Ethanol-Induced Barrier Dysfunction and Its Prevention by Growth Factors in Human Intestinal Monolayers: Evidence for Oxidative and Cytoskeletal Mechanisms

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ABSTRACT

Exposure of intestinal mucosa to ethanol (EtOH) disrupts barrier function and growth factors [epidermal growth factor (EGF) and transforming growth factor-α (TGF-α)] are protective, but the mechanisms remain obscure. Accordingly, we sought to determine whether the molecular mechanism of EtOH-induced intestinal barrier dysfunction involves oxidative stress and disassembly of microtubules and whether the mechanism of protection by EGF or TGF-α involves prevention of these alterations. To this end, human colonic (Caco-2) monolayers were exposed to 0 to 15% EtOH with or without pretreatment with EGF or TGF-α (10 ng/ml) or with oxidative or cytoskeletal modulators. Effects on cell viability, barrier function, tubulin (microtubules), and oxidative stress were then determined. Cells were also processed for immunoblots of polymerized tubulin (S2; index of stability) and the monomeric tubulin (S1; index of disruption). EtOH dose-dependently decreased the stable S2 polymerized tubulin and concomitantly increased measures of oxidative stress, including oxidation and nitration of tubulin, fluorescence of dichlorofluorescein, and inducible nitric oxide synthase activity. EtOH also dose-dependently disrupted barrier function and extensively damaged microtubules, and these effects were prevented by pretreatment with antioxidant scavengers: l-cysteine, superoxide dismutase, and l-N-alpha-imidoethyl-lysine (an inducible nitric oxide synthase inhibitor).

In monolayers exposed to EtOH, pretreatment with EGF or TGF-α prevented the oxidation and nitration of tubulin, increases in the levels of the unstable S1 tubulin, disruption of microtubules, and barrier dysfunction. A microtubule stabilizer (paclitaxel, Taxol) mimicked, in part, the effects of EGF and TGF-α, whereas a microtubule disruptive drug (colchicine) prevented the protective effects of these growth factors. We concluded that mucosal barrier dysfunction induced by EtOH involves oxidative stress, which causes the disassembly of the microtubule cytoskeleton. Protection by EGF and TGF-α involves the prevention of these EtOH-induced alterations in microtubules.

Intestinal epithelium is a highly selective barrier that permits the absorption of nutrients from the gut lumen into the circulation but normally restricts the passage of harmful and potentially toxic compounds such as products of the luminal microflora (e.g., endotoxin) and proinflammatory molecules (Bode et al., 1987; Hollander, 1988, 1992). Indeed, the epithelium is the most important biological barrier against the toxic dietary and luminal substances. An abnormal gut barrier, in contrast, may promote the initiating of inflammatory processes and mucosal damage after the penetration of normally excluded luminal substances. It is thus not surprising that an abnormal intestinal barrier integrity has been implicated in a wide range of illnesses, including alcoholic cirrhosis (Bode et al., 1987; Hollander, 1988, 1992; Keshavarzian et al., 1994, 1999).

Alcohol consumption can deleteriously affect both the functional and the anatomic integrity of the intestinal mucosa (Carter et al., 1987). Both acute and chronic alcohol consumption can cause intestinal barrier dysfunction (Robinson et al., 1981; Talbot et al., 1984; Keshavarzian et al., 1994). Although the pathophysiological mechanisms have remained elusive, it has been suggested that oxidative stress may contribute to the abnormal mucosal barrier function associated with ethanol (EtOH) administration in vivo (Kvie et al., 1990; Dinka et al., 1996), as well as in several gastrointestinal (GI) inflammatory disorders (Lih-Brody et al., 1996; McKenzie et al., 1996; Singer et al., 1996).

Recent studies of several systemic and inflammatory intestinal disorders have suggested that protein carbonylation

ABBREVIATIONS: EtOH, ethanol; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; GI, gastrointestinal; DCF, dichlorofluorescein; FSA, fluorescein sulfonic acid; DMEM, Dulbecco’s modified Eagle’s medium; EGF, epidermal growth factor; TGF-α, transforming growth factor-α; DNP, dinitrophenylhydrazine; SOD, superoxide dismutase; l-NIL, l-N-alpha-imidoethyl-lysine; EGFR, epