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Reduced Severity of a Mouse Colitis Model With Angiotensin Converting Enzyme Inhibition

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Abstract

Ulcerative colitis is characterized by elevated rates of epithelial cell apoptosis, and an up-regulation of pro-apoptotic cytokines including tumor necrosis factor alpha (TNF- α). Recently, angiotensin converting enzyme (ACE) has been shown to promote apoptosis. In addition, pharmacologic ACE inhibition (ACE-I) both prevents apoptosis and reduces TNF- α expression in vitro. We hypothesized that ACE-I, using enalaprilat, would decrease colonic epithelial cell apoptosis and reduce colitis severity in the dextran sulfate sodium (DSS)-induced colitis model in mice. We assessed the severity of colitis, and colonic epithelial cell apoptosis, after administration of DSS. Mice were given either daily ACE-I treatment or daily placebo. ACE-I treatment markedly improved clinical outcomes. In addition, ACE-I treatment significantly reduced the maximum histopathologic colitis grade. ACE-I also dramatically reduced the epithelial apoptotic rate. To investigate the mechanism by which ACE-I reduced apoptosis; we measured TNF- α , Bcl-2, and Bax expression. TNF- α mRNA was significantly lower with ACE-I treatment compared to placebo at every time point; as was the ratio of Bax to Bcl-2. We conclude that ACE-I reduces the severity of DSS-induced colitis and reduces epithelial cell apoptosis.

Keywords

Dextran sulfate sodium; ulcerative colitis; angiotensin converting enzyme; tumor necrosis factor-alpha; apoptosis; ACE-inhibitor

INTRODUCTION

Classical features of ulcerative colitis (UC) include chronic relapsing diarrhea, rectal bleeding and inflammation, and abdominal pain, in addition to mucosal ulceration and microscopic crypt abscesses. The areas of mucosal ulceration seen on gross exam of UC specimens demonstrate markedly increased epithelial cell apoptosis and necrosis on histologic examination, both in human UC (1) as well as in animal models of this disease (2,3). Importantly, the epithelial cell apoptotic rate correlates with the severity of the disease (1). Although the pathophysiology of UC is believed to be multifactorial, the cytokine tumor necrosis factor-alpha (TNF- α) has been implicated as a critical inflammatory mediator in UC (4–7). TNF- α is well established as a major trigger of apoptosis in numerous cell lines (8), and in addition, the high rate of apoptosis and mucosal injury in UC appears to be mediated by an increase in TNF- α (9,10). Therapies

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that reduce epithelial cell apoptosis and TNF- α levels have led to clinical and histologic improvements in experimental models of colitis (2,11).

Recently, angiotensin converting enzyme (ACE) has been shown to play a critical role in apoptosis (19–23). ACE is a zinc carboxypeptidase best known for its effects on blood pressure control (24,25). Its primary substrate is the decapeptide angiotensin I, which is cleaved by ACE to form the octapeptide angiotensin II (Ang II). ACE inhibition (ACE-I) prevents the formation of Ang II. ACE is expressed at particularly high levels on pulmonary vascular endothelium, renal tubules, and the myocardium; but also - surprisingly - in both the colonic and small intestinal epithelium (26–28). In addition, ACE is shed from the gut epithelium and can be detected in stool (49). Although the hypertensive effects of Ang II are well known, the function of ACE in the small and large intestine is far less well understood. The effects of ACE and Ang II are mediated through a series of cell-surface Ang II receptors; and remarkably, these receptors are also found in the intestinal mucosa - in particular, colonic epithelial cells (26, 29).

Recent studies demonstrate that ACE is required to initiate apoptosis in a number of tissues, including alveolar epithelial cells and cardiac myocytes (20,30–32). Studies using pharmacologic ACE inhibition (ACE-I) have shown that ACE-I significantly reduces apoptosis in pulmonary and cardiac cells (31–34); and this reduction of apoptosis exhibits a concentration dependent effect (34). In addition, investigators have shown that Ang II is required for the induction of apoptosis in alveolar epithelial cells and cardiac myocytes (31–36). The production of Ang II is precisely what ACE-I prevents. Further studies have strengthened the evidence for the pro-apoptotic effect of ACE. For example, apoptosis induced by Ang II could be completely prevented by Ang II neutralizing antibodies or by an Ang II receptor antagonist (31). ACE plays a role in apoptosis in a wide variety of tissues, including neural tissues, vascular endothelium, smooth muscle, and fetal adrenals (21,37–39). Interestingly, ACE and Ang II have also been linked to upregulation of TNF- α (40). In fact, apoptosis attributable to TNF- α was actually shown to be dependent on the binding of Ang II to its receptors on alveolar epithelial cells (8). TNF- α is therefore probably linked to the pro-apoptotic effects of ACE.

