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INTESTINAL INTRAEPITHELIAL LYMPHOCYTE DERIVED ANGIOTENSIN CONVERTING ENZYME MODULATES EPITHELIAL CELL APOPTOSIS

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Abstract

Background & Aims—Intestinal adaptation in short bowel syndrome (SBS) consists of increased epithelial cell (EC) proliferation as well as apoptosis. Previous microarray analyses of intraepithelial lymphocytes (IEL) gene expression after SBS showed an increased expression of angiotensin converting enzyme (ACE). Because ACE has been shown to promote *alveolar* EC apoptosis, we examined if IEL-derived ACE plays a role in *intestinal* EC apoptosis.

Methods—Mice underwent either a 70% mid-intestinal resection (SBS group) or a transection (Sham group) and were studied at 7 days. ACE expression was measured, and ACE inhibition (ACE-I, enalaprilat) was used to assess ACE function.

Results—IEL-derived ACE was significantly elevated in SBS mice. The addition of an ACE-I to SBS mice resulted in a significant decline in EC apoptosis. To address a possible mechanism, tumor necrosis factor alpha (TNF- α) mRNA expression was measured. TNF- α was significantly increased in SBS mice, and decreased with ACE-I. Interestingly, ACE-I was not able to decrease EC apoptosis in TNF- α knockout mice.

Conclusions—This study shows a previously undescribed expression of ACE by IEL. SBS was associated with an increase in IEL-derived ACE. ACE appears to be associated with an up-regulation of intestinal EC apoptosis. ACE-I significantly decreased EC apoptosis.

Keywords

angiotensin converting enzyme; intraepithelial lymphocytes; apoptosis; tumor necrosis factor alpha; epithelial cells

INTRODUCTION

Short bowel syndrome (SBS) after a massive small bowel resection results in a series of adaptive processes^{1–3}. A number of growth factors and nutrients may help to mediate these changes^{1, 4–7}. In addition to an increase in functional absorption, adaptation also consists of an increase in villus length and crypt depth, and an increased number of microvilli. This adaptation requires a remodeling of the cryptvillus complex and includes significant increases in both epithelial cell (EC) proliferation as well as EC apoptosis^{8–10}. The regulation of gastrointestinal adaptation is complex and the precise factors which guide this adaptive process remain unclear¹¹. A number of local and distant trophic factors contribute to this adaptation

5, 12–15. Intraepithelial lymphocytes (IEL) also have a role in this adaptive process. The gamma delta T-cell receptor sub-population of the IEL, as an example, express the trophic molecule keratinocyte growth factor (KGF), which is up-regulated during intestinal mucosal injury¹⁶, and following massive small bowel resection¹⁷. To further elucidate the relationship between IEL and the homeostasis of the intestinal epithelium, and to identify IEL-derived factors contributing to structural intestinal adaptation in SBS, IEL-gene analysis (microarray assays) were performed 1 week after massive small bowel resection using a mouse model. The focus of analysis was on genes which have a role in affecting EC proliferation or apoptosis pathways. A detailed analysis of these findings has been previously reported¹⁸. In this previous report, IEL-derived expression of angiotensin converting enzyme (ACE) was examined, as it was found to be significantly elevated after SBS creation. Interestingly, ACE has been shown to have a significant role in promoting apoptosis in a number of organs including pulmonary alveolar epithelial cells¹⁹, renal epithelial cells²⁰ and cardiac myocytes²¹. ACE has been previously detected in the mucosal epithelium in human, swine, rat, as well as mouse intestine^{18, 22–27}; however, whether ACE has a functional role in mediating EC apoptosis in the gastrointestinal mucosa has not been evaluated. This study was designed to confirm the expression of ACE and understand its role in small bowel during EC turnover.

METHODS

Animals: Male, 2 month old, C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were maintained in a 12-hour day-night rhythm at 23° C and a relative humidity of 40%–60%. 24 hours before surgery chow was exchanged to micro-stabilized rodent liquid diet (TestDiet, Richmond, IN). Anesthesia was achieved using an injection of ketamine (87 mg/kg) and rompun (13 mg/kg) given together intramuscularly. For some experiments B6;129S6-*Tnf^{tm1Gkl}* (TNF- α -knockout mice, Jackson Labs) were used. Experiments were approved by the University Committee on Use and Care of Animals at the University of Michigan.

Animal Model

Short Bowel Syndrome (SBS) Model: A 70% mid-small bowel resection was performed similar to that previously described²⁸. This consisted of a resection of the bowel between 3.5cm distal to the ligament of Treitz and 3.5cm proximal to the ileocecal valve, and was followed by an end-to-end jejunoileal anastomosis with 8-0 absorbable suture (Vicryl, Ethicon Corporation, USA),.

Sham operation: Mid-small bowel was transected and re-anastomosed without bowel resection.

Post-Surgical Care: Postoperatively a subcutaneous bolus of 3 ml 0.9% saline solution was given to both groups in order to maintain hydration status. Mice were allowed *ad libitum* water and liquid diet after surgery²⁸. Mice were sacrificed using CO₂ at 7 days post-surgery, and the intestine was harvested.

Treatment with ACE inhibitor: The ACE inhibitor (ACE-I) enalaprilat, (0.6 mg/kg/day i.p. Abbott Laboratories, North Chicago, IL) was given to a separate group of Sham and SBS mice; starting one day prior to the surgery. In some experiments, mice were matched to a control group of non-operated mice; and were studied after a 7 day period.

Histology: A 0.5 cm segment of mid-small bowel (at least 2 cm away from the anastomosis) was fixed in 10% formaldehyde, and processed for hematoxylin and eosin staining. Villus

height and crypt depth were measured using a calibrated micrometer. Each measurement consisted of a mean of 16 different low power fields.

