

Equal amounts of protein were loaded into each well and electrophoresed on 10% SDS-polyacrylamide gels, then transferred to nitrocellulose membranes. After the membranes were blocked in 5% skim milk dissolved in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.1% Tween-20, blots were probed with primary antibody. Antigen-antibody complexes were visualized using horseradish peroxidase-conjugated antibody and an ECL kit.

Immunohistochemistry

Tissue samples were fixed in 10% buffered formalin and prepared for routine paraffin embedding. After paraffin removal and dehydration, sections were rinsed in PBS and incubated in blocking buffer (PBS containing 1% bovine serum albumin and 2% normal goat serum) for 1 h at room temperature. They were pretreated by microwave heating for antigen retrieval. Slides were then incubated with primary antibody at 1.2 μg/ml for 1 h

at room temperature. The slides were washed in PBS and successively incubated with biotinylated goat antiserum to mouse immunoglobulin G (DAKO, Carpinteria, CA) at a 1 : 500 dilution, and then a complex of streptavidin-biotin-alkaline phosphatase (DAKO). The localization of alkaline phosphatase was revealed using Fast Red substrate solution (DAKO).

Statistical analysis

Data were analysed by one way analysis of variance. $p<0.05$ was considered significant. SPSS statistical software (SPSS inc., Chicago, USA) was used for analyses.

RESULTS

Administration of gliotoxin ameliorates TNBS-induced colitis

To investigate any potential protective effects of gliotoxin on experimental colitis, the condition was induced in BALB/c mice by intra-rectal administration of two doses (at days 0 and 7) of TNBS, which haptenates autologuous colonic proteins with trinitrophenol. Gliotoxin (1 μg/100 μl) was administered at day 1 before TNBS treatment and days 3, 6 after TNBS treatment. The general condition of the mice was checked daily. After the first TNBS challenge, a dramatic, rapid decrease in body weight was observed (Fig. 1A). Colon weights were increased (Fig. 1B), and the entire colonic wall became thicker due to edema (Fig. 1C). In addition, TNBS treatment significantly increased colonic MPO levels (Fig. 1D). In contrast, gliotoxin protected TNBS-induced damages in colonic tissues (Fig. 1A, B, C, D).

HO-1 induction by Gliotoxin ameliorates TNBS induced colitis

HO-1 is strongly and rapidly induced during inflammation (Maines, 1988; Keyse and Tyrrell, 1989). However the exact roles of HO-1 in colitis remain largely unknown. To investigate the effects of gliotoxin on HO-1 expression, we treated HT-29 cells with gliotoxin for 0, 3, 6, 12, and 24 h. Western blot analysis revealed that gliotoxin increased HO-1 expression (Fig. 2A). In HT-29 cells, TNF-α led to a remarkable increase in ICAM-1 expression, whereas gliotoxin suppressed increased ICAM-1 expression by TNF-α (Fig. 2B). To examine the effect of gliotoxin-mediated HO-1 induction on ICAM-1