In a model of the short bowel syndrome, we found that ACE-I reduces small intestinal epithelial cell apoptosis, and reduces the expression of TNF- α as well (22). But despite the fact that ACE has been found at high levels in colonic epithelium, the effect of ACE-I on colonic epithelial cell apoptosis has not yet been reported. We hypothesized that ACE-I, using enalaprilat, would decrease epithelial cell apoptosis in the DSS-induced colitis model, and reduce the severity of ulcerative colitis. Finally, we investigated whether modulation of TNF- α , Bax, and Bcl-2 expression was involved in this action.

MATERIALS AND METHODS

Animals

Specific-pathogen-free male C57BL/6 mice 2–3 months of age (Taconic Farms, Inc., Germantown, NY) were used in accordance with the *Guide for the Care and Use of Laboratory Animals* (41) and with the approval of the University Committee on Use and Care of Animals at the University of Michigan. In each set of experiments, littermates were randomly assigned to each of the various treatment groups. All animals were kept in the University of Michigan vivarium under temperature- and humidity-controlled conditions with light/dark cycles of 12/12 hours. Animals were fed standard rodent chow *ad libitum* (LabDiet® 5001 Rodent Diet, PMI Nutrition International, LLC, Brentwood, MO).

Colitis Model

Colitis was induced by administration of 2.5% (w/v) reagent-grade dextran sulfate sodium (DSS) (M.W. 36,000 – 50,000, ICN Biomedicals, Inc., Aurora, OH) dissolved in drinking water provided *ad libitum*, as described previously (12–17).

ACE-Inhibition or Placebo

Mice were randomly assigned to receive once-per-day intraperitoneal injection of either Placebo (normal saline, 0.5 ml) or else the ACE-inhibitor enalaprilat (Abbott Laboratories, North Chicago, IL; diluted in normal saline solution to a final concentration of 0.03 mg/ml at 14.5 micrograms per dose = injected volume 0.5 ml). The treatment (Placebo or ACE-I) was administered daily for as many days as the mice were receiving DSS. This dose of enalaprilat was chosen based on previous work in this laboratory that demonstrated a reduction in small intestinal epithelial cell apoptosis with enalaprilat in a model of the short bowel syndrome (22).

Time course of the experiment

DSS was administered for either three consecutive days followed by euthanasia, five days followed by euthanasia, or for seven days plus one day returned to plain drinking water, followed by euthanasia. For each series (3, 5, and 7 days of DSS), identical mice from the same lots were randomly assigned to either Placebo (n = 6 at each time point) or ACE-I (n = 6 at each time point), as described above.

Besides Placebo and ACE-I groups, two control groups were also used. A “Naïve” group (n = 5) received plain drinking water *ad libitum* and no other treatment. An “ACE-I-only” group (n = 5) received daily intraperitoneal injection of enalaprilat at the same dose as the ACE-I treated groups (14.5 micrograms/dose) for a total of seven days in addition to plain drinking water.

Data and Specimen Collection

Body weight of each mouse was recorded daily. In experimental groups administered DSS for seven days. Stool was also assessed daily for the presence of occult blood using a guaiac-card test (Cenogenics Stool Blood Test, Cenogenics Corp., Morganville, NJ). After the specified number of days, mice were euthanized with carbon dioxide asphyxiation, and laparotomy with total colectomy was immediately performed. Colons, not including cecum, were placed into RPMI cell culture medium on ice, and fecal contents were gently flushed out. Several 5–10 mm sections taken from the distal half of the colon were excised and placed into 10% neutral buffered formalin. Colonic epithelium was isolated for RNA and mucosal protein isolation, as described previously (42). Briefly, the remainder of the colon was opened longitudinally and rinsed with fresh 4 °C RPMI, then placed mucosal-surface-upwards on a glass slide. The epithelium was mechanically scraped off and epithelial cells were collected in fresh RPMI with glutamine on ice. These epithelial cells were then rapidly pelleted in RPMI by centrifugation at $330 \times g$ at 4 °C for 3 minutes. The supernatant was decanted and the epithelial cell pellet was then immediately snap-frozen in liquid nitrogen and processed for RNA and protein extraction. Sections of colon after epithelial scraping were also examined microscopically, confirming that the mechanical removal of epithelial cells was accurate and did not include submucosa or muscularis.

