NHE3 modulates the severity of colitis in IL-10-deficient mice

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Larmorier CB, Laubitz D, Thurston RD, Bucknam AL, Hill FM, Midura-Kiela M, Ramalingam R, Kiela PR, Ghishan FK. NHE3 modulates the severity of colitis in IL-10-deficient mice. Am J Physiol Gastrointest Liver Physiol 300: G998–G1009, 2011. First published March 17, 2011; doi:10.1152/ajpgi.00073.2011.—NHE3, the major intestinal Na+/H+ exchanger, was shown to be downregulated and/or inhibited in patients with inflammatory bowel disease (IBD), a phenomenon believed to contribute to inflammation-associated diarrhea. NHE3−/− mice spontaneously develop colitis and demonstrate high susceptibility to dextran sulfate-induced mucosal injury. We investigated the effects of NHE3 deficiency on the development of chronic colitis in an IL-10 knockout (KO) mouse model of Crohn’s disease. NHE3−/− mice were first backcrossed to 129/SvEv mice for >10 generations, with no apparent changes in their survival or phenotype. These mice were crossed with IL-10−/− mice on the same genetic background, and the phenotypes of 10-wk-old wild-type (WT), IL-10−/−, NHE3−/−, and IL-10−/−/NHE3−/− (double-KO) mice were studied. Histological and immunohistochemical examination of the colon established important functional alterations, including increased neutrophilic and mononuclear cell infiltration in double- compared with single-KO mice. Double-KO mice demonstrated increased expression of neutrophil collagenase matrix metalloproteinase-8 and the chemokines macrophage inflammatory protein-2, CXCL1, CXCL10, and CXCL11. Colonic IFNγ, IL-17, and IL-12/23 p40 protein secretion was significantly increased in double-compared with single-KO mice. IL-10−/−/NHE3−/− mouse colonic epithelium exhibited increased hallmarks of apoptosis, including a significantly increased number of cleaved caspase-3-positive surface epithelial cells. These results highlight the importance of NHE3 in the maintenance of intestinal barrier integrity and in modulating the inflammatory process in IL-10-deficient mice. Chronic NHE3 inhibition or underexpression observed in IBD may therefore contribute to the pathogenesis of IBD by influencing the extent of the epithelial barrier defect and affect the ultimate degree of inflammation.

Diarrhea, observed commonly in inflammatory bowel disease (IBD), is a direct result of perturbations in colonic absorptive and secretory processes (4, 21). Electroneutral Na+/H+ exchange (NHE), mediated primarily by NHE3, plays a critical role in intestinal Na+ and water absorption, regulation of intracellular pH, and acidity of the apical microenvironment, and its inhibition is believed to significantly contribute to IBD-associated diarrhea. NHE3 is a target of proinflammatory cytokines such as TNFα and IFNγ, major proinflammatory cytokines associated with intestinal inflammation (1, 21), as well as of the enteropathogenic bacteria (13). In vivo, NHE3 activity and transport function are severely impaired in different models of spontaneous colitis, such as IL-2 knockout (KO) and IL-10 KO mice, with negligible changes in NHE3 gene expression (2, 27). In a model of systemic T cell activation driven by anti-CD3 injection, TNFα was shown to simultaneously inhibit NHE3-mediated Na+ transport and promote myosin light chain kinase-mediated barrier dysfunction (5). NHE3 expression has been shown to be significantly downregulated (>87% decrease) in the mucosa of patients with active IBD (30). Other recent studies with biopsy specimens obtained from control and ulcerative colitis patients demonstrated a significant impairment of colonic NHE3 activity without changes in mRNA or protein expression (11, 34).