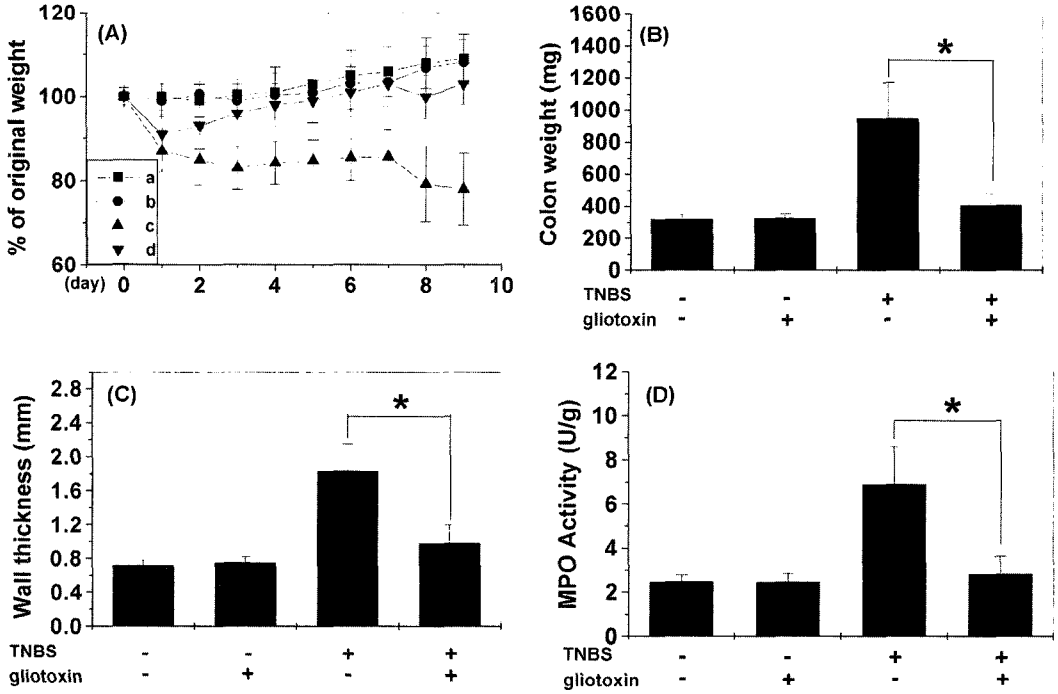

Fig. 1. Gliotoxin ameliorates TNBS induced colitis in mice. Body weight was recorded daily from day 0 to day 9. The weight change is expressed as a percentage of body weight on day 0 (A) in a) EtOH-treated control mice, (b) gliotoxin/EtOH-treated mice, (c) TNBS/EtOH-treated mice, and (d) gliotoxin/TNBS/EtOH-treated mice. The wet weight of distal 5-cm colon portions (B) and the wall thickness of colon portions at the distal 2.5 cm (C) were analyzed. The MPO activities of the distal 5-cm colon portions at day 9 after TNBS treatment was plotted (D). Data represent the mean values ± SEM for 5 mice per group (*$P<0.05$).

