Colon Epithelial Cell Death in 2,4,6-Trinitrobenzenesulfonic Acid-Induced Colitis Is Associated with Increased Inducible Nitric-Oxide Synthase Expression and Peroxynitrite Production

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Under physiological conditions, normal colon epithelia proliferate from the lower portions of the crypts of Lieberkuhn and migrate upward before dying by apoptosis (Jones and Gores, 1997). The frequency of apoptosis in both lamina propria leukocytes and epithelia was increased significantly during inflammation associated with a large increase in the number of interstitial CD95L+ cells (Strater et al., 1997). Increased apoptosis was the main cause of epithelial loss in crypts of active ulcerative colitis patients where Fas/Fas-L interaction is an important mediator (Iwamoto et al., 1996). These findings suggest that the epithelial barrier in the intestine may be compromised by the augmented cell death, leading to the invasion of pathogenic microorganisms in ulcerative colitis (Strater et al., 1997). This notion is supported by the observation that doxorubicin-enhanced epithelial cell apoptosis in the rat intestine is associated with an increase of bidirectional permeability of the intestinal barrier (Sun et al., 1998).

Although the Fas/Fas-L interaction is considered as the principal mediator of intestinal epithelial death, other substances generated in the inflammatory lesion have also been implicated as causative agents for epithelial death. For example, in vitro studies have demonstrated that the reactive nitrogen species such as peroxynitrite can induce apoptosis in gastrointestinal epithelial cells (Sandoval et al., 1997; Kim et al., 1998). Our previous work (Lin et al., 1995) showed that peroxynitrite induced apoptosis in HL-60 human leukemia cells in a time- and concentration-dependent manner. These studies however, did not demonstrate the role of peroxynitri
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trite in colon epithelial apoptosis in vivo. Peroxynitrite is a potent oxidant formed by the reaction of NO\textsuperscript{-} and O_2\textsuperscript{-}, which causes DNA strand breakage, leading to cell necrosis and/or apoptosis. Ford et al. (1997) observed that extensive apoptosis of enterocytes in the apical villi of infants with necrotizing enterocolitis was strongly associated with the presence of nitrotyrosine staining, suggesting peroxynitrite action. In addition, Rachmilewitz et al. (1993) reported that administration of peroxynitrite intrarectally to rats caused a colon inflammation that had histological similarity with IBD. In the colon tissue of patients with active ulcerative colitis, elevated iNOS protein was found in epithelial cells (Kolios et al., 1998), neutrophils, and macrophages at the base of the ulcer; in the inflammatory infiltrate of the lamina propria; and within the cytoplasm of epithelial cells lining the colon (Godkin et al., 1996; Kimura et al., 1998). Singer et al. (1996) reported intense focal iNOS immunoreactivity in the inflamed colon epithelium as well as in lamina propria leukocytes in ulcerative colitis, Crohn’s disease, and diverticulitis. Sites of increased iNOS expression appeared to be coincident with sites showing increased nitrotyrosine immunostaining, which is a marker of peroxynitrite-induced protein modification (Singer et al., 1996). These data indicate that the increased level of reactive nitrogen species produced by elevated iNOS expression could contribute to the intestinal tissue injury during inflammatory bowel disease. On the other hand, Dijkstra et al. (1998) found that nitrotyrosine formation was only on CD-15-positive cells (monocytes/granulocytes), but not on epithelial cells in biopsy specimens from patients with IBD. The discrepancy in these findings indicates that the association between endogenous peroxynitrite formation and epithelial apoptosis remains to be elucidated.

The use of iNOS-deficient mice to examine the effects of high levels of NO\textsuperscript{-} production has produced conflicting results. McCafferty and coworkers reported that iNOS-deficient mice had significantly increased macroscopic inflammation and granulocyte infiltration compared with wild-type mice at early time points in an acetic acid model of colitis (McCafferty et al., 1997) and a TNBS model of colitis (McCafferty et al., 1998). On the other hand, Zingarelli et al. (1999a) found that iNOS ablation conferred substantial reduction in colon injury and nitrotyrosine staining in a model of TNBS-induced colitis. The discrepancy of these results may be related to model systems used in the experiments and the diversity of TNBS doses to induce colitis. iNOS-deficient mice had an exacerbated colon injury in a model where a high dose (6 mg/mouse) of TNBS was given (McCafferty et al., 1999) compared with an improvement in injury when a lower dose (1 mg/mouse) of TNBS was given (Zingarelli et al., 1999a). In a study using the pharmacological agent mercaptopetoethyuanidine, a combined inhibitor of iNOS and peroxynitrite scavenger, colonic injury was reduced in a TNBS-induced colitis model (Zingarelli et al., 1998). Again, a mechanism was not clearly elucidated. These results suggest that the specific role of iNOS at least partially depends on the model system that is used. A crucial question is to what extent iNOS and its products such as peroxynitrite are associated with epithelial death during colitis. In fact, there has been little in vivo work that demonstrates that iNOS or its related metabolites such as peroxynitrite are directly associated with epithelial apoptosis during colitis.

In this communication, we show that epithelial cell death is associated with apoptosis in the colon lesion of a rat model of TNBS-induced colitis. Importantly, we provide evidence that reactive NO\textsuperscript{-} metabolites such as peroxynitrite are directly related to the induction of epithelial apoptosis-like death and that selective inhibition of iNOS can reduce peroxynitrite formation and ameliorate epithelial apoptosis in an in vivo model of colitis.

**Experimental Procedures**

**Materials.** Rabbit polyclonal antibody against cytokeratin used to identify colon epithelial cells was obtained from Biomedical Technologies (Stoughton, MA). A rabbit polyclonal antibody against nitrotyrosine was purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal antibodies recognizing iNOS were purchased from Santa Cruz Biochemicals (Santa Cruz, CA). Cy3-conjugated donkey anti-rabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA) was used to detect rabbit IgG antibodies against cytokeratin and against nitrotyrosine. A fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch) was used to detect rabbit IgG against iNOS. The in situ terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) stain was used in the in situ Apoptag Plus Fluorescein kit (Intergen, Purchase, NY or Oncor, Gaithersburg, MD). TACS Apoptotic DNA Laddering kit was purchased from Trevigen (Gaithersburg, MD).

**Animal Model of Colitis.** Rat colitis was induced as previously described (Yue et al., 1996). Sprague-Dawley rats (300–350 g) were lightly anesthetized with metofane and a rubber catheter was inserted into the colon to a distance of 8 cm from the anus. A small volume (1.2 ml) of TNBS, 25 mg/ml dissolved in ethanol (50% v/v), was administered into the lumen of the colon through the catheter. Control rats received an equal volume of saline. Animals were sacrificed at 24 h after induction of disease. The colon from cecum to anus was removed and opened by a longitudinal incision. The feces and other colon contents were removed by a gentle flushing with saline, and the tissue damage was examined at low magnification. Tissue specimens were taken for measuring tissue superoxide, myeloperoxidase activity (MPO), enzyme activity of NOS, H&E staining, immunohistochemical staining, RNA extraction for RT-PCR to determine NOS gene expression, and mucosal cell apoptosis or necrosis. Rat plasma was collected for the NO\textsuperscript{-} assay. Each experimental group consisted of a minimum of five animals.

**1-L-N\textsuperscript{6}-(1-Iminoethyl)lysine (L-NIL) Treatment.** L-NIL (10 mg/kg i.p.) was given immediately after administration of TNBS and repeated every 3 h for a total of four treatments. Normal controls and positive controls that received TNBS were given sterilized phosphate-buffered saline instead of L-NIL. Rats were sacrificed 24 h after induction of colitis. L-NIL has been commonly used in vivo experiments and is known for its high specificity for iNOS inhibition (Schwartz et al., 1997; Cockrell et al., 1999).

**Immunohistochemistry and Histological Analyses.** Tissue specimens were embedded in cryomatrix (Shandon, Pittsburgh, PA). Embedded specimens were quickly frozen in liquid nitrogen and then kept at −80°C. Sections of 5-μm thickness were made at −22°C with a cryostat. Sections were stained with H&E for histological examination under a light microscope. Sections for immunohistochemical staining were incubated with 5% BSA before incubation with primary antibodies to decrease nonspecific binding. Primary and secondary antibodies were suspended in 1% BSA. Specimens were incubated with first antibodies for 1 h at 37°C and then washed three times with 0.1% Tween-phosphate-buffered saline for 5 min each time. Specimens were incubated with second antibody for 1 h in room temperature and then washed three times with 0.1% Tween/phosphate-buffered saline for 5 min each time. The primary antibodies consisted of antibody against cytokeratin to detect epithelial cells, antibody against nitrotyrosine to detect action of peroxynitrite, and antibody against iNOS to detect inducible nitric-oxide synthase ex-
pression. Cell nuclei were counterstained with DAPI. Specimens were examined with a Nikon Photomat microscope equipped with epifluorescence optics (Micron Optics, Cedar Knolls, NJ) and images were captured using a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI). Digital images were pressed using Image-Pro Plus imaging analysis software (Media Cybernetics, Silver Spring, MD).

Detection of TUNEL-Positive Cells. The in situ TUNEL assay was performed on frozen sections with thickness of 5 μm using the in situ Apoptag Plus Fluorescein kit according to the manufacturer’s instructions. Apoptotic cells were identified by virtue of their FITC-fluorescence intensity compared with cells in positive control slides pretreated with DNase. The number of stained cells in 10 high-power fields (400× magnification) was counted. Results from each slide were the mean of the number of positively stained cells per 10 high-power fields.

Double Immunohistological Staining. To localize nitrotyrosine in apoptotic cells or apobodies, and to immunohistochemically identify epithelial cells undergoing apoptotic-like cell death, double immunohistological staining was applied to frozen sections. Cryostat sections were first incubated with an unconjugated primary antibody to detect the first epitope followed by detection with a Cy3-labeled second antibody. Both primary and secondary antibodies were suspended in cytopore. Cytopore is a detergent used to permeabilize the cell membrane for TUNEL staining. The TUNEL staining was performed with the FITC-conjugated anti-digoxigenin antibody of the Apoptag Plus Fluorescein kit. The green (FITC) and/or red (Cy3) fluorescence was observed under a Nikon Photomat epifluorescence microscope and analyzed by Image-Pro Plus image analysis software. To confirm the specificity of these antibodies, control slides were prepared with the omission of primary antibodies and terminal deoxynucleotidyl transferase enzyme, instead of 1% BSA and reaction buffer, respectively.

DNA Extraction and Analysis of DNA Fragmentation by Gel Electrophoresis. Colon tissues were minced, frozen in liquid nitrogen, ground into powder, and suspended in 0.5 ml of lysis buffer (50 mM Tris-HCl, pH 8; 20 mM EDTA, pH 8; 1% SDS; 0.1 mg/ml proteinase K) for 200- to 400-mg samples. The samples were incubated for 5 min at 50°C with constant shaking. DNA (200 μl) was extracted with 700 μl of extraction solution 2 (Trevigen) and 400 μl of extraction buffer 3 (Trevigen). After centrifugation for 5 min at 12,000g, the aqueous layer was collected and supplemented with one-tenth volume of 3 M sodium acetate, pH 5.2 and 1 volume of 2-propanol. The precipitated DNA was collected by centrifugation, washed with 70% ethanol, and dissolved in 100 μl of DNase-free water. The concentration of DNA was estimated by measurement of optical density at 260 nm. Specimens containing 5 μg of DNA were then applied to 1.5% agarose gels. DNA ladder (100 bp) was used as standard. Electrophoresis was carried out in TAE buffer (20 mM Tris-acetate, 1 mM EDTA) and DNA was stained with ethidium bromide and quantified by computerized image densitometry.

Semiquantitative RT-PCR Analysis. Semiquantitative RT-PCR analysis to determine Bcl2, Bax, and iNOS gene expression was performed according to the protocols described previously (Yin et al., 1999). Specific primer sets for rat Bcl2, Bax, and iNOS were devised by the primer design software program Primer Designer (CLON-TECH, Palo Alto, CA). Oligonucleotide primers were purchased from Integrated DNA Technology (Corvalle, IA). The sequences of the primers were as follows: 1) primers for Bcl2, 5′-TAT-GAT-AAC-CGG-GAG-ATC-GTG-3′ (sense) and 5′-CAT-ATG-CCG-GTG-CAG-TCA-CTC-3′ (antisense); 2) primers for Bax, 5′-CAAGA-GCT-GAG-CGA-GCT-GTG-TCT-3′ (sense) and 5′-GGT-TCT-GAT-CGA-GTG-CAC-3′ (antisense); 3) primers for iNOS, 5′-AGAGG-AGA-ACTG-TGA-CCA-TCA-TGG-ACC-ACC-3′ (sense) and 5′-AGAGA-GCA-GAA-AAG-ACC-GCT-TTC-ACC-3′ (antisense); and 4) primers for GAPDH, 5′-GGT-GGA-GTGA-CGG-GTG-CAG-GAT-3′ (sense) and 5′-CAT-GGC-AAA-GTT-CTC-ATG-GAT-GAC-C-3′ (antisense). The optimum number of amplification cycles within the exponential amplification phase for each primer set was predetermined by running cycle studies. The band intensity was expressed as absolute integrated optical density, i.e., the volume of the band in the lane profile, subtracting local background, which was defined as the average optical density of the open space parallel to the lanes. The integrated optical density of each PCR product was normalized to that of GAPDH for the same animal. Data are expressed as the mean normalized values ± S.E.M.

Enzymatic Activity of cNOS and iNOS Enzyme. Enzymatic activity of cNOS and iNOS was determined by conversion of L-[3H]arginine to L-[3H]citrulline. Enzyme conversion of L-[3H]arginine to L-[3H]citrulline was performed by a modified method described by McNaughton et al. (1998). Briefly, frozen colon specimens were homogenized in an ice-cold HEPES buffer (100 mM) with a Takmer Ultra-Turrax homogenizer at maximum speed for 20 s. The HEPES buffer consisted of the following components: 0.02 M HEPES, pH 7.4; 0.25 M sucrose; 1 mM EDTA; 1 mM diethiothreitol; 10 μg/ml soybean trypsin inhibitor; 10 μg/ml leupeptin; 10 μg/ml pepstatin A; and 0.1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 11,300g for 10 min and supernatants were collected. Aliquots (50 μl) of the supernatant were mixed with assay mixture in a final volume of 200 μl and controls were prepared with distilled water instead of homogenate supernatants. The calcium-dependent assay mixture for total NOS activity contained the following components: 50 mM HEPES, pH 7.4; 1 mM valine; 10 μM arginine containing 0.15 μCi/assay [3H]arginine; 1 mM NADPH; 1 mM CaCl₂; and 0.16 mM MgCl₂. The calcium-free assay mixture for iNOS contained 1 mM EDTA instead of CaCl₂. The incubation was performed at 37°C for 10 min and the reaction was stopped by addition of ice-cold Dowex AG 50W-X8 resin to remove the arginine. Resin was washed with 5 N NaOH overnight and washed with deionized water until the pH was neutral. This pretreatment strengthened the capacity of the resin to remove free arginine. The final mixture was incubated for 30 min at 4°C after addition of another 0.5 ml of distilled water. The tubes with the final mixture were centrifuged at 1500 rpm and 0.7-ml aliquots of supernatant were counted in a Beckman LS-7500 scintillation counter. The difference between the calcium-dependent conversion and calcium-independent conversion was considered as cNOS activity. The activity was calculated as picomoles of citrulline produced per minute per milligram of protein.

MPO Measurement. Colon tissue MPO activity was determined as previously reported (Yue et al., 1996). Briefly, the tissue strips were suspended in potassium phosphate buffer containing 0.5% hexadecyltrimethyl-ammonium bromide (pH 6.0; 50 mg of tissue per milliliter) and then homogenized for 30 s using a Polytron homogenizer. After homogenates were centrifuged at 40,000g for 15 min, the supernatants were collected to determine the tissue levels of MPO activity using a technique described by Bradley et al. (1982). Theoretically, a unit of MPO activity was defined as that converting 1 μmol of hydrogen peroxide to water in 1 min at 22°C.

Detection of Superoxide. Detection of superoxide levels in colon tissues was performed as previously reported (Yue et al., 1996). Briefly, the tissue strips (0.2 × 4 cm) were incubated in Krebs’ bicarbonate buffer (pH 7.4), gassed with 95% O₂, 5% CO₂ at room temperature for 30 min. The tissue strip was placed in plastic scintillation vials containing 0.25 mM lucigenin in a final volume of 1 ml of Krebs’ buffer with 10 mM HEPES-NaOH (pH 7.4). The chemiluminescence elicited by superoxide in the presence of lucigenin was measured using the Mark 5303 scintillation counter (TM Analytic, Elk Grove Village, IL). The instrument was sensitive enough to collect sufficient light emission indicative of superoxide in 6 s.

Plasma Nitric Oxide Measurement. Blood samples from rats were centrifuged at 1200g to obtain plasma. To convert nitrate to nitrite, 200 μl of plasma was incubated with nitrate reductase (0.25 units/ml) at 37°C for 30 min in the presence of 3 mM NADPH. Samples (50 μl) were then injected into a purge vessel (Sievers NO analyzer 270B) containing 1% sodium iodide in glacial acetic acid.
(5-ml volume) in an atmosphere of nitrogen to reduce all nitrite to NO\textsuperscript{z} gas. Gaseous NO\textsuperscript{z} was purged from the vessel and detected in a cell reaction chamber after coming in contact with ozone to give off a chemiluminescent signal. Nitric oxide concentrations were determined by comparison of values obtained using standard concentrations of sodium nitrite (20–400 pmol).

**Crypt Epithelial Cell Counts.** Crypt epithelial cell counts were based on the methods described previously (Hall et al., 1994; Sträter et al., 1995). Double immunofluorescence-stained slides were examined using a fluorescence microscope. A minimum of 40 full-length and well oriented crypts on each specimen was examined. Data were expressed as number of cells per crypt. TUNEL-positive epithelial cells were identified by comparing the FITC brightness with positive control. Nitrotyrosine-positive cells were identified by their bright red (Cy3) cytosol with either blue (DAPI-positive) nuclei or green (TUNEL-positive) nuclei. Normal cells were identified by virtue of their intact cell shape and the normal appearance of their nuclei. Infiltrated neutrophils were clearly distinguished by the presence of a multilobed nucleus.

**Statistical Analysis of Data.** Data were analyzed with one-way ANOVA with the Sigma Stat program (Jandel Scientific, Chicago, IL). Differences among groups were then determined with the Student-Newman-Keuls test. Groups were deemed to be significantly different from one another when \( P < 0.05 \).

**Results**

**Colon Cell Apoptosis.** When frozen sections of control rat colons were examined for DNA fragmentation by TUNEL,
only a few TUNEL-stained cells were localized at the top of crypts (Fig. 1A). A large number of TUNEL-positive cells was observed in frozen sections of colons from rats at 24 h after administration of TNBS (Fig. 1, B and D). The TUNEL-positive cell counts significantly increased 24 h after administration of TNBS (Fig. 2A). These TUNEL-stained cells were seen in the crypts and lamina propria 24 h after treatment with TNBS (Fig. 1B). The TUNEL-stained cells in the crypts had the morphology of epithelial cells (Fig. 1D), and those in the lamina propria appeared to be infiltrated granulocytes. Some cells clearly appeared to be apoptotic bodies (Fig. 1, D–F), which provides further evidence of cell apoptosis. Above the zone of intensive TUNEL staining, cells were not stained with DAPI or TUNEL, suggesting that these cells had already undergone necrosis (Fig. 1B). Figure 1, E and F, are the same view of a specimen 24 h after administration of TNBS. Photo was taken with a triple-wavelength filter (propium iodide/DAPI/FITC) and analyzed by Image Pro Plus image analysis software. After diminishing red and green lights, blue light represents DAPI staining (Fig. 1E). After diminishing red and blue light, green light represents TUNEL staining (Fig. 1F). Arrows point out coincident individual cells undergoing apoptosis. Arrow and number give rise to a sequential process of nuclear morphologic change during apoptosis (Fig. 1, E and F).

Consistent with in situ TUNEL, agarose gel electrophoresis of DNA isolated from the 24-h inflamed colon showed a typical DNA laddering pattern (Fig. 2B), demonstrating DNA fragmentation characteristic of apoptosis. Also, cells that were double stained with both cytokeratin and TUNEL (confirming apoptosis of epithelial cells) increased dramatically 24 h after administration of TNBS, indicating that epithelial cells had increased their rate of apoptosis. Most double-stained cells were located as a monolayer lining the luminal side of crypts (Fig. 1C).

**Bcl2 and Bax Expression.** Bcl2 and Bax are important proteins involved in cell apoptosis. Bax can induce cytochrome c release and is inhibited by Bcl2/Bcl-xL. Thus, the ratio of Bcl2/Bax is a marker of cytochrome c and cell apoptosis. In this TNBS-induced colitis, Bcl2 mRNA showed a significant decrease by 41% compared with control rats at

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**Fig. 2.** A, TUNEL-positive cell counts. Values are means ± S.E.M. for n = 5 in each group. *Significantly different from saline-treated control rats (P < 0.05). B, gel electrophoresis of colon tissue DNA. Agarose gels (1.5%) were used. A typical DNA ladder appeared 24 h after TNBS administration. A 100-base pair interval DNA ladder was used as standard.

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**Fig. 3.** Bcl2 and Bax gene expression in colon tissue determined by RT-PCR analysis. A, Bcl2 gene expression. B, Bax gene expression. Values are mean ± S.E.M. for n = 6 animals for each group. *Significantly different from saline-treated control rats (P < 0.05).
24 h after TNBS administration and Bax mRNA remained at control levels, indicating a relatively higher level of Bax in inflamed colon at 24 h after TNBS administration (Fig. 3).

**iNOS Expression.** To confirm elevated expression of iNOS in inflamed colon tissue, we measured iNOS mRNA expression in colon tissue. We found that iNOS mRNA expression was significantly increased in the inflamed colon at 24 h after TNBS administration, whereas it was undetectable in saline control animals (Fig. 4). To determine changes in iNOS protein expression, we compared inflamed colon tissue with that of corresponding controls using immunohistochemistry. Intense iNOS immunoreactivity was seen in both epithelial cells and cells in the lamina propria at 24 h after administration of TNBS (Fig. 5B). Most of the latter were morphologically identified as polymorphonuclear neutrophils and monocytes (Fig. 5C). No iNOS staining was observed in colon tissue taken from saline control animals (Fig. 5A).

**Plasma NO and Tissue Superoxide.** Consistent with the observed increase in iNOS expression and enzyme activity, there was a 6-fold increase in plasma nitric oxide level at 24 h after TNBS administration (Fig. 6A). Tissue superoxide generation, as measured by chemiluminescence, showed a 5-fold increase over basal levels in colon tissue taken from rats 24 h after TNBS administration (Fig. 6B).

**Enzymatic Activity of cNOS and iNOS.** Enzymatic activity of cNOS and iNOS were measured in rat colon tissues. iNOS activity was very low in the normal control colon tissue. Administration of TNBS caused a 7-fold increase in iNOS activity at 24 h. In contrast, colon tissue cNOS activity decreased by 70% 24 h after TNBS administration. L-NIL administration blocked the elevation in iNOS activity in 24-h lesions by 92% compared with TNBS-treated rats. The inhibitor was found to have no effect on cNOS (Fig. 7).

**Double Immunostaining for Apoptosis and Nitrotyrosine.** Double immunofluorescence of anti-nitrotyrosine/TUNEL (Fig. 8) was performed on cryosections of colon tissue. The stained epithelial cells were counted under epifluorescence microscopy. In the colon tissue taken from normal control rats, approximately every two crypts contained one TUNEL-positive epithelial cell. Most of them were

![Fig. 4. iNOS gene expression in colonic tissue determined by RT-PCR analysis. A, gel-electrophoresis of RT-PCR products. B, quantification of RT-PCR product. Volumes were normalized to that of GAPDH. Values and presented as means ± S.E.M. for n = 6 animals in each group. *Significantly different from saline-treated control rats (P < 0.05).](image-url)
located at or near the top of crypts. A 50-fold increase in TUNEL-positive epithelial cells was observed in tissues taken from rats 24 h after administration of TNBS. However, in rats that received L-NIL, TUNEL-positive epithelial cell counts were decreased by 66% in 24-h lesions (P < 0.05). In parallel with the TNBS-induced increase in TUNEL-positive cell counts, nitrotyrosine-positive epithelial cell counts also were elevated in 24-h lesions and were significantly decreased after L-NIL treatment. Interestingly, dual-stained nitrotyrosine-TUNEL-positive epithelial cells were rarely observed in normal control animals. By contrast, nitrotyrosine-TUNEL-positive epithelial cells appeared in 24-h inflamed tissues at a rate of 15 nitrotyrosine-TUNEL-positive epithelial cells per crypt. L-NIL treatment significantly decreased the nitrotyrosine-TUNEL-positive epithelial cell count by 82% and significantly increased the normal cell counts in 24-h inflamed tissues. This finding suggests that there is an intrinsic association between nitrotyrosine-positive staining and DNA fragmentation in individual cells. These data indicate that peroxynitrite is an important endogenous contrib-

![Fig. 6. A, chemiluminescence measurements of plasma nitric oxide. Values are means ± S.E.M. for n = 5 in the control group and n = 7 for TNBS treated. *Significantly different from saline-treated control rats (P < 0.05). B, chemiluminescence measurements of superoxide generation in colonic tissue. Values are means ± S.E.M. for n = 6 in the control group and n = 11 for TNBS-treated rats. *Significantly different from saline-treated control rats (P < 0.05).](image1)

![Fig. 7. Colon tissue iNOS and cNOS activities were measured by enzyme conversion. A, iNOS activity. B, cNOS activity. Values are means ± S.E.M. for n = 8 in the control and TNBS-treated groups, whereas the L-NIL-treated group had n = 10. *Significantly different from saline-treated control rats (P < 0.05).](image2)

![Fig. 8. Colon tissue iNOS and cNOS activities were measured by enzyme conversion. A, iNOS activity. B, cNOS activity. Values are means ± S.E.M. for n = 8 in the control and TNBS-treated groups, whereas the L-NIL-treated group had n = 10. *Significantly different from saline-treated control rats (P < 0.05).](image3)

utator to epithelial cell death. Results obtained from the double-positive cell counts, nitrotyrosine-positive cell counts, TUNEL-stained cell counts, and counts of cells with normal nuclear shape revealed by DAPI staining are summarized in Fig. 9.

**Tissue MPO Activity.** Coincident with the increased activity of iNOS, colon tissue MPO activity was elevated significantly 24 h after administration of TNBS and was decreased by 50% by L-NIL treatment, indicating a significant decrease in the infiltration of neutrophils after inhibition of iNOS staining (Fig. 10).