Epithelial cell apoptosis assays: TUNEL staining was used for EC apoptosis assays. Briefly, paraffin-embedded tissue was assayed with TUNEL (Terminal deoxynucleotidyl transferase Biotin-dUTP Nick End Labeling) staining, according to manufacturer's instructions (ApopTag InSitu Apoptosis Detection Kit, Serological Corporation, Norcross, GA), with slight modification. Slides were incubated with only one-third of the recommended concentration of TdT enzyme, in order to avoid over-staining. Apoptosis rate (percent of EC showing apoptosis) was assessed with TUNEL staining and by morphological criteria (nuclear margination, chromatin and cytoplasmic condensation, shrinkage from neighboring cells, and formation of apoptotic bodies with nuclear and cytoplasmic fragmentation)²⁹. Each assessment of apoptosis consisted of the mean of a minimum of 16 different crypt-villus-complexes per animal, and a minimum of 5 animals per experimental set. Scoring of apoptosis was similar to that described by Marshman, et³⁰. A Nikon TS-100 microscope was used at 400X power, and images were digitally recorded with an Evolution MP 5.1 CCD camera. Sections were selected that were completely longitudinal along the crypt/villus axis. In general, expression of EC apoptosis is referred to as the total number of apoptotic cells along this axis per 100 EC. In some cases a more detailed analysis of the location of apoptosis was performed using previously established techniques³⁰. For this, apoptosis along the villi were differentiated between the lower one-third of the villi and upper one-third of the villi. Apoptosis was recorded as the number of apoptotic cells per 100 EC. For apoptosis in the crypts, cells in each crypt were numbered starting at the base of the crypt column, and the number of apoptotic cells at each position was recorded as a ratio of all counted cells at this position³⁰.

Epithelial cell proliferation: Mice were injected intraperitoneally with 5-bromo-2-deoxyuridine (BrdU, 50 mg/kg, Roche Diagnostic Corporation, IN) 1 h before mice were sacrificed. Paraffin-embedded sections of 5 μ m thickness were deparaffinized with xylene. Immunohistochemistry was done by using a BrdU In-Situ detection Kit according manufacturer's guidelines (BD PharMingen, San Diego, CA). Briefly, endogenous peroxidase was quenched with 3% H₂O₂. Slides were then incubated with biotinylated anti-BrdU antibody in a 1:10 dilution, washed and then incubated with Streptavidin-horse radish peroxidase. Slides were then exposed to DBA substrate and counter-stained with hematoxylin. An index of the crypt cell proliferation rate was calculated by the ratio of the number of crypt cells incorporating BrdU to the total number of crypt cells. The total number of proliferating cells per crypt was defined as a mean of proliferating cells in 10 crypts (counted at 45X magnification).

Mucosal cell isolation: Isolation of mucosal cells was performed using a previously described protocol³¹. This included isolation of cells with an extraction buffer (1 mM EDTA, 1mM dithiotheritol in phosphate buffer saline), and purification in 30% isotonic Percoll (Pharmacia, Piscataway, NJ). Viability exceeded 95% using trypan blue exclusion staining.

IEL purification: Isolation of purified IEL from mucosal cell isolates was performed by direct magnetic separation. This allowed the detection of mRNA changes in a purified IEL population. Magnetic beads conjugated with antibody to CD45 (pan-lymphoid marker) were used to segregate IEL from EC (BioMag SelectaPure Anti-Mouse CD 45R Antibody Particles, Polyscience Inc., Warrington, PA). Magnetic separation was performed twice to further deplete EC. Final IEL purity was greater than 99% by flow cytometry.

IEL staining and sorting: IEL were stained with antibodies to T-cell receptor (TCR)- α β (H57, Invitrogen Corporation Life Technologies, Carlsbad, CA), TCR- γ δ (GL3, Invitrogen), CD4 (RM4-5, BD PharMingen, San Diego, CA) or CD8 α (53-6.7, PharMingen). Isotype control antibodies were used to adjust gating. IEL sub-populations were sorted using an EPICS

Elite (Coulter, Miami, Florida) flow cytometer. Sorted IEL were kept at 4°C until RNA isolation.

Isolation of total RNA: A guanidine isothiocyanate/chloroform extraction method was performed using Trizol (Gibco BRL, Gaithersburg, MD) according to manufacturer's directions.

Reverse transcriptase polymerase chain reaction (RT-PCR): mRNA (poly-A positive) was reversed transcribed into cDNA following a standard protocol³¹. Specific primers for selected gene sequences were designed using proprietary software (LaserGene, DNASTar, Inc, Madison, WI). PCR and gel were run under standard conditions³². To ensure that DNA product was generated at the exponential portion of the product curve 28 cycles were used for somatic ACE, 32 cycles for TNF- α ; and 34 cycles for angiotensin II receptors. Gel bands were analyzed by DNA sequencing to ensure the correct product. Kodak EDAS System (Rochester, NY) was used for imaging and quantification. Results were expressed as the ratio of the investigated mRNA over β -actin mRNA expression.

Real-time PCR: Real-time PCR was run to better quantify actual changes in ACE expression. cDNA was also used for real time PCR. The same primers for somatic ACE and β -actin were used as for conventional PCR, SYBR Green I was utilized for fluorescence. PCR was run with the following steps: 94° C for 15 seconds, 66° C for 15 seconds, and 72 ° C for 25 seconds. A cDNA standard curve was created with defined concentrations of cDNA. This allowed for an extrapolation of the numbers of copies using the following formula: cDNA copies/ml = DNA concentration (mg/ml) x (10⁶ pg/mg) x (pmol/660 pg) x (1/sequence size [bp]) x (10¹² mol/10³ ml) x 6.023 x 10²³. Ct- values of ACE and β -actin were converted to numbers of cDNA copies, and results expressed as the ratio of ACE over β -actin expression.

Immunoblot analysis: Protein was extracted from isolated IEL, and immunoblots performed using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) with standard methods³³. Purified biotinylated anti-mouse ACE (R&D Systems, Inc., Mineapolis, MN) (at a concentration of 0.1 μ g/ml diluted in blocking solution (Zymed Laboratories, Inc., San Francisco, CA) was used for ACE detection. This was followed by an application of horse radish peroxidase-streptavidin conjugate (1:5000 dilution; Zymed). Expression for β -actin was determined in the same fashion by re-probing membranes with purified anti-mouse β -actin (1:8000, Sigma-Aldrich, St. Louis, MO). The peroxidase-conjugated second antibody is goat anti-mouse IgG (1:5000 in blocking solution) from Santa Cruz Biotechnology (Santa Cruz, CA). Results are expressed as the ratio of ACE over β -actin protein expression.

Statistical analysis: Data are expressed as mean \pm standard deviation. These results were analyzed using ANOVA with least significant difference testing for post-hoc analysis of significance. For assessment of changes in apoptosis between crypt cells using an apoptotic index, a Fisher Exact test was used. Statistical significance was set at $P < 0.05$.

RESULTS

Changes in ACE expression after bowel resection

Increased ACE expression after bowel resection—To confirm previous microarray observations, IEL-derived ACE expression was measured after bowel resection using both real-time PCR and Western blot. Real-time PCR showed a significantly increased expression of IEL-derived ACE mRNA in the SBS group when compared to the Sham group ($P < 0.05$, $N = 5$) (Figure 1). Western immunoblotting showed a 24% increase in the SBS (2.07 ± 0.59) group, and was significantly ($P < 0.05$) higher than the Sham (1.69 ± 0.14) group (Figure 2).

Changes of ACE expression in IEL sub-populations—Because of the unique phenotype of the IEL, cells were sorted by phenotype markers. This allowed a detailed examination of the specific subpopulations of the IEL that expressed or did not express ACE, and the differential expression of ACE in IEL sub-population after bowel resection. mRNA expression of ACE was detected in all sorted IEL sub-populations (Table 1). Although a greater expression of ACE was noted in the CD8 α sub-population, no statistical difference was noted among groups.

Effects of ACE inhibition on small intestine

To understand the significance of increased ACE expression on SBS adaptive changes, the ACE inhibitor (ACE-I) enalaprilat was given to a separate group of Sham and SBS mice. These mice were matched to a control group of untreated mice, and were studied after a 7 day period.

Weight changes in the SBS model—All groups of mice showed some degree of weight loss following surgery; however, the loss in each of these plateaued by the latter two days of the study period. Pre-operative weights in the Sham, SBS and SBS+ACE-I groups were (grams): 25.5 \pm 1.3, 25.0 \pm 1.5 and 24.1 \pm 1.2, respectively. One week following surgery, weights were (grams): 24.2 \pm 2.6, 20.8 \pm 1.7, and 21.6 \pm 1.7, respectively; and represented a -4.7%, -16.5% and a -10.7% weight loss, respectively. The loss of weight in the SBS group was significantly greater than the Sham or SBS+ACE-I groups ($p=0.001$ and $P=0.01$, respectively); however, the loss of weight in the SBS+ACE-I group was not significantly different than the Sham operated group ($P=0.07$).

ACE-I administration significantly altered mucosal structural adaptation—Bowel adaptation in the SBS model: Bowel resection (i.e., the SBS model) led to significant intestinal hypertrophy (Table 2). The villus height was noted to significantly increase in the SBS group when compared with the Sham group. The crypt depth increased significantly after bowel resection ($P=$).

Alteration of adaptation with ACE-I: ACE-I administration led to a further increase in this intestinal hypertrophy (Table 2). ACE-I administration to SBS mice (SBS+ACE-I) resulted in a significant ($P=0.02$), additional, increase in crypt depth beyond that seen in untreated SBS mice. Villus height was unchanged compared to the untreated SBS group. Those mice undergoing a sham resection and treated with ACE-I (Sham+ACE-I) showed no significant alteration in histological features in either crypt depth or villus height when compared with the Sham group.

ACE-I significantly decreased SBS-associated apoptosis—Changes in EC apoptosis with surgical manipulation: Rates of EC apoptosis significantly increased in both the Sham and SBS groups compared to non-operated mice (Figures 3 and 4, and Table 2). Rates of apoptosis were highest in the SBS group (Table 2), however, both forms of surgical manipulation (Sham and SBS groups) led to increases in EC apoptosis compared to non-operated mice. Apoptosis was increased both in the crypt (Figure 3) as well as the villi (Figure 4).

Alteration of EC apoptosis with ACE-I: Because of the known action of ACE-I on apoptosis in the cardiac and pulmonary systems, intestinal EC apoptosis was examined by using ACE-I. ACE-I resulted in a significant decline in EC apoptosis rates was observed in both the Sham and SBS groups. EC apoptosis decreased 2.4-fold when compared with the untreated SBS group (Table 2). These levels declined to those similar to that observed in non-operated mice (Table 2). The action of ACE-I on lowering the rate of EC apoptosis varied with the location along the crypt-villus axis. A significant decline in the rate of apoptosis was noted in both the

crypt and lower one-third of the intestinal villi (Figures 3 and 4). Whereas, only a slight decline in EC apoptosis was noted with ACE-I in the distal one-third of the intestinal villi (Figures 3 and 4).

ACE-I administration significantly increased epithelial cell proliferation—To better understand the implications of ACE-I administration on the adaptation of ECs during the early stages of SBS, EC proliferation was assessed with BrdU incorporation. EC proliferation was much greater ($P < 0.01$) in SBS mice (0.24 ± 0.07) compared to the Sham group (0.12 ± 0.02 (number of crypt cells incorporating BrdU to the total number of crypt cells)). Administration of ACE-I led to a significant ($P < 0.001$) increase in EC proliferation in SBS mice (0.39 ± 0.06) compared to SBS mice which did not receive ACE-I (0.24 ± 0.07).

ACE-I administration significantly increased ACE expression—Administration of ACE-I led to a significant increase in both IEL-derived ACE mRNA expression in both Sham (0.66 ± 0.22) and SBS (0.63 ± 0.25) groups of treated mice as compared to untreated Sham and non-operated mice ($P < 0.05$, $P < 0.01$, respectively, see Figure 1). A 24% increase in ACE mRNA expression between SBS and SBS+ACE-I groups, but the difference was not significant.

Mechanism of ACE-mediated EC apoptosis

Alteration in angiotensin II receptor expression—ACE is mediated via angiotensin II (ANG II) via type 1 (subtypes 1a and 1b) and type 2 receptors²¹. To address an ANG II-mediated mechanism of ACE and/or ACE-I on EC apoptosis, EC mRNA expression of type 1a, type 1b and type 2 ANG II receptors were measured (Table 3). For this purpose 3 groups of mice were investigated, Sham, SBS and SBS+ACE-I. ANG II receptor type 2 expression decreased significantly with administration of ACE-I ($P < 0.05$ SBS vs. SBS+ACE-I). No significant difference in mRNA expression was found between untreated Sham and SBS mice. ANG II receptor type 1a mRNA expression showed a decrease in the SBS+ACE-I group compared to the other groups; however, this difference did not approach significance. Type 1b receptor significantly decreased ($P = 0.01$) with ACE-I treatment in SBS mice compared to untreated SBS mice. No significant difference in mRNA expression was found between untreated Sham and SBS mice.

Alteration of pro-inflammatory cytokine gene expression—Because ACE-I may decrease apoptosis in the cardio-pulmonary system via a down-regulation of pro-inflammatory cytokine expression^{34, 35}, a similar mechanism was examined in the intestinal mucosa. To address this, expression of mRNA from a number of potential pro-inflammatory cytokines were examined: IL-1a, IL-6, IL-12a and 12b, tumor necrosis factor alpha (TNF- α) and interferon gamma (Figure 5). Three groups were studied: Sham, SBS and SBS+ACE-I. Of note, TNF- α showed a significant rise in mRNA expression in the SBS group (0.70 ± 0.19) compared to Sham levels (0.41 ± 0.13 , $P < 0.01$). SBS mice treated with ACE-I showed a significant decline in TNF- α mRNA (0.51 ± 0.07 , $P < 0.05$) compared to untreated SBS mice; and levels were not significantly different than Sham levels. A small decline in IL-1a was also noted, but this was not significant.

TNF- α and ACE-mediated EC apoptosis—The increase in TNF- α in SBS mice, and the observed decline in TNF- α expression with ACE-I treatment suggested this pathway may be needed for ACE-I to mediate its blockade of EC apoptosis. To further evaluate the mechanism by which ACE-I treatment blocks EC apoptosis, TNF- α -knockout (TNF- $\alpha^{-/-}$) mice were used. For this, a series of Sham operated mice (intestinal transection and re-anastomosis of the mid-small bowel) were studied in this section. This model was used as TNF- $\alpha^{-/-}$ mice were less tolerant of major bowel resections, yet increased levels of EC apoptosis were still observed in

Sham mice. Two groups of mice were investigated: 1) Sham operated TNF- $\alpha^{-/-}$ mice; and 2) Sham operated TNF- $\alpha^{-/-}$ mice treated with ACE-I. Rates of apoptosis in all TNF- $\alpha^{-/-}$ mice were consistently lower than wild-type mice (compare to Table 2). Interestingly, we found that ACE-I administration had no effect on the EC apoptosis in TNF- $\alpha^{-/-}$ mice. Rates of apoptosis were determined in each group. Sham TNF- $\alpha^{-/-}$ mice treated with ACE-I showed an EC apoptotic rate of $1.7 \pm 0.3\%$ with TUNEL assay. Sham TNF- $\alpha^{-/-}$ mice which did not receive ACE-I had an EC apoptotic rate of $1.8 \pm 0.3\%$ with TUNEL staining ($P > 0.05$). This suggests that the presence of TNF- α was essential for ACE-mediation of EC apoptosis.

Alteration of Fas and Fas ligand expression—Based on cardio-pulmonary literature, Fas and Fas ligand (FasL) have been suggested to mediate ACE apoptotic actions³⁵. EC mRNA expression of Fas and IEL mRNA expression of FasL were measured (using magnetic bead isolation, Table 4). EC-derived Fas-expression increased slightly (but not significantly) in SBS mice (1.12 ± 0.13) compared to Sham mice (0.96 ± 0.08). The expression of Fas decreased with ACE-I treatment in SBS mice (Table 4), but the decline was also not significant. IEL-derived FasL did not significantly change between Sham; SBS; and SBS +ACE-I groups.

DISCUSSION

In this study, several novel findings are reported (Summarized in Table 5). First, the increase in IEL-derived ACE expression after formation of SBS was confirmed with both real-time PCR and Western immunoblots. Additionally, ACE expression was found in all four major T-lymphocyte IEL sub-populations: $\alpha\beta$ -TCR⁺ and $\gamma\delta$ -TCR⁺, as well as both CD4⁺ and CD8⁺ IEL. This increase in IEL-derived ACE may be associated with an up-regulation of intestinal EC apoptosis after bowel resection. ACE-I was used to assess the functional role of ACE in the mucosa. We observed that the administration of ACE-I significantly decreased EC apoptosis. Additionally, the finding that ACE-I was ineffective in reducing EC apoptosis in TNF- α knockout mice suggests that TNF- α may be involved in this ACE-mediated EC apoptosis.

ACE has been identified as a membrane-bound enzyme (peptidyl dipeptidase) in several immunologic cells, including lymphocytes and macrophages. T-lymphocytes have been shown to contain high levels of ACE, approximately 28 times more per cell than monocytes³⁶. High levels of ACE have also been found along the intestinal mucosa in human, swine, rat, as well as mouse intestine^{22, 23 24–27}. Immunohistochemical studies^{23, 25} have shown that there is strong expression of ACE identified in non-vascular sites of human and mouse intestinal mucosa; however, ACE expression has not been previously described in the IEL. ACE protein is located on the microvillus surface of the epithelium, and notably on the luminal surface of the small intestine. Despite this well-appreciated presence of ACE within the gastrointestinal mucosa – the functional role of the RAS in the intestinal mucosa has not been well defined. ACE has been recently found to mediate the induction of alveolar EC apoptosis^{19, 37}. Administration of ACE-I has led to reduced apoptotic rates in alveolar EC, renal EC and cardiac myocytes^{20, 35, 38}. Such action may have great importance in preservation of the function of these organs in a number of disease processes. Based on our finding of increased ACE mRNA and protein expression within the IEL, we then hypothesized that ACE may also have a relevant role in the modulation of EC apoptosis within the gastrointestinal mucosa. Administration of the ACE-I enalaprilat to SBS mice reversed the increase in EC apoptosis observed with SBS alone, bringing levels to that of non-operated mice. Interestingly, this observed ACE-I-associated decline in EC apoptosis was predominately in the crypt region and lower one-third of the villi. Morphologically, this decline in EC apoptosis was reflected in a significant increase in crypt depth; whereas there was relatively little change in total villus length. Crypt cell apoptosis has been shown to progress quite differently compared to apoptosis along the villi³⁰. This also suggests that ACE may play a key role in the remodeling of the