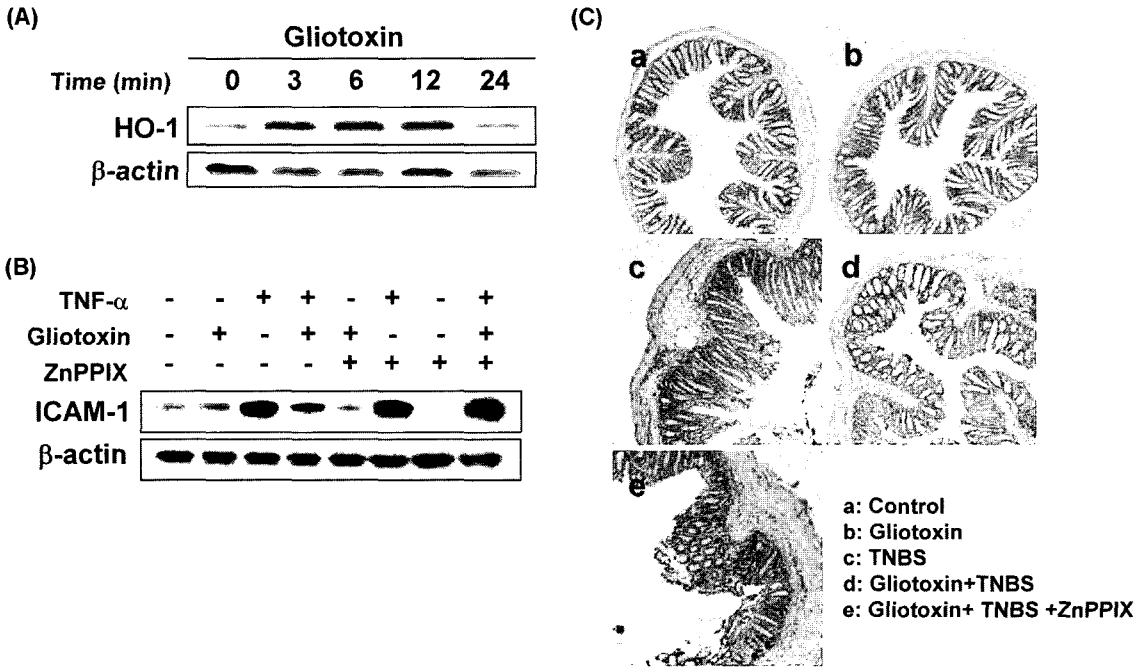

Fig. 2. Gliotoxin induced the increase of HO-1 expression in HT-29 cells. ZnPPIX, HO-1 inhibitor, reversed the effects of gliotoxin *in vivo* and *in vitro*. HT-29 cells were stimulated with gliotoxin (0.2 μg/ml) for 0, 3, 6, 12, and 24 h, and then HO-1 induction was measured by western blot analysis (A). Western blot analysis of ICAM-1 expression was carried out in HT-29 cells after pretreatment with and without 0.2 μg/ml gliotoxin, ZnPPIX (20 μM) for 3 h and then cells were stimulated with TNF-α (1 ng/ml) for 24 h (B). Immunohistochemistry of ICAM-1 (C) in (a) EtOH-treated mice, (b) gliotoxin/EtOH-treated mice, (c) TNBS/EtOH-treated mice, (d) gliotoxin/TNBS/EtOH-treated mice, and (e) ZnPPIX/gliotoxin/TNBS/EtOH-treated mice was performed. Specimen was microscopically observed at a magnification of 200×.

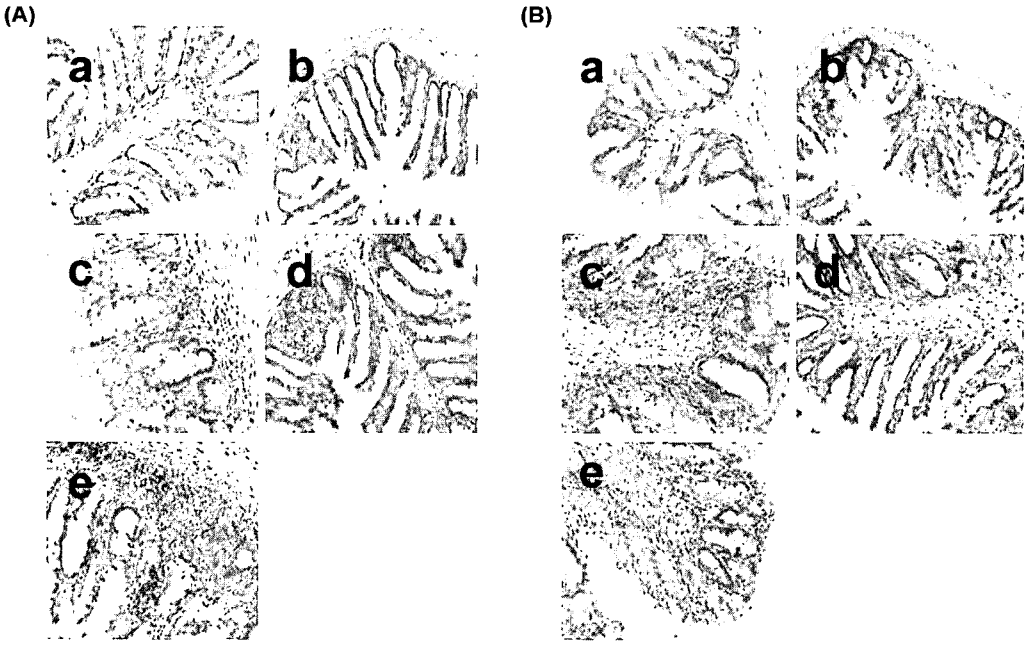

Fig. 3. ZnPPIX reversed the effects of HO-1 inducer CoPPIX on down regulating cytokine expression by TNBS. The immunohistochemical stainings against TNF-α (A) and IL-1β (B) were carried out with the colons of (a) EtOH-treated control mice, (b) CoPPIX/EtOH treated, (c) TNBS/EtOH-treated mice, (d) CoPPIX/TNBS/EtOH-treated mice, (e) CoPPIX/ZnPPIX/TNBS/EtOH-treated mice. Specimen was microscopically observed at a magnification of 200×.

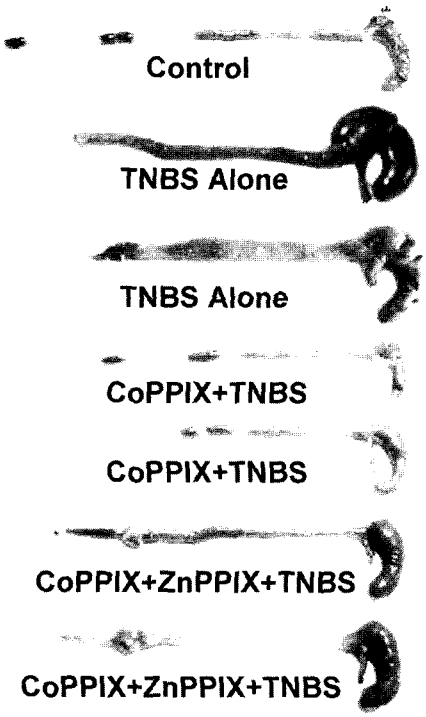

Fig. 4. ZnPPIX abolishes the protective effects of CoPPIX on TNBS-induced colitis in mice. CoPPIX and ZnPPIX (each 20 μM/100 μl) were treated into the peritoneum in mice every day. Macroscopic appearances of the colons of EtOH-treated control mice, TNBS/EtOH-treated mice, CoPPIX/TNBS/EtOH-treated mice, CoPPIX/ZnPPIX/TNBS/EtOH-treated mice were observed.