Assessment of Colitis

Formalin-preserved sections of distal colon, as described above, were processed in paraffin with standard technique. Transverse sections 5 µm thick were stained with hematoxylin and eosin (H&E) and were examined independently in duplicate by two observers blinded to the experimental group from which each section came. All grading of colitis was done according

to the method described by Cooper et al. (15). In this method, crypt shortening and distortion, together with inflammatory infiltrative thickening of the lamina propria, are assigned a score of 0 (normal) through 4 (complete loss of crypts, ulceration, and severe thickening of the lamina propria) (15). The individual scores (0 – 4) from four different areas of each transverse colonic section were summed, such that the maximum colitis score for a given section is 16, and the minimum score is 0. Several sections were assessed in this manner for each mouse. The final score for each section was the mean of the duplicate independent assessments for that section; and the final histopathologic colitis score for each mouse was therefore the mean of the scores of all the colon sections from that mouse.

Assessment of Apoptosis

Tissue sections 5 μ m thick from the same regions of colon used to assess colitis severity were stained with the TUNEL (terminal deoxynucleotidyl transferase (TdT) dUTP-nick end labeling) method, using the ApopTag In Situ Peroxidase Cell Death Detection Kit (Serologicals Corporation, Norcross, GA), using a modified technique previously described (43). Briefly, to prevent false positives, all tissue sections were incubated in 3% H_2O_2 for 30 minutes to quench endogenous peroxidases, and TdT enzyme was used at half the supplied concentration to avoid over-staining (43). A light hematoxylin counterstain was then applied. Specimens were processed simultaneously under identical conditions to guarantee identical staining of slides from different experimental groups. Specimens from the two control groups (Naïve and ACE-I-only mice) underwent identical TUNEL staining as well, to determine baseline apoptotic rates. For negative controls (to demonstrate specificity of the TUNEL stain for apoptotic nuclei) parallel samples underwent the same process but with omission of TdT enzyme. Subsequently, apoptosis was quantified by an observer blinded to the identity of the tissue sections. Independent assessments were performed in duplicate. TUNEL-positive nuclei were clearly identified as brown-stained nuclei, indicating the presence of DNA fragmentation due to apoptosis. TUNEL-positive staining correlated with morphological features of apoptosis (nuclear and cytoplasmic blebbing, chromatin margination) (1,38,43). The apoptotic index was quantified as the number of apoptotic nuclei per crypt, that is:

$$(\text{number of apoptotic nuclei counted}) / (\text{number of contiguous colonic crypt units evaluated})$$

A minimum of 24 colonic crypt units (one crypt plus adjacent surface epithelium) were assessed for every tissue section. The final score for each section was the mean of the independent assessments for that section; the final apoptosis score for each mouse was therefore the mean of the scores of colon sections from that mouse.

Flow Cytometry

As an additional assessment of apoptotic rates, epithelial cell surface staining with Annexin V was performed (Annexin V-FITC Apoptosis Detection Kit I, BD Pharmingen, Palo Alto, CA). Propidium iodide was used to distinguish necrotic cells from apoptotic cells, according to the manufacturer's instructions. Annexin V staining was analyzed using flow cytometry (FACSCalibur, Becton Dickinson Immunocytometry Systems, San Jose, CA). Data were analyzed using CELLQuest software (Version 3.2.1f1, Becton Dickinson).

RNA Isolation

Total epithelial cell RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Purity of RNA was confirmed by spectrophotometric determination of the 260/280 λ absorbance ratio.

Reverse Transcription (RT) and Polymerase Chain Reaction (PCR)

Poly-A positive mRNA was reverse transcribed into first-strand cDNA using oligo dT₍₁₂₋₁₈₎ (Invitrogen) as the primer. All reactions were carried out with the protection of RNase Inhibitor (Roche, Penzberg, Germany) and RT was performed using Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase (Invitrogen). RT reaction was carried out at 40 °C for 70 minutes according to a standard protocol (43). TNF- α , Bcl-2, and Bax expression was quantified using previously validated oligonucleotide primers specific for each mRNA (22). PCR conditions have been previously described (43). PCR product was generated at the exponential portion of the product curve. The Kodak EDAS System (Rochester, NY) was used for imaging and quantification of PCR products. All results were normalized to the level of β -actin mRNA expression.

TNF- α Mucosal Concentration

An enzyme immunometric assay (TiterZyme EIA mouse TNF- α , Assay Designs Inc., Ann Arbor, MI) was used to determine the concentration of TNF- α in the mucosal protein isolate. Samples were performed in duplicate and were standardized to a known concentration of mouse TNF- α (Assay Designs Inc.). Mucosal TNF- α was measured with this technique after 3 and 4 days of DSS, but not later in the course of colitis, due to severe mucosal destruction and mucosal loss later in the course of the disease (see results, below).

Western Blot

The mucosal concentration of ACE protein was assessed using standard Western blot techniques. Briefly, mucosal epithelial cell isolates were homogenized in nonidet P-40 based (NP-40) buffer at 4 °C. Protein determination was performed by using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Approximately 60 μ g of total protein in loading buffer was loaded per lane in a SDS-polyacrylamide-gel (13%), and separated using electrophoresis. Proteins were then transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA). Non-specific binding to the membrane was controlled with a blocking solution (Zymed Laboratories Inc, CA), and probed with goat anti-mouse biotinylated-ACE Ab (R&D Systems, Inc. Minneapolis, MN) (0.15 μ g/ml in blocking solution) for 1 hour. Bound antibodies were exposed to a Streptavidin-HRP conjugate (1:10000, Zymed Laboratories Inc, CA), detected on X-ray film and quantified using Kodak 1D image quantification software (Kodak Co, Rochester, NY).

Mucosal and mesenteric blood flow

To evaluate the effects of administration of ACE-I on mesenteric, as well as colonic mucosal blood flow the laser Doppler perfusion imager (LDPI, Perimed Inc., North Royalton, OH) with computer software was used. In anesthetized mice (n=5, in each group) a median laparotomy was performed and the mesentery exposed. The LDPI 670nm helium-neon laser beam was placed 12 cm above the mesentery to sequentially scan the surface of the mesentery and detect moving blood cells. In the presence of moving blood cells, the frequency shifts of the back-scattered light are detected by a photo-detector and processed to determine a percentage perfusion value (<http://www.perimed.se>). The microvascular blood flow (recorded in arbitrary units) was derived from the power spectra of backscattered laser light representing the distribution of Doppler shifts of the erythrocyte velocities.

Maximum, minimum and mean percent perfusion was normalized to total pixel area. After measurement of mesenteric perfusion was completed, the colonic mucosa was exposed by anti-mesenteric longitudinal opening of the mid-colon over a length of 2 cm. After gentle removal of fecal material with a cotton tip the percent perfusion of colonic mucosa was determined. At the end of the measurements the mice were sacrificed.

Statistical Analysis

Data are reported as mean $\pm$ standard deviation (SD). Statistics were performed using SPSS 12.0 software (SPSS Inc., Chicago, IL). Analysis of variance (ANOVA) with the post-hoc Bonferroni test was used for comparisons between multiple groups. In cases when there were only two groups for comparison, the two-tailed Students' *t* test was used. Results were considered significant if $P < 0.05$.

RESULTS

Effect of ACE-I on Clinical Parameters

Body weights—Body weight change (stated as percent change from baseline body weight on day 0) is shown in Figure 1. In agreement with other studies characterizing the time course of DSS colitis (3,12–13), significant weight loss occurred toward the end of one week of DSS administration. However, ACE-I treatment significantly protected against this weight loss (Figure 1). The difference between Placebo and ACE-I treated groups became significant after day 5 of DSS. After one week, weight loss was severe in the Placebo group ($23.7 \pm 4.2\%$ weight loss) but this was significantly attenuated in the ACE-I group ($16.6 \pm 2.7\%$) ($P < 0.01$, two-tailed unpaired *t* test).

Fecal blood—The onset of heme-positive stools corresponded closely to the development of weight loss. Mice in the ACE-I group experienced a significantly longer period before developing heme-positive stools (5.0 ± 0.7 days) than mice in the Placebo group (3.5 ± 0.8 days, $P < 0.02$, two-tailed *t* test).

Effect of ACE-I on Histopathology

As outlined in Table 1, naïve control mice, as well as control mice receiving ACE-I but not DSS, had no evidence of colitis. In mice receiving DSS, the most severe ulcerative lesions were consistently found in the distal colon, which is consistent with previous studies using the DSS model (2,3). In addition, lesions did not fully develop until near the end of one week of DSS, which is also in agreement with previous reports (2,3,12,44). Thus, in both the Placebo group, as well as ACE-I treated mice, histology was essentially normal at Day 3 of DSS, and minor lesions had developed by Day 5 (Figure 2). There was a trend towards more crypt disruption on Day 5 in the Placebo group in comparison to the ACE-I group, although this difference was not significant. However, severe ulcerative lesions rapidly developed by Day 7 of DSS in the Placebo group; and these changes were significantly attenuated in the ACE-I treated mice (data shown in Table 1, histopathology in Figure 2).

Effect of ACE-I on Epithelial Apoptosis

Although histological crypt disruption and ulcerations were not visible early in the course of the experiment, we questioned whether an increase in apoptosis preceded the development of these lesions. TUNEL staining demonstrated that, in fact, a very early and marked increase in epithelial cell apoptosis did occur in the development of colitis. Apoptosis was maximal at Day 3 in Placebo-treated mice receiving DSS. In contrast, ACE-I treatment reduced the apoptotic rate at Day 3 by 42.5% ($P < 0.0005$, Figure 3). This marked increase in apoptosis occurred early in the course of DSS and decreased later, as epithelial ulcerative lesions developed (Figure 4). Thus, in Placebo treated DSS mice by day 7, epithelial ulceration was so severe that essentially no normal crypts or surface epithelial cells remained. Apoptotic rates could not be accurately measured at the final time point in the Placebo group due to epithelial disintegration (large numbers of apoptotic cells, however, are seen in the lumen, Figure 4). These results demonstrate the progression of ulcerative lesions, beginning with an early rise in apoptosis (*prior to* disruption of crypt architecture), leading to a subsequent loss of epithelial cells, and

finally resulting in the severe lesions of DSS-induced colitis. In ACE-I treated mice, the rate of apoptosis was *significantly* reduced at Day 3 (the time point with the highest apoptotic rate), although apoptosis was greater than Controls. By Day 5 the apoptotic index was reduced in both DSS groups. However, the rate of apoptosis remained significantly lower in the ACE-I treated group compared to saline treated DSS mice (Figure 3). Thus, a sharp reduction in apoptosis with ACE-I treatment may well have contributed to the quantitative improvement in colitis grade by Day 7.

To further validate these results, Annexin V staining - a very early marker of apoptosis (52) - was performed on samples at Day 3, the time point with the highest rate of apoptosis on TUNEL staining. Annexin V staining confirmed that ACE-I dramatically reduced the percentage of apoptotic epithelial cells, in contrast to Placebo (Table 2). Interestingly, although rates of cellular necrosis (assessed with Propidium iodide) were very low at Day 3, even at this early time point, epithelial cell necrosis was significantly lower in the ACE-I group than the Placebo group (Table 2), and cellular viability was increased with ACE-I treatment.

Effect of DSS on ACE Expression

ACE expression was investigated with Western immunoblotting to assess the effect the administration of DSS. Interestingly, mice receiving DSS showed a significantly ($P < 0.001$) elevated expression of mucosal levels of ACE (0.91 ± 0.20) compared to Control mice (0.27 ± 0.07). Thus, it is possible that increased ACE expression may have a role in the formation of inflammatory or apoptotic changes observed in DSS mice.

Effect of ACE-I on TNF- α Expression

TNF- α expression was investigated because previous work has demonstrated a reduction in TNF- α with ACE-I, with a corresponding reduction in apoptosis (22). As expected, DSS-induced colitis treated with Placebo was associated with a significant increase in TNF- α expression. However, in ACE-I treated mice, TNF- α mRNA expression was significantly reduced at every time point in the study (Figure 5). In fact, although the level of TNF- α was somewhat elevated in the ACE-I group, it was not significantly greater than that in Control mice at any time point (Figure 5). Thus, ACE-I achieved a significant reduction in TNF- α mRNA throughout the entire course of the study. Consistent with this finding, the mucosal protein concentration (by immunometric assay) of TNF- α in ACE-I colons was reduced by more than two-fold compared to Placebo (ACE-I, 22.4 ± 28.3 picograms of TNF- α per mg total protein, versus Placebo, 58.2 ± 51.0 pg/mg). This difference approached significance ($P = 0.07$).

Effect of ACE-I on Bcl-2 and Bax Expression

Bax and Bcl-2 mRNA expression was measured as these factors are also critical to the modulation of apoptosis in a number of cell types, including intestinal epithelium (43). Results (Figure 6) showed predominant expression of bax (pro-apoptotic) over bcl-2 (anti-apoptotic) in naïve non-manipulated controls (bax to bcl-2 ratio 22.8 ± 45.2). Treatment of control mice with ACE-I resulted in an increased expression in both bcl-2 and bax; however, the relative changes result in a marked increase in bcl-2 expression (bax to bcl-2 ratio 1.72 ± 0.14), suggesting that a modulation in the expression of these factors are greatly influenced by the inhibition of ACE activity. This further suggests that ACE-I mediated decline in apoptosis may be due to this relative increase in bcl-2 expression. DSS treatment resulted in a shift in the expression of these apoptotic factors toward an increased expression of bax. This increase in bax was noted in both placebo and ACE-I treated DSS mice, and the differences were not significantly different. The most profound effect of ACE-I on DSS mice was noted at 5 days. At this time point, the largest decline in bcl-2 was seen in placebo (saline) treated mice; whereas at 5 days in the ACE-I treated group a significant ($P < 0.05$) rise in bcl-2 expression was noted

(Figure 6). The bax to bcl-2 ratios were essentially the same in all treatment groups at days 3 and 8 (not shown). However, a significant difference in this ratio was observed at 5 days between the placebo versus ACE-I treated DSS mice (bax to bcl-2 ratio 101.0 ± 77.8 versus 2.73 ± 0.87 , respectively). This again suggests that the reduction in apoptosis by ACE-I may be mediated at least in part by an increased expression of bcl-2.

Effect of mucosal blood flow

Mucosal blood flow measurements of colonic tissue showed slightly lower levels in ACE-I treated mice compared to untreated controls; however, the values were not significantly different between the two groups (0.976 ± 0.218 versus 0.760 ± 0.151 , respectively; expressed in arbitrary units).

DISCUSSION

In this study, ACE-inhibition (ACE-I) significantly reduced epithelial cell apoptosis, decreased the mucosal expression of TNF- α , and attenuated the course of DSS-induced colitis in mice. Weight loss was significantly lower and heme-positive stools were reduced in ACE-I treated mice. The histopathologic grade of colitis was also significantly lower in ACE-I treated mice. Importantly, we found that the rate of colonic epithelial cell apoptosis, which was markedly elevated in Placebo-treated DSS mice, was significantly reduced with ACE inhibition. This finding was confirmed using two independent techniques (Annexin V flow cytometry, and TUNEL immunohistochemistry).

By following the development of the ulcerative lesions in this model over time, throughout the very early phases of DSS-induced colitis, we were able to demonstrate that a sharp increase in apoptosis precedes the development of severe mucosal damage. Although it has limitations, the dextran sulfate sodium (DSS) model is a very well established, reproducible model used extensively in the study of ulcerative colitis (12–17). DSS-induced colitis resembles human UC in several important clinical and histopathologic features, including the high rates of apoptosis characteristic of UC (3,16). Improvements in DSS-induced colitis in our study correlated with a reduction in apoptotic rates (2,3,12,13,18). The reduction in apoptosis by ACE-I may well have contributed to a subsequent decrease in the severity of mucosal lesions. Our data indicate that epithelial cell apoptosis is actually a very early phenomenon. The degree of apoptosis, at the first time point (Day 3) in this study, correlated with the severity of colitis subsequently. These data agree with the observation in human UC patients that increasing rates of colonic epithelial apoptosis correlate with increasing severity of disease (1).

Multiple lines of evidence now implicate ACE as a promoter of apoptosis (51–54). Small intestinal epithelial apoptosis is elevated in a mouse model of short-bowel syndrome; and ACE-I reduces apoptosis in this setting (22). Extensive studies in alveolar epithelial cells have demonstrated that ACE activity is necessary for apoptosis in these cells (20,34–35). Angiotensin II, produced by ACE, induces apoptosis of pulmonary alveolar epithelial cells in a dose-dependent manner (34). The ACE/Angiotensin system is highly expressed in the developing fetal cardiopulmonary system and adrenal glands, where tissue remodeling is mediated by high levels of apoptosis (21,37). However, we are unaware of any previous study examining the role of ACE in apoptosis in experimental colitis. Although it has been demonstrated that ACE is highly expressed in the small and large intestinal epithelium of rodents and of humans (26,29,49), the functional role of this expression is uncertain. It was interesting to note that we observed a marked increase in the expression of ACE protein in the placebo-treated DSS mice.

We chose enalaprilat because of its proven anti-apoptotic effect in the small bowel (22). However, a number of other ACE-inhibitors have also been shown to inhibit apoptosis in other

tissues, including enalapril (32), quinaprilat (36), and captopril (35), as well as other inhibitors of components of the ACE/Angiotensin signaling cascade, such as angiotensin receptor blockers (30,40).

In addition, studies have shown that ACE upregulates TNF- α expression, and that ACE-I downregulates TNF- α (22,45,48,50). Because TNF- α is not only a potent promoter of epithelial apoptosis, but also is potentially involved in the pathophysiology of the inflammatory process seen in UC, we questioned whether ACE-I would affect TNF- α expression in the DSS model. Interestingly, we found that ACE-I did significantly downregulate TNF- α mRNA expression at every time point in this study, as well as a nearly significant two-fold reduction in protein expression at 3 days. Our results suggest that ACE within the colonic mucosa is involved in downregulation of TNF- α , and this action may be involved in the mechanism by which ACE-I reduced both apoptosis and the inflammatory process. However, TNF- α is known to be controlled (both at the transcriptional and post-transcriptional level) by a very complex network of signaling cascades, and it is likely that other factors in addition to ACE contributed to levels of TNF- α in our study. The effect of ACE inhibition on apoptosis may involve other signaling cascades, as well, such as ERK, JNK, NF B, and others, which may affect TNF- α expression (33,46–47). Further work will be necessary to more fully elucidate the mechanisms involved in ACE signaling in the colonic epithelium.

The finding that bax expression increased with DSS treatment suggests that the Bcl-2 family members could be another one of the mediators of the observed increase in epithelial cell apoptosis in this colitis model. The finding that treatment of control mice with ACE-I results in a reduction in the bax to bcl-2 ratio, supports the findings of others that suggests alteration in bax and bcl-2 expression may be one of the mechanisms which account for ACE-I mediated decrease in apoptosis (55). Similarly, we found a marked increase in the bax to bcl-2 ratio in 5 day DSS mice treated with saline and a significant decline in this ratio in ACE-I treated DSS mice.

ACE-I can clearly affect blood flow and blood pressure of these mice. In order to rule out the possibility that major alterations in mesenteric blood flow could account for the observed changes in apoptosis and colitis Doppler blood flows were determined. We found no significant change in blood flow between naïve controls and ACE-I treated mice, suggesting that alterations in blood flow had little to no effect on ACE-I associated decline in apoptosis.

Although ACE inhibition produced significant clinical and histological improvements, it is noteworthy that colitis was not completely prevented by ACE-I treatment. Furthermore, apoptosis was significantly reduced, but not entirely prevented. Colitis is likely to be multifactorial, and our data indicate that ACE-I prevented some, but not all of the pro-apoptotic and inflammatory stimuli in this model. It is important to note that our findings are supported by other investigators who have recently identified a potential benefit in blocking the rennin-angiotensin (RAS) pathway in inflammatory bowel disease. Wengrower, et al showed that fibrotic changes were significantly diminished in a trinitrobenzene sulphonic acid (TNBS) induced model of colitis in rats (56). Blockade of the RAS pathway using angiotensinogen-knock out mice also resulted in a diminution of the level of inflammation in this TNBS model (57). In another recent study, captopril and lisinopril were used in a TNBS rat model. These investigators failed to see an improvement in the histology with lisinopril, but saw improvement with captopril. Additionally, they noted a diminution of the serum levels of TNF- α (58). While the observations made by these recent publications confirm many of the findings of our work, they do not completely address the potential mechanism by which inhibition of the RAS acts to prevent colitis. Our study supports the contention that action of ACE-I in a rodent model of ulcerative colitis may be mediated by a diminution of both pro-inflammatory cytokines as well as a marked decrease in epithelial cell apoptosis.

Patients with ulcerative colitis suffer from a complex inflammatory disease, with a primary etiology that is probably multifactorial, yet incompletely understood despite decades of research. The most effective therapies to date - short of proctocolectomy - have been based on anti-inflammatory pharmacology. The idea of targeting epithelial apoptosis and blockade of TNF- α hence, ulceration - the sequelae of inflammation - could potentially provide a novel approach to the treatment of UC. Further, the fact that the use of ACE inhibition does not have any associated immunosuppressive side effects may allow clinicians to eventually provide this therapy in conjunction with reduced doses of immunomodulating therapies.

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Figure 1.

Body weight loss is shown as percent change from baseline. ACE-inhibitor treatment significantly reduced the loss of body weight associated with DSS-induced colitis. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.01$, ACE-I versus Placebo. Error bars indicate mean $\pm$ SEM.

Figure 2.

Histopathology of DSS-induced colitis treated with Placebo (saline) or ACE-inhibitor, shown after 3, 5, or 7 days of DSS. ACE-inhibitor treatment significantly reduced the severity of colitis. While only minor lesions are found at Day 5, mucosal architecture rapidly disintegrates thereafter in Placebo mice. In contrast, ACE-I treatment significantly ameliorated these lesions, although crypt distortion and some ulceration was present. Original magnification $\times 200$ at Days 3 and 5 (top panels); and $\times 100$ at Day 7 to show wider field of view. Note the complete loss of epithelium and dramatic thickening of the lamina propria in Placebo colon at Day 7 (bottom left), in contrast to ACE-I colon (bottom right). Further examples shown in Figure 4.

Figure 3.

The apoptotic index decreased significantly with ACE-inhibitor treatment. Apoptotic index is quantified as number of TUNEL-positive nuclei per colonic crypt unit (crypt and surface epithelium adjacent to crypt). Observers were blinded to the identity of tissue sections, and all observations were performed in duplicate. In DSS-induced colitis with Placebo treatment, the maximum rate of apoptosis was seen at Day 3, and apoptosis continued to be significantly elevated at Day 5 (black bars). In contrast, ACE-inhibitor treatment (white bars) significantly reduced the maximum apoptotic rate at Day 3, compared to Placebo, although it was still greater than Controls. By Day 5, apoptosis declined in both groups, but apoptosis in ACE-I treated colons remained significantly less than saline treated DSS mice. Comparisons made using ANOVA with post-hoc Bonferroni test. Error bars show mean $\pm$ SEM.

Figure 4.

Apoptotic epithelial cells were clearly identified (brown) using TUNEL staining. Apoptosis was most prominent on Day 3 of DSS, before any visible ulcerations or disruption of crypt architecture had occurred. Furthermore, apoptosis was significantly reduced with ACE-inhibitor treatment (ACE-I). The level of apoptosis early in the course of DSS-induced colitis correlated with the degree of ulceration subsequently. As a result, the reduced apoptotic rate in ACE-I colon resulted in less severe epithelial damage (Day 7). Note the complete loss of epithelium on Day 7 in Placebo colon, where only sloughed epithelial cells (brown) are seen overlying the greatly thickened lamina propria. Original magnification $\times 200$. The quantitative apoptotic index is shown in Figure 3.

Figure 5.

Mucosal tumor necrosis factor (TNF- α) expression was assessed with RT-PCR. At every time point in the study, TNF- α mRNA levels were significantly less in the ACE-inhibitor treated group (DSS+ACE-I) than in the Placebo (DSS+Placebo) group. For comparison, the baseline level of TNF- α expression in Naïve Controls is shown (dotted line). At every time point, the ACE-inhibitor treated group is not significantly different than Controls. TNF- α values are expressed relative to beta-actin. Error bars indicate mean $\pm$ SEM. Comparisons made using ANOVA with post-hoc Bonferroni test. * $P < 0.05$, ** $P < 0.01$, DSS+ACE-I versus DSS +Placebo.

Figure 6.

Mucosal expression of bax and bcl-2 mRNA expression in DSS treated mice given either saline (Placebo) or an ACE-I. Controls are Naïve non-treated mice and control mice treated with ACE-I. mRNA values expressed relative to beta-actin. Error bars indicate mean $\pm$ SD. Comparisons made using ANOVA with post-hoc Bonferroni test. * $P < 0.05$.

TABLE 1

Histologic Colitis Grade

<i>Duration of DSS (days)</i>	DSS + Placebo	DSS + ACE-I
3	0.1 ± 0.0	0.1 ± 0.1
5	0.6 ± 0.6	0.4 ± 0.4
7	14.7 ± 1.4	11.8 ± 1.5 *
<i>Control Groups</i> (No DSS)	Naïve Control 0.1 ± 0.1	Control + ACE-I 0.0 ± 0.1

Abbreviations: DSS, dextran sulfate sodium; ACE-I, angiotensin converting enzyme inhibitor.

Only minor histopathologic changes were seen at day 5, but colitis rapidly developed thereafter.

* $P < 0.005$ (ANOVA), Placebo versus ACE-I group. Data are mean ± SD.

TABLE 2

Apoptotic Index by Flow Cytometry

(% of Epithelial cells)	DSS + Placebo	DSS + ACE-I
Annexin V + (Apoptotic)	14.7 ± 7.9	3.8 ± 3.1 *
Annexin V & PI negative (Viable cells)	82.0 ± 8.6	94.7 ± 4.1 *
PI + (Necrotic)	3.3 ± 1.0	1.5 ± 1.0 *

Apoptosis was assessed by Annexin V staining, with PI (propidium iodide) to distinguish necrotic cells. Cells positive for Annexin V only were considered apoptotic; cells negative for both Annexin V and PI were considered viable. ACE-I treatment significantly reduced apoptosis and necrosis, and enhanced cellular viability; flow cytometry was performed at Day 3 when apoptotic rates were highest, but very little necrosis had yet occurred.

* $P < 0.01$, ACE-I versus Placebo.