**Discussion**

In these experiments, we observed that administration of TNBS caused significant increase in TUNEL staining, nitrotyrosine staining, and colocalization of TUNEL-nitrotyrosine in colon epithelial cells, which were consistent with increased iNOS expression and activity in the rat colon. In separate studies, administration of a selective iNOS inhibitor, L-NIL, suppressed iNOS activity by 92%. In these latter studies, TUNEL staining, nitrotyrosine staining, and TUNEL-nitrotyrosine dual staining were significantly decreased, and were accompanied by a significant increase in the number of normal epithelial cells in crypts at 24 h after TNBS treatment. TUNEL-positive colon epithelial cells were decreased by 77%, whereas nitrotyrosine-positive colon epithelial cells and TUNEL-nitrotyrosine-positive colon epithelial cells were de-
creased by 73 and by 82%, respectively. Normal epithelial cell counts were elevated 33-fold compared with cell counts in the crypts in rats 24 h after administration of TNBS. MPO, as an inflammatory index, was decreased by 50%. To address the fate of epithelial cells, the time point of 24 h after TNBS treatment was selected because a relatively intact mucosal structure, even in the positive control rats, can be distinguished under light and fluorescence microscopy. In cell apoptosis, cytochrome $c$ released from mitochondria forms “apoptosome” containing Apaf-1 (apoptotic protease activating factor-1) and procaspase-9 to initiate downstream caspase activation. Cytochrome $c$ release can be induced by Bax and is inhibited by Bcl2/Bcl-xL (Green and Reed, 1998). Thus, we have used the ratio of Bcl2/Bax expression as an indirect index to present the cytochrome $c$ release and apoptosis. In our experiments, a significant decrease in Bcl2 mRNA expression was coincident with apoptosis of colon epithelial cells, whereas Bax remained at control levels at 24 h after TNBS administration. An imbalance of Bcl2/Bax may lead to release of cytochrome $c$ from mitochondria and initiate downstream caspase activation.

The role of NOS inhibitors in inflammation is still controversial. Miller et al. (1993) used a TNBS-induced guinea pig ileitis model to test the effect of $N^\omega$-nitro-arginine-methyl ester (L-NAME), a nonselective inhibitor of NOS activity, on the inflammation response. They found that L-NAME could prevent tissue injury caused by TNBS. Rachmilewitz et al. (1995) reported similar results when they applied an NOS inhibitor to treat TNBS-induced colitis. In the latter study, L-NAME was added to the drinking water at the time of colitis induction and resulted in significant decrease in the extent of tissue injury in TNBS-treated rats. Conversely, Pfeiffer and Qiu (1995) found that L-NAME enhanced lesions in TNBS-induced rat colitis in a dose-dependent manner, whereas low doses (0.042 mg/kg/h) showed slight (insignificant) reduction in lesion formation (Pfeiffer and Qiu, 1995). They suggested that variations in the response to various doses of L-NAME might reflect the differences in iNOS and cNOS activity. Later, Miller et al. (1995) tried to selectively inhibit iNOS with aminoguanidine, a relatively selective iNOS inhibitor in TNBS-induced guinea pig ileitis, and this treatment led to reduced inflammation and restored tissue morphology (Miller et al., 1995). These results suggest that iNOS may play a different role than cNOS in colitis, and that preservation of cNOS activity is beneficial in the cytoprotection of colon tissue. In our experiments, selective inhibition with L-NIL significantly decreased tissue iNOS activity in TNBS-treated rats without affecting cNOS activity. It should be noted that cNOS activity was already significantly reduced in TNBS-treated rats in the absence of L-NIL. Thus, L-NIL would not be expected to alter the already reduced activity of cNOS. Administration of L-NIL, however, did have a pronounced effect on reducing the amount of nitrotyrosine staining. These results suggest that NO derived from increased iNOS activity contributed to peroxynitrite formation. Additionally, the results provide the first direct evidence in
an in vivo model that peroxynitrite formation is associated with colonic epithelial cell death. Because the data show that an increase in cell apoptosis was associated with increased nitrotyrosine staining, whereas pharmacological inhibition of iNOS was associated with a reduction in nitrotyrosine and TUNEL colocalization, we speculate that may have been a causal relationship between increased NO/peroxynitrite formation and colonic epithelial cell apoptosis.

In the present experiments, we found that TNBS administration not only elevated iNOS activity by 87% in colon lesions but also suppressed cNOS activity by 68%. Our data were consistent with another study showing mucosal injury accompanied by elevated expression of iNOS and reduced expression of cNOS in lesions from acetic acid-induced rat gastric ulcer (Akiba et al., 1997). In the animal model of Helicobacter pylori lipopolysaccharide-induced gastritis, a marked increase in epithelial cell apoptosis was accompanied by a 6.5-fold increase in mucosa expression of iNOS and a 2.2-fold decline in cNOS (Slomiany et al., 1999). In TNBS-induced colitis model in mice, an increase in iNOS activity and a concomitant decrease in cNOS activity (McNaughton et al., 1998) were reported. These data suggest that gastrointestinal inflammation may cause a decline in cNOS expression and activity. Therefore, administration of nonselective NOS inhibitors may cause oversuppression of cNOS in gastrointestinal inflammation, leading to exacerbation of mucosal injury. Subsequently, selective inhibition of cNOS or iNOS is required to study the role of NOS in gastrointestinal inflammation.

Elevated expression of iNOS and the formation of per-
oxynitrite are considered to at least partially mediate the cell death and tissue injury in inflamed tissues. Miller et al. (1995) found that nitrosoguanidine prevented nitrotyrosine formation, suggesting that iNOS activation is responsible for the formation of peroxynitrite during intestinal inflammation. Furthermore, Singer et al. (1996) observed that nitrotyrosine labeling was located in the inflamed colon epithelium of patients with inflammatory bowel disease and was closely associated with iNOS staining, concluding that iNOS in the inflamed colon epithelium is associated with the formation of peroxynitrite and the nitrination of cellular proteins.

As a potent oxidant, peroxynitrite may react with proteins, lipids, and DNA. When peroxynitrite reacts with tyrosine residues yielding 3-nitrotyrosine, the new product changes the hydrophilic group to a hydrophobic group, leading to protein destruction. In addition, tyrosine nitration is a convenient marker for the presence of peroxynitrite. Although reactive species such as nitrogen dioxide and acidified nitrite can also produce nitrotyrosine, the amounts are too low to cause significant nitration in vivo (Beckman and Koppenol, 1996; Hughes, 1999). In vitro studies have shown that nitration of tyrosine may be due to reactions with other oxidative species in addition to peroxynitrite. Eiserich et al. (1996) reported that nitration of tyrosine is independent of peroxynitrite. The reaction of nitrite ([NO2]−) with hypochlorous acid (HOCl) forms the intermediate species nitryl chloride ([NO2Cl]) and nitrogen dioxide ([NO2]), which are capable of nitrating phenolic substrates such as tyrosine with maximum yields at physiological pH. They also showed that activated human polymorphonuclear leukocytes can convert NO2 into NO2Cl and NO2 through an MPO-dependent pathway (Eiserich et al., 1998). The key reaction in this biochemical cascade is the reaction of hydrogen peroxide with chloride to form HOCl catalyzed by MPO. But, the reaction of O2 with NO2 to form peroxynitrite occurs 150 to 1300 times faster than O2 with all other pathways combined (Squadrito and Pryor, 1995). This reaction will overwhelm the O2 decaying mechanism catalyzed by superoxide dismutase to generate H2O2. Moreover, NO2 and acidified nitrite are also capable of nitrosation of tyrosine. However, the reactivity of these species is not high enough to compete with that of peroxynitrite in vivo (Beckman and Koppenol, 1994; Beckman et al., 1996). Therefore, peroxynitrite is the most likely agent for tyrosine nitration in vivo, although the other reactive species can also form nitrotyrosine in vitro (Beckman et al., 1996). Consequently, double immunofluorescence of anti-nitrotyrosine/TUNEL appears to indicate the peroxynitrite action, rather than other reactive species.

There is other evidence that suggests that peroxynitrite is a potent initiator of DNA strand breakage, which acts as an obligatory stimulus to activate the nuclear enzyme poly ADP ribosyl synthetase (PARS). The peroxynitrite-PARS pathway contributes to cell death, including necrosis and apoptosis in shock and inflammation, pancreatic islet cell destruction, diabetes, stroke, and neurodegenerative disorders (Szabo, 1996). Recently, Zingarelli et al. (1999b) reported that blockade of PARS inhibited inflammation and mucosal injury in murine colitis induced by TNBS. Our data provide compelling evidence demonstrating that nitrotyrosine is localized in apoptotic epithelial cells and can be suppressed by the selective iNOS inhibitor l-NIL.

These studies for the first time show that there is an increase in colon epithelial cells in TNBS-induced colitis, and nitrotyrosine as a marker for peroxynitrite colocalized extensively with apoptotic epithelial cells 24 h after TNBS administration. Use of a specific iNOS inhibitor (l-NIL) reduced the number of apoptotic epithelial cells, the number of dual nitrotyrosine-TUNEL-staining cells and significantly increased recovery of normal colon epithelial cells in TNBS-treated rats. These results strongly suggest that peroxynitrite is a major contributor to colon epithelial cell apoptosis at the acute phase of inflammation in the TNBS model of colitis. Selective inhibition of iNOS reduced peroxynitrite formation and consequently reduced epithelial cell apoptosis as well as enhanced the epithelial cell recovery. Importantly, our data demonstrate that the TNBS-induced colitis model can be used to study epithelial cell apoptosis, a cell death process that occurs in human IBd. It is plausible that reduction of epithelial cell apoptosis by selective inhibition of iNOS and peroxynitrite formation may have therapeutically significant in IBd.

References


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