Chronic NHE3 inhibition in intestinal inflammation may have consequences beyond alteration of ion transport. There is a significant upregulation of IFNγ and IFNγ-inducible genes in the jejunal mucosa of NHE3-deficient mice (18, 32), with the infiltrating CD8+ and asialoGM1+ NK cells as the primary sources of the cytokine (16). Moreover, NHE3-deficient mice spontaneously develop colitis restricted to distal colonic mucosa (18). NHE3−/− mice housed in a conventional facility exhibited phenotypic features such as mild diarrhea, occasional rectal prolapse, and reduced body weight. Furthermore, NHE3−/− mice display alterations in epithelial gene and protein expression patterns that predispose them to a high susceptibility to dextran sulfate sodium (DSS), with accelerated mortality resulting from intestinal injury and bleeding, hypovolemic shock, and sepsis (16). Our studies suggest that NHE3 participates in mucosal responses to epithelial damage, acting as a modifier gene determining the extent of the gut inflammatory responses in the face of intestinal injury.

The aim of this study was to investigate the contribution of NHE3 to the development of colitis, immune activation, and mucosal homeostasis in IL-10-deficient mice, an established Crohn’s disease (CD) model of unrestrained activation of a T-helper (Th)-1/Th-17-mediated immune response (8, 33). Severe IBD has been described to be more prominent in patients with IL-10 receptor deficiency. We created double-KO mice lacking both IL-10 and NHE3, which represents a combination of several key factors implicated in the pathogenesis of IBD: genetic predisposition (22), loss of immune tolerance to colonic commensal bacteria (24, 28), and defective intestinal NHE with the ensuing consequences (16, 18). Examination of proximal and distal colon established important histological alterations, such as a dramatically increased neutrophilic and mononuclear cell infiltration in NHE3−/−×IL-10−/− double-KO mice compared with single-KO littermates. Leukocyte infiltration was accompanied by elevated expression of macrophage inflammatory protein-2 (MIP-2) and keratinocyte chemoattractant (KC) neutrophil chemokines and the IFNγ-inducible T cell chemokines CXCL10 and CXCL11. Colonic IFNγ and IL-17

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NHE3 Care and Use Committee. The procedures were approved by the University of Arizona Animal Care and Use Committee. Sendai, epizootic diarrhea of infant mice, mouse minute virus, Theiler’s murine encephalomyelitis, murine pathogens (mouse hepatitis virus, mouse parvovirus, minute virus), and compound-homozygous mice are IL-10 deficient.

MATERIALS AND METHODS

Experimental animals. Slc9a3-deficient (NHE3−/−) mice on a mixed genetic background (129/Black Swiss) (26) were obtained from Dr. Gary Shull (University of Cincinnati, Cincinnati, OH). They were backcrossed for >10 generations into the 129/SvEv background and then bred to IL-10−/− (also 129/SvEv) mice obtained from the laboratory of Ballour Sartor (University of North Carolina at Chapel Hill, Chapel Hill, NC). The mice were maintained in the animal facility at the University of Arizona Health Sciences Center as described previously (18). All mice were maintained in identical conditions as one colony. Wild-type (WT) mice are homozygous IL-10−/− or NHE3−/− (single-KO) mice, and compound-homozygous mice are IL-10−/−/NHE3−/− (double-KO) mice. Sentinel mice were routinely monitored and determined as free from common murine pathogens (mouse hepatitis virus, mouse parvovirus, minute virus, Thielер’s murine encephalomyelitis, Mycoplasma pneumoniae, Sendai, epizootic diarrhea of infant mice, mouse minute virus, and ecto- and endoparasites). All animal protocols and procedures were approved by the University of Arizona Animal Care and Use Committee.

Histology and scoring. Proximal and distal colons from WT, NHE3−/−, IL-10−/−, and NHE3−/−/IL-10−/− mice were harvested and fixed in 10% neutral buffered formalin (Fisher Scientific, Tustin, CA). Fixed tissues were embedded in paraffin, and 5-μm-thick sections were stained with hematoxylin and eosin for light-microscopic examination. Sections were graded according to previously published criteria (17) by a veterinary pathologist blinded to the study design.

Hematology. Hematologic profile analysis with complete differential was done by an experienced pathologist at the University Animal Care and Use Committee.

Immunohistochemistry. Sections of proximal and distal colon were prepared as described above. After deparaffinization and rehydration, antigen retrieval was performed by heating slides in citrate buffer (10 mM sodium citrate and 0.05% Tween 20, pH 6.0), Slides were washed in PBS, and residual endogenous peroxidase activity was quenched by 3% H2O2 in water for 10 min. Slides were then incubated for 1 h in normal goat serum blocking buffer (KPL, Gaithersburg, MD) or normal rabbit serum blocking buffer (Vector Laboratories, Burlingame, CA), depending on the secondary antibody. Next, sections were incubated with a primary antibody against cleaved caspase-3 (1:50 dilution; Cell Signaling Technology, Danvers, MA) or mouse neutrophil polymorphic 40-kDa antigen (rat anti-mouse MCA771GA antibody, 1:50 dilution; Serotec, Raleigh, NC) in 1× PBS with 1% BSA for 1 h. Slides were then incubated with biotinylated secondary antibody and avidin-peroxidase complex according to the manufacturer’s recommendation (KPL or Vector Laboratories). Slides were then washed in 0.1% PBS, incubated with 3,3′-diaminobenzidine (Vector Laboratories) and mounted with VectaMount medium (Vector Laboratories). Slides were examined independently by two experienced scientists in a blind manner using a Zeiss Axioplan microscope (Carl Zeiss MicroImaging, Thornwood, NY). Images were captured with a Nikon Digital Sight DS-Fi1 camera and NIS-Element software (Nikon Instruments, Melville, NY). Staining was scored by counting the number of cleaved caspase-3-positive cells per high-power field (×200 magnification). A total of five fields in three randomly chosen sections were analyzed for each group.

Real-time RT-PCR. Real-time RT-PCR was used to evaluate colonic expression of IFNγ, inducible nitric oxide synthase, IL-17, IL-12/23 p40, MIP-2, CXCL1 (KC), matrix metalloproteinase (MMP)-8, CXCL9, CXCL10, and CXCL11 mRNA. Total RNA was isolated from mouse colon using TRIzol reagent (Invitrogen, Carlsbad, CA), and its integrity was confirmed by denaturing agarose gel electrophoresis and calculated as the densitometric 28S-to-18S ratio. Total RNA (250 ng) was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Subsequently, 20 μl of the PCRs were set up in 96-well plates containing 10 μl of 2× iQ Supermix (Bio-Rad), 1 μl of TaqMan primer-probe set (Applied Biosystems, Foster City, CA), 2 μl of the cDNA synthesis reaction (10% of RT reaction), and 7 μl of nuclease-free water. Reactions were run and analyzed on a Bio-Rad iCycler iQ real-time PCR detection system. Cycling parameters were determined, and resulting data were analyzed using the comparative cycle threshold (Ct) method as a means of relative quantification, normalized to an endogenous reference (TATA box-bonding protein) and relative to a calibrator (normalized Ct value obtained from control mice) and expressed as 2−ΔΔCt.

Cytokine assays. IFNγ, inducible nitric oxide synthase, IL-17, and IL-12/23 p40 concentrations in the supernatant of colonic explant and mesenteric lymph node cultures were measured by ELISA (eBioscience, San Diego, CA) according to the manufacturer’s protocols.

Statistical analysis. Statistical significance was determined by ANOVA followed by Fisher’s protected least significant difference post hoc test with the StatView software package (version 4.53, SAS Institute, Cary, NC). Data are expressed as means ± SE.