crypt during the early phases of SBS adaptation. This location of EC apoptosis was different from the finding of apoptosis in the villus tips, as seen with the normal process of villus growth and in some models of intestinal ischemia³⁹. Importantly, however, EC apoptosis in other mouse models of intestinal injury as well as in other studies of SBS have been shown to predominate in the crypt region^{30, 40}. Thus it appears that different processes will greatly affect the location of apoptosis along the crypt-villus axis. The observed changes in weight loss – and particularly the lower degree of weight loss in the SBS+ACE-I group may suggest an improvement in SBS adaptation in the ACE-I treated group, although specific absorption studies would need to be performed in future studies to prove this. It was also interesting to note that EC proliferation was also augmented in the SBS+ACE-I group compared to the SBS group. Interestingly, ACE may also demonstrate effects on proliferation, as well as apoptosis²⁰. This ACE-I associated augmentation of EC proliferation, may help to augment SBS adaptation, although this will need future work to confirm.

The mechanism by which ACE-I affects apoptosis is not fully understood. Several studies have shown a change in lymphocyte cytokine expression with the use of ACE-I. Schindler, et al. investigated the *in vitro* synthesis of TNF- α from human peripheral blood mononuclear cells in the presence of the ACE-I captopril. These investigators showed a dose-dependent suppression of the synthesis of TNF- α ⁴¹. Our group has previously showed that IEL-derived TNF- α is increased after the creation of a SBS model in mice³². Our current study confirmed this finding. Interestingly, we further observed a significant decline in TNF- α with ACE-I. To further address the possibility of a TNF- α -dependent mechanism for the reduction of apoptosis with ACE-I, TNF- α ^{-/-} mice were used. TNF- α ^{-/-} mice subjected to a Sham (transection) operation demonstrated no change in EC apoptotic rates with or without the administration of the ACE-I enalaprilat. This strongly suggests that ACE-mediated EC apoptosis within the gastrointestinal tract is dependent on TNF- α . It also suggests that the observed down-regulation in TNF- α expression by enalaprilat could be a major mechanism by which ACE-I mediates its anti-apoptotic action. This effect of ACE-I on TNF- α expression has also been shown by Fukuzawa, et al using blood mononuclear cells. In their study the administration of captopril, delapril or cilazapril was shown to inhibit the expression of TNF- α ⁴². Ortiz, et al studied modulation of TNF- α expression in a mouse model of pulmonary hypertension induced by bleomycin. In this model there was an enhanced expression of TNF- α expression in the lung. Administration of enalaprilat inhibited lung-TNF- α expression⁴³.

ACE may work via the renin-angiotensin-system by producing angiotensin II (ANG II). Wang, et al⁴⁴ found that ANG II was a potent inducer of apoptosis in alveolar EC. It has been shown that ANG II acts through ANG II type 1 and type 2 receptors²¹. Although variable responses may be seen in different tissues, type 1 receptors can mediate the effects of smooth muscle cell proliferation and growth^{20, 45}. Others have also associated type 1 receptors are also important mediators for apoptosis, although the type of mediation may vary with different tissues. In vascular smooth muscles cells, apoptosis was significantly reduced in ANG II type 1a knockout mice⁴⁶. Conversely, others have shown that the type 1a receptor is needed for the mediation of alveolar EC apoptosis⁴⁷. Type 2 receptors may enhance cell differentiation⁴⁸ as well as stimulate apoptosis⁴⁹. In our study we found that administration of enalaprilat to SBS mice had led to a significant decline in type 1b and type 2 receptors, as well as a large, but non-significant decline in the type 1a receptor. This may imply that ACE mediation of EC apoptosis, and its prevention with enalaprilat, is via a modulation of the expression of ANG II receptors. Further assessment of this pathway will be needed in the future to confirm this observation. ACE may be expressed by both IEL (as observed in our current study) as well as by EC, themselves⁵⁰. Although there was a marked increase in ACE expression by IEL in our SBS model, future studies will need to be done to better define the amount of apoptosis mediated by IEL-derived versus EC-derived ACE in the modulation of EC apoptosis following SBS formation.

Another signaling pathway for EC apoptosis is via the Fas/Fas ligand (FasL) system⁵¹. Wang, et al showed that activation of Fas stimulates alveolar EC to synthesize ANG II de novo⁴⁴. Moreover, the autocrine synthesis of ANG II was found to be required for the induction of apoptosis by Fas. Apoptosis could be abrogated by ANG receptor antagonists or other blockers of ANG II function. In our study, we did not find any significant changes in Fas or FasL mRNA expression after bowel resection. Additionally, ACE-I administration after bowel resection did not lead to any significant change in either Fas, or FasL expression. These results are supported by work from Knott et al⁵² and a study from Tang et al⁵³. Both of these investigators reported that there were no significant changes in Fas and FasL after bowel resection. These results may suggest that Fas/FasL extrinsic pathway is not involved in this SBS associated EC apoptosis.

The intrinsic pathway via the Bcl-2 family of proteins have been well studied and found to play an important role in the SBS related EC apoptosis⁵²⁻⁵⁵. An increase in intestinal bax (pro-apoptotic gene) expression and a decrease in bcl-2 (anti-apoptotic gene) were found in a mouse SBS model^{55, 56}. Interestingly, the bcl-2 family of proteins appears to be another mediator of apoptosis by the RAS^{46, 57-60}. Studies have identified a link between ANGII signaling and apoptosis through this family of proteins⁵⁷⁻⁶¹, including a decrease in the bcl-2 : bax protein ratio in other systems. Whether IEL-derived ACE mediates apoptosis via the intrinsic pathway, including changes in the bcl-2 family, will be important areas for future investigations

Conclusion

In conclusion, SBS was associated with a previously undescribed expression of ACE by intestinal IEL. Another novel finding was that administration of the ACE-I enalaprilat reversed the increase in SBS-associated EC apoptosis; suggesting that, as observed in cardiac and pulmonary tissue, ACE-I may have a similar action on intestinal epithelial cells. Possible anti-apoptotic mechanisms of the ACE-I enalaprilat in the intestinal tract may be through RAS via suppression of TNF- α expression. It is potentially possible that SBS patients may benefit from the use of ACE-I, via a reduction of the apoptotic rate – thus facilitating the degree of adaptation. Future investigation will hopefully offer greater insight into the action of ACE on the gastrointestinal mucosa.

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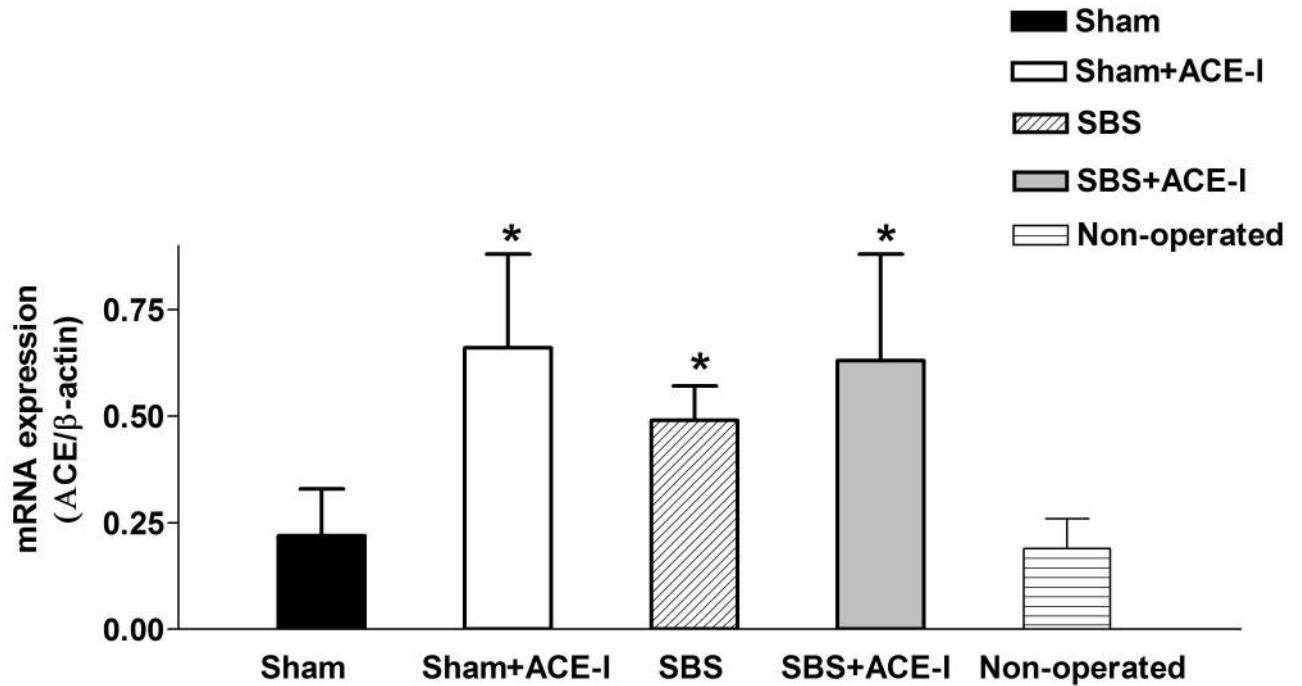


Figure 1. ACE mRNA expression: mRNA expression of angiotensin converting enzyme (ACE) by real-time polymerase chain reaction techniques. Results are the number of ACE-cDNA copies over β -actin-cDNA copies (mean \pm SD, data from at least 5 mice per group). Abbreviations: IEL, intraepithelial lymphocytes; ACE-I, angiotensin converting enzyme inhibitor, SBS, short bowel syndrome. * $P < 0.05$ Sham vs. SBS.