expression, cells were pretreated with 0.2 μg/ml gliotoxin and the HO-1 inhibitor ZnPPIX for 3 h and were stimulated with TNF-α for 24 h. Interestingly, ZnPPIX could reverse the action of gliotoxin on ICAM-1 induction by TNF-α *in vitro* and by TNBS *in vivo* (Fig. 2B, C). These data suggest that gliotoxin suppresses ICAM-1 induction produced by TNF-α in cells or by TNBS in mice through inducing HO-1 production. Furthermore, TNBS led to a remarkable increase in TNF-α and IL-1β expression in mice, whereas HO-1 inducer CoPPIX suppressed increased TNF-α and IL-1β expression by TNBS. In addition, ZnPPIX reversed the suppressive effects of CoPPIX by TNBS (Fig. 3A, B). Furthermore, CoPPIX mimicked the protective effects of gliotoxin in macroscopic findings (Fig. 4). These findings strongly suggest that gliotoxin protects TNBS induced colitis through HO-1 induction.

DISCUSSION

Heme oxygenase-1 (HO-1) is the rate-limiting enzyme for heme catabolism. Recent studies with HO-1-knockout mice have shown that induction of HO-1 helps amelioration of tissue injury and inflammatory changes in a variety of experimental models (Vogt *et al.*, 1996; Laniado-Schwartzman *et al.*, 1997). While the exact role of HO-1 in Crohn's disease is not largely known, this report is the first to deal with the potential mechanism for the observed protective effects of gliotoxin and HO-1 in chronic colitis. Gliotoxin improved clinical and histopathological symptoms by reducing TNF-α, IL-1β, and ICAM-1 levels in TNBS induced colitis and CoPPIX, a potent HO-1 inducer, mimicked the protective effects of gliotoxin in mice with TNBS-induced colitis. The effects of gliotoxin and CoPPIX were abolished by ZnPPIX, a potent inhibitor of HO-1 enzyme activity. Together, these data suggest that gliotoxin protects against TNBS induced colitis by inducing HO-1 expression.

Heme may oxidize low-density lipoproteins (ox-LDL) that form potent chemo-attractants (Balla *et al.*, 1991; Ishikawa *et al.*, 1997). In agreement with previous reports, our study demonstrated that gliotoxin and CoPPIX suppressed leukocyte infiltration and MPO activity in colon tissue treated with TNBS. In addition, ZnPPIX abolished the suppressive effects of gliotoxin and CoPPIX on leukocyte infiltration. It was recently discovered that heme strongly induces monocyte chemo-attractant protein 1 (MCP-1) (Nath *et al.*, 2001; Kanakiriya *et al.*, 2003) and that exposure of granulocytes to heme induces expression of IL-8 (Graca-Souza *et al.*, 2002). These heme-mediated inflammatory processes may be counteracted by HO activity. In fact, it has been observed that heme breakdown products down-modulate ox-LDL mediated leukocyte chemotaxis (Ishikawa *et al.*, 1997). Moreover, pro-inflammatory cytokines and chemokines are significantly suppressed in HO-1 transgenic mice (Minamino *et al.*, 2001). Heme-induced inflammation in the rat model demonstrated that administration of lysed blood, hemoglobin, or heme caused an increase in expression of adhesion molecules (Wagener *et al.*, 1997; Wagener *et al.*, 2001), influx of leukocytes (Letarte *et al.*, 1993), NFκB activation (Lavrovsky *et al.*, 1994), and chemokine production (Nath *et al.*, 2001). Taken together, our data show that gliotoxin protects against TNBS-induced colonic damage by inducing HO-1. Finally, we believe that gliotoxin could be an appropriate therapeutic agent for treatment of patients with IBD, and that HO-1 is an important target for treatment of IBD.

ACKNOWLEDGEMENT

This paper was supported by Wonkwang University in 2003.

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