RESULTS

NHE3 deficiency in 129/SvEv mice. The original colony of NHE3−/− mice was maintained on a mixed 129/Black Swiss background (26). Breeding of these mice into the C57BL/6 background results in increased severity of diarrhea and higher rates of spontaneous mortality, particularly around weaning age (G. Shull, personal communication; P. R. Kiela et al., unpublished observations). There was therefore a legitimate concern about the health status of NHE3-deficient mice when they were transferred into an inbred 129/SvEv background compatible with future cross with IL-10−/− mice. Evaluation of the symptoms in generations 5–10 on the 129/SvEv background indicated no increased mortality, small intestine length, or symptoms of diarrhea (intestinal distension, appearance of loose stools, or frequency of rectal prolapse). Breeding efficiency was also not altered on the 129/SvEv genetic background (data not shown).

Histological alterations in proximal and distal colon of IL-10−/−/NHE3−/− mice. Proximal and distal colonic morphology was analyzed from WT, NHE3−/−, IL-10−/−, and IL-10−/−/NHE3−/− mice. Consistent with our previously published observations (18), histological analysis of the distal colon of NHE3−/− mice showed a characteristic pattern of inflammation, including crypt hyperplasia, apparent loss of goblet cells, neutrophil infiltrate, and mild edematous...
Fig. 1. Histology of proximal and distal colon of wild-type (WT), IL-10−/−, NHE3−/−, and IL-10−/−/NHE3−/− mice. A: histological analysis (hematoxylin-eosin staining) of colon morphology in WT, IL-10−/−, NHE3−/−, and IL-10−/−/NHE3−/− mice. Sections were scored on a scale of 0–5, based on degree of lamina propria mononuclear cell infiltration, crypt hyperplasia, and architectural distortion, by an unbiased pathologist according to previously described criteria (17). B and C: penetration of colitis, expressed as percentage of mice with histological score >2, in proximal and distal colon.
changes. IL-10−/− mice showed signs of colonic inflammation consistent with those described by others (7). Proximal and distal colon were affected, with degenerative lesions and excessive regenerative mucosal hyperplasia, leading to a marked thickening of the intestinal wall, accompanied by marked mixed leukocytic infiltrate in the lamina propria. Colonic sections were evaluated in a blinded manner by a veterinary pathologist according to the previously published criteria (17), taking into consideration lymphocytic and neutrophilic infiltration, crypt hyperplasia, mucosal ulcerations, focal or multifocal transmural necrosis, and penetration of the colonic wall. Double-KO mice exhibited a worsening of the inflammation pattern in distal and proximal colon (Fig. 1A). We observed significantly increased penetration of the disease, with significantly more mice exceeding the threshold of inflammation arbitrarily set to 2 (Fig. 1, B and C).

Hematologic parameters in WT, single-KO, and double-KO mice. To further characterize the contribution of NHE3 deficiency in IL-10 KO mice, basic hematologic parameters were evaluated. Although the neutrophil count was elevated in IL-10−/− and double-KO mice compared with WT or NHE3−/− mice, the overall white blood cell count showed only a tendency for increase, without reaching statistical significance (Fig. 2A). A small, but significant increase in the numbers of erythrocytes was observed in IL-10−/− mice but was normalized in double-KO mice. No significant differences in monocyte, basophil, or eosinophil counts were observed between groups (Fig. 2B). Hematologic parameters of the double-KO mice did not demonstrate any significant difference from homozygous single-KO mice.

Increased neutrophil and mononuclear cell recruitment and related chemokine expression in the colon of IL-10−/−NHE3−/− mice. Immunohistochemical staining of neutrophils was performed using the Ly-6B.2 antibody, which specifically recognizes a 40-kDa antigen expressed by polymorphonuclear cells but is absent in resident tissue macrophages. A significant increase in neutrophil infiltration in IL-10−/−NHE3−/− compared with WT, IL-10−/−, or NHE3−/− mice was observed in proximal (Fig. 3A) and distal (Fig. 4A) colon. This observation was further confirmed by real-time RT-PCR analysis of the mucosal expression of MMP8, a neutrophil-specific collagenase. In proximal colon, MMP8 mRNA expression was increased 10-, 17-, and 89-fold in IL-10−/−, NHE3−/−, and double-KO mice, respectively (Fig. 3B). In distal colon, the differences in MMP8 expression were also highly pronounced: 8-, 2.5-, and 137-fold in IL-10−/−, NHE3−/−, and double-KO mice, respectively (Fig. 4B). Consistent with this observation, expression of MIP-2 (CXCL2), a potent murine neutrophil chemokine and a functional IL-8 homolog, was also significantly elevated in double-KO mice. In proximal colon, MIP-2 transcript increased 5-, 3, and 71-fold in IL-10−/−, NHE3−/−, and double-KO mice, respectively (Fig. 3C), while in distal colon the respective differences were 32-, 16-, and 84-fold, respectively (Fig. 4C). We also assessed the expression of CXCL1 (KC), which, similar to IL-8, has been shown to positively correlate with disease activity in IBD patients (14). In agreement with increased neutrophil infiltration and MIP-2 expression data, KC mRNA expression was significantly increased in proximal and distal colon of IL-10−/−NHE3−/− mice compared with WT or single-KO littermates (Fig. 5).

To investigate the mechanism responsible for the histologically apparent increase in mononuclear cell infiltration in both colonic segments in double-KO mice, we analyzed the mRNA expression of major IFNγ-inducible T cell chemocytokines: CXCL9 (MIG), CXCL10 (IP10), and CXCL11 (IP9). Mucosal CXCL9 expression was not significantly increased in any of the mouse genotypes or in either colonic segment (Figs. 6A and 7A). In proximal colon, CXCL10 and CXCL11 were significantly increased in IL-10−/− mice, but not in NHE3−/− littermates, and there was no significant effect of the double-KO status in this colonic segment (Fig. 6). However, distal colonic expression of CXCL10 was enhanced in IL-10−/−NHE3−/− mice (16- and 28-fold over WT in IL-10−/− and IL-10−/−NHE3−/− mice, respectively; Fig. 7B), whereas CXCL11 mRNA in IL-10−/− and IL-10−/−NHE3−/− mice was increased 25- and 84-fold, respectively (Fig. 7C).

Elevated IFNγ, IL-17, and IL-12/23 p40 protein secretion in the colon of IL-10−/−NHE3−/− mice. IFNγ, IL-17, and IL-12/23 p40 were chosen as key cytokines involved in the Th-1/Th-17 T cell differentiation pathways implicated in the pathogenesis of Crohn’s disease and IL-10-deficiency colitis. Cytokine secretion was evaluated by ELISA in colonic explant culture supernatants. Consistent with previously published data (18), colonic expression of IL-17, IFNγ, and IL-12/23 p40 subunit was not elevated in NHE3−/− mice (Fig. 8). Production of all three cytokines in the colon of 10-wk-old IL-10−/− mice was elevated, although IL-17 did not reach the level of statistical significance (Fig. 8A). In IL-10−/−NHE3−/− mice, colonic secretion of IL-17, IFNγ, and IL-12/23 p40 was sig-
Fig. 3. Neutrophilic infiltration and macrophage inflammatory protein (MIP)-2 chemokine expression in proximal colon of WT, IL-10−/−, NHE3−/−, and IL-10−/−NHE3−/− mice. 

A: neutrophil-specific immunohistochemistry staining with MCA771GA antibody recognizing a polymorphic 40-kDa antigen expressed by murine polymorphonuclear (PMN) cells (brown staining; ×200 magnification). B and C: quantitative assessment of PMN infiltration by real-time RT-PCR analysis of matrix metalloproteinase-8 (MMP8), a neutrophil collagenase, and MIP-2, a potent neutrophil chemoattractant, in colonic mucosa of WT, IL-10−/−, NHE3−/−, and IL-10−/−NHE3−/− mice. Values were normalized to TATA box-binding protein (TBP) mRNA as an internal control and calculated using the cycle threshold (ΔΔCT) method, with WT mice used as a calibrator. Statistical significance was determined by ANOVA followed by Fisher’s protected least significant difference post hoc test, with P ≤ 0.05 considered significant; horizontal lines above bars show P values.

In our initial description of spontaneous colitis in NHE3−/− mice, microarray analysis of colonic gene expression identified a significant number of genes associated with regulation of programmed cell death (18). Although NHE3−/− mice were found to be highly susceptible to DSS-induced epithelial injury (18), which is typically associated with increased epithelial cell apoptosis (31), NHE3 status in mice did not influence the numbers of cleaved caspase-3-positive cells after short-term exposure to DSS (18). However, a chronically increased level of proinflammatory cytokines, such as IFNγ, is significantly higher than in WT mice. Moreover, secretion of IL-17 and IFNγ by the colonic explants was significantly higher in double-KO than IL-10−/− mice (Fig. 8). Real-time RT-PCR analysis of the cytokine mRNA level in colonic mucosa followed the same pattern (data not shown).
believed to significantly contribute to the pathology of IBD, in part by interfering with antiapoptotic signals in the epithelium as well as by modulating the integrity of the epithelial barrier (9). Therefore, we investigated whether loss of NHE3 could contribute to the worsening of the symptoms observed in double-KO mice through increased epithelial cell apoptosis. Proximal and distal colonic sections were stained for cleaved caspase-3, and the numbers of cleaved caspase-3-positive cells were evaluated (Figs. 9 and 10). The number of cleaved caspase-3-positive cells was significantly increased in proximal (Fig. 9) and distal (Fig. 10) colon of the IL-10−/− NHE3−/− mice. The differences were more dramatic in proximal colon (typically more affected in IL-10 deficiency), where the number of apoptotic epithelial cells was increased ~20-fold in double-KO mice compared with IL-10−/− mice (Fig. 9). Interestingly, in proximal colon, the majority of the cleaved caspase-3-positive cells stained with MCA771GA antibody (brown staining; ×200 magnification).
caspase-3 staining was observed in surface epithelial cells, where expression of NHE3 is normally the highest.

**DISCUSSION**

Under physiological conditions, the intestinal epithelium serves as a strict barrier between luminal content and the intestinal mucosa. As a very dynamic tissue, the epithelial barrier requires the maintenance of a balance between intestinal cell proliferation, differentiation, and apoptosis. At the interface between the intestinal microflora and the gastrointestinal-associated lymphoid tissue, the intestinal epithelium not only serves as a physical barrier, but it also plays an essential role in shaping the mucosal immune system, actively participating in the sensing of commensal and pathogenic bacteria, alongside its critical role in transport and nutrient absorption. Decreased expression of NHE3 or its key accessory proteins or greatly depressed NHE3 activity without changes in its expression has been reported in IBD (11, 30, 34), likely contributing to inflammation-associated diarrhea. However, loss of NHE3 function in Slc9a3 KO mice also leads to spontaneous distal colitis and extreme susceptibility to DSS-mediated mucosal injury (16, 18). Since NHE3 deficiency could not be studied in chronic settings using chemically induced models of IBD, we investigated its contribution to the development of colitis in IL-10−/− mice, an accepted model of chronic Crohn’s-like disease. To this end, we crossed NHE3 mice into the 129/SvEv genetic background for >10 generations with no adverse effects on their phenotype. We also evaluated the penetration and severity of colitis in 10-wk-old single-KO (NHE3−/− or IL-10−/−) and double-KO (IL-10−/− NHE3−/−) mice.

Histological, immunohistochemical, and gene expression analyses of proximal and distal colon established important architectural features consistent with exacerbated colitis in IL-10−/− NHE3−/− mice compared with WT or single-KO mice. Increased lamina propria infiltration with granulocytes and mononuclear cells was accompanied by elevated expression of key neutrophil and T cell chemokines (MIP-2, CXCL1, CXCL9, and CXCL11).

Fig. 5. Real-time PCR analysis of CXCL1 [keratinocyte chemoattractant (KC)] mRNA expression in proximal (A) and distal (B) colon of WT, IL-10−/−, NHE3−/−, and IL-10−/− NHE3−/− mice. Values were normalized and calculated as described for MMP8 in Fig. 3B legend. Statistical significance was determined as described in Fig. 3 legend.

Fig. 6. Real-time RT-PCR analysis of CXCL9 (A), CXCL10 (B), and CXCL11 (C) mRNA expression in proximal colon of WT, IL-10−/−, NHE3−/−, and IL-10−/− NHE3−/− mice. Values were normalized and calculated as described for MMP8 in Fig. 3B legend. Statistical significance was determined as described in Fig. 3 legend.
and it is believed that regulation of intestinal cell survival and related signaling pathways may represent a therapeutic approach for the treatment of IBD. It is very likely that NHE3 deficiency leads to lowering of the threshold for inflammation-induced apoptosis, and several hypotheses could be put for-

Fig. 7. Real-time RT-PCR analysis of CXCL9 (A), CXCL10 (B), and CXCL11 (C) mRNA expression in distal colon of WT, IL-10−/−, NHE3−/−, and IL-10−/−/NHE3−/− mice. Values were normalized and calculated as described for MMP8 in Fig. 3B legend. Statistical significance was determined as described in Fig. 3 legend.

CXCL10, and CXCL11). Accordingly, colonic secretion of IFNγ, IL-17, and IL-12/23 p40 was increased in the colon of IL-10−/−/NHE3−/− mice above the levels observed for single-KO littermates. We also established that the colonic epithelium of the double-KO mice exhibited increased hallmarks of apoptosis, including a significant increase in the number of cleaved caspase-3-positive epithelial cells.

Tightly regulated epithelial cell apoptosis plays a key role in the maintenance of epithelial integrity and restitution. In patients with IBD, increased apoptosis has been found in the acute inflammatory sites throughout the entire crypt-villus axis,
ward that could explain increased epithelial cell apoptosis in mice lacking both NHE3 and IL-10.

Changes in ionic fluxes in the NHE3-deficient colonocytes may contribute to increased epithelial cell apoptosis. The magnitude and scope of changes in the expression of membrane transport-related genes in the colon of NHE3−/− mice seem to support this hypothesis (18). Na+ deprivation experienced by NHE3-deficient cells could lead to a decreased apoptotic threshold similar to that reported in HeLa cells, in which medium Na+ depletion leads to increased Na+ efflux, normotonic shrinkage, and increased caspase-3 cleavage (23). However, the results from an acute in vitro study are unlikely to be replicated in a model of chronic NHE3 deficiency, where numerous compensatory mechanisms are expected to be activated. Some of these include cation-sparing processes, such as downregulation of the Kcnq4 K+ channel and upregulation of the nongastric H+−K+−ATPase (Atp12a) and Na+−K+−ATPase (Atp1a1) (18). Interestingly, although this was not investigated in the gut epithelial cells, inhibition of K+ efflux in neutrophils prevented mitochondrial dysfunction, caspase-3 activation, and apoptosis (10). If applicable to the intestinal epithelial cells (IECs), this finding could explain why we do not observe increased constitutive apoptosis in the colonic epithelium in NHE3−/− mice (16) and would imply that other superimposed stimuli are necessary to elicit such a response.

IL-10 has been shown to prevent loss of intestinal barrier integrity (20), whereas proinflammatory cytokines, such as IFNγ and TNFα, have been shown to significantly impair mucosal integrity (6, 19, 25). In the case of double-KO mice, the significant increase of proinflammatory cytokines could be the triggering factor leading to a dramatic increase in CEC apoptosis. IL-10 has also been described to modulate Fas

Fig. 9. Immunohistochemical analysis of cleaved caspase-3-positive cells in proximal colon of WT, IL-10−/−, NHE3−/−, and IL-10−/− NHE3−/− mice. A: brown staining indicates cells positive for cleaved caspase-3 (×200 magnification). B: staining was scored by counting the number of cleaved caspase-3-positive cells per high-power field of vision. Total of 5 fields in 3 randomly chosen sections were analyzed for each mouse. Statistical significance was determined as described in Fig. 3 legend.
expression on IECs (3). Fas is a member of the TNF death-domain-containing receptor family and is expressed constitutively in epithelial cells, whereas Fas-ligand is only expressed by CD8$^+$ cytolytic T cells under inflammatory conditions. In IL-10$^{-/-}$ NHE3$^{-/-}$ mice, proinflammatory cytokines could upregulate the expression of Fas on IECs, which in the absence of IL-10 regulation, would sensitize CECs to Fas ligand-induced apoptosis. We evaluated colonic Fas expression by real-time RT-PCR in all four mouse genotypes and found it to be elevated in proximal and distal colon of double-KO mice compared with NHE3$^{-/-}$ or IL-10$^{-/-}$ NHE3$^{-/-}$ mice, although the difference did not reach statistical significance (data not shown). Therefore, Fas involvement can only partially explain the changes observed in double-KO mice.

Cytotoxic T cells can also trigger CEC apoptosis degranulation of granzymes, serine proteases that, together with the pore-forming protein perforin, induce cell death. IL-10-deficient mice overexpress granzyme A in the large intestine (12). Granzyme A and perforin 1 are upregulated in the colon of NHE3$^{-/-}$ mice (18). However, expression of neither showed an additive or synergistic trend in IL-10$^{-/-}$ NHE3$^{-/-}$ mice (data not shown), suggesting that CEC apoptosis in this model is not mediated via the granzyme-perforin axis.

In IBD, endoplasmic reticulum (ER) stress plays a role in the initiation and propagation of the inflammatory condition, ultimately leading to epithelial cell apoptosis (15). In addition, it has been shown that IL-10 is a potent inhibitor of the inflammation-induced ER stress response by modulating activating...
transcription factor 6 nuclear recruitment to the grp78 gene promoter (29). As part of this complex process, ER stress results in the splicing of a 26-bp fragment from the mRNA encoding the transcription of transcription factor X-box-binding protein 1, which is a strong inducer of a subset of unfolded protein response target genes (15). Although the role of ER stress appears to be more significant in secretory cells than in the absorptive surface epithelium, we explored the possibility that, in double-KO mice, the combined absence of NHE3 and IL-10 could contribute to the exacerbation of ER stress and, ultimately, to IEC apoptosis. However, the analysis of X-box-binding protein 1 splicing in the colon (whole colonic extract), as well as grp78 mRNA expression, did not confirm the involvement of ER stress as the major inducer of IEC death (data not shown). Similarly, analysis of the markers of hypoxia, accumulation of hypoxia-inducible factor-α and the subsequent upregulation of intestinal trefoil factor, did not yield conclusive answers, with no significant differences between single-KO and IL-10−/−NHE3−/− mice (data not shown).

In summary, NHE3 deficiency superimposed on chronic colitis in IL-10−/− mice leads to exacerbation of colitis, likely precipitated by increased predisposition to programmed cell death in CECs. Although we were not able to determine the precise cause of CEC apoptosis in IL-10−/−NHE3−/− mice, it is plausible that a combination of multiple factors contributed by impaired electrolyte and immune homeostasis is necessary. Our results highlight the importance of NHE3 in the maintenance of intestinal barrier integrity and in modulating the inflammatory process in IL-10-deficient mice. These novel observations may be of high significance for the overall IBD pathogenesis, where NHE3 inhibition, associated with genetic predisposition (such as IL-10 or IL-10 receptor polymorphisms), may influence the extent of the epithelial barrier defect and contribute to the ultimate degree of inflammation.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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