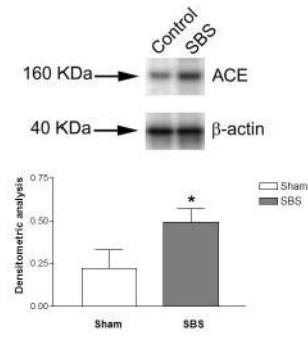
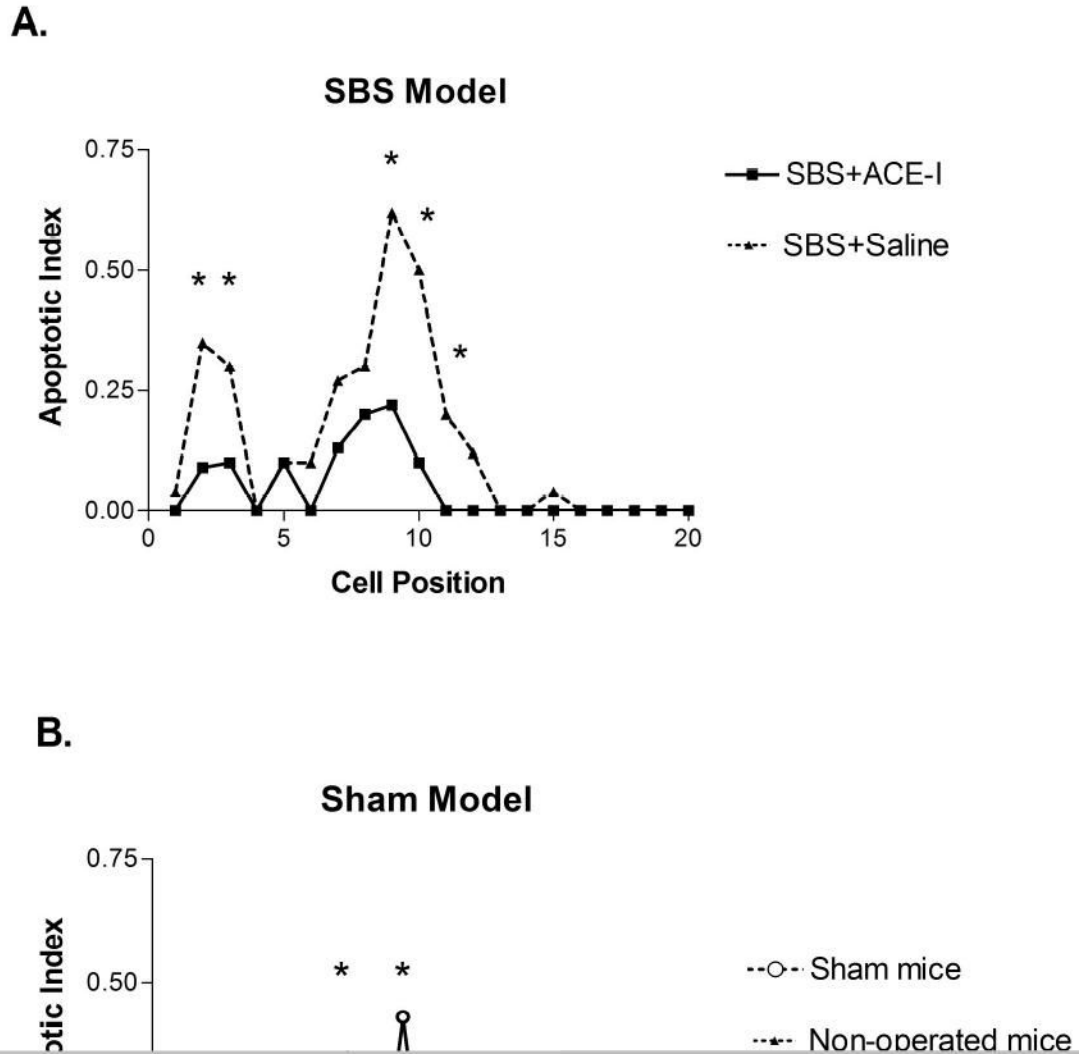


Figure 2. ACE protein expression: Western immunoblots from a representative mouse in the Sham (Control) and SBS groups. Both ACE (at 160 KDa) and beta actin (β -actin, 40 KDa), used as a control, are shown.

**Figure 3.**

Crypt cell apoptosis: Cell positional distribution of apoptosis in small intestinal crypts from mice in each treatment group. The first cell position is at the base of the crypt, and apoptosis is reported as an index of apoptotic cells to non-apoptotic cells found in that cell position. A minimum of 10 half-crypts (one side of a crypt) were counted per animal, and at least 5 animals for each study group were assessed. * $P < 0.05$ comparing the two groups of mice.

A) Apoptotic index is shown for the short bowel syndrome (SBS) group with or without angiotensin converting enzyme (ACE-I) treatment.

B) Apoptotic index is shown for the Sham treated (transection without resection) and non-operated mouse groups.

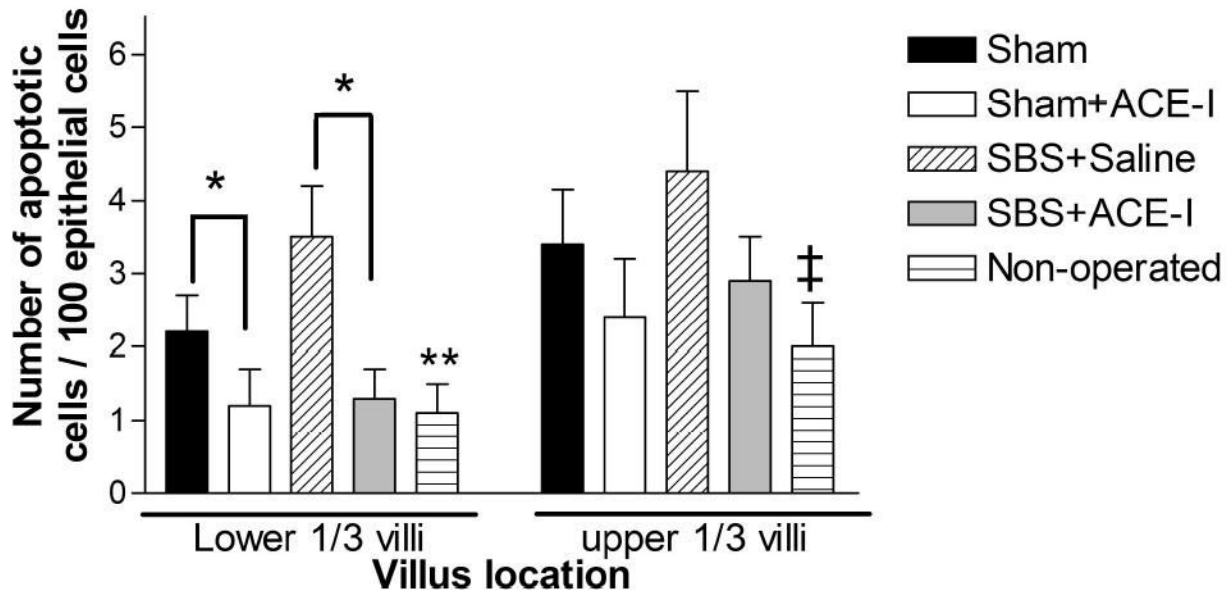


Figure 4.

Villus apoptosis: Apoptotic rate (number of apoptotic cells per 100 epithelial cells) is shown for the experimental groups. Data is from a minimum of 5 mice in each group, and at least 10 villi from each mouse.

Left portion of the graph: Data from the lower one-third of the villi, * $P < 0.05$ Sham vs. Sham + ACE-I, and SBS group vs. SBS+ACE-I. ** $P < 0.01$ Non-operated mice compared to Sham operated and SBS mice.

Right portion of the graph: Data from the upper one-third of the villi. ‡ $P < 0.05$ Non-operated mice vs. SBS and Sham groups. ACE-I resulted in a reduction in apoptosis for the SBS and Sham groups, but this was not significant ($P = 0.15$).

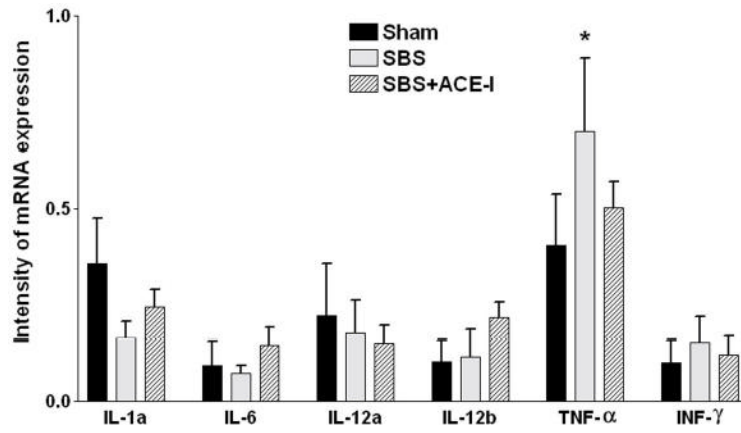


Figure 5.

IEL Cytokine expression: Cytokine modulation of intraepithelial lymphocytes (IEL) 1 week postoperatively in Sham, SBS and SBS+ACE-I groups. Intensity of mRNA expression is given as the relative value compared to beta actin expression. Of note, TNF- α showed a significant rise in mRNA expression in the SBS group compared to Sham levels. SBS mice treated with ACE-I showed a decline in TNF- α mRNA to Sham levels. A small decline in IL-1a was also noted, but this was not significant. * $P < 0.01$ Sham vs. SBS, and $P < 0.05$ for SBS vs. SBS+ACE-I.

Table 1

ACE mRNA expression in IEL sub-populations.

IEL Sub-population	ACE mRNA expression
$\alpha\beta$ -TCR	0.63 \pm 0.01
$\gamma\delta$ -TCR	0.57 \pm 0.15
CD4	0.77 \pm 0.13
CD8 α	0.86 \pm 0.13

IEL were derived from adult, untreated mice, and were purified using flow cytometric sorting. Note the fairly uniform expression of ACE in each sub-population. Abbreviations: TCR, T-cell receptor. Data is derived from semi-quantitative PCR based on the relative expression of beta actin, and is a mean (\pm SD) of IEL derived from 3 individual sortings.

Table 2
Epithelial cell apoptosis and crypt-villus dimensions

Group	EC Apoptosis ^a (%)	Villus height (μm)	Crypt depth (μm)
Non-operated Control	1.6 ± 0.7	310 ± 42	93 ± 18
Sham	2.7 ± 1.0	353 ± 40	77 ± 10
Sham+ACE-I	1.9 ± 0.4 [†]	340 ± 20	87 ± 6
SBS	4.3 ± 1.3 [*]	556 ± 66 [*]	113 ± 14 [*]
SBS+ACE-I	1.8 ± 0.5 [‡]	552 ± 56 [*]	145 ± 22 [‡]

Summary (mean ±SD, from at least 6 mice per group) of results of ACE-inhibitor (ACE-I) studies. Sham+ACE-I was used as an additional control to assess the effects of ACE-I in the absence of a SBS.

^aResults of TUNEL analysis (number of apoptotic cells per 100 epithelial cells).

Abbreviations: IEL, intraepithelial lymphocytes; ACE-I angiotensin converting enzyme inhibitor; EC, epithelial cells.

* P<0.05 sham vs. SBS

[†] P<0.05 Sham vs. Sham + ACE-I

[‡] P<0.05 SBS vs. SBS+ACE-I.

Table 3
Angiotensin II (ANG II) receptor type 1 and type 2 mRNA expression

Group	ANG II receptor, type 1a	ANG II receptor, type 1b	ANG II receptor, type 2
Sham	0.13 ± 0.14	0.14 ± 0.17	0.41 ± 0.19
SBS	0.05 ± 0.05	0.14 ± 0.03	0.44 ± 0.09
SBS+ACE-I	0.03 ± 0.03	0.03 ± 0.03 [†]	0.30 ± 0.20*

Expression of the ANGII receptor type 1a and 2 were significantly decreased with administration of ACE-I (P<0.05 for SBS vs. SBS + ACE-I). Data are from semi-quantitative RT-PCR based on the relative expression of the target gene to beta actin expression (mean ±SD, N=6).

* P<0.05 for SBS+ACE-I group vs. SBS group.

[†] P=0.001 for SBS+ACE-I group vs. SBS group.

Table 4

Fas and Fas ligand mRNA expression

Group	Fas	FasL
Sham	0.96 ± 0.08	0.36 ± 0.25
SBS	1.12 ± 0.13	0.24 ± 0.20
SBS+ACE-I	1.00 ± 0.17	0.24 ± 0.11

Data shown are Fas and Fas ligand mRNA expression. Fas is expressed by epithelial cells and FasL is expressed by intraepithelial lymphocytes. Note there was a slight increase in Fas in the SBS group, and a decline with ACE-I treatment. This approached, but did not reach significance (P=0.07). FasL expression did not appreciably change in any of the groups. Data (mean ± SD, N=6 per group) are from semi-quantitative RT-PCR analysis based on the relative expression of beta actin

Table 5
Summary of the major findings of ACE-I action after SBS formation

Parameter studied after formation of SBS	Action of ACE-I
Crypt-villus architecture	Increased crypt depth
Epithelial cell apoptosis	Decreased
Epithelial cell apoptosis in TNF- α knockout mice	No effect
Epithelial cell proliferation	Increased
ACE expression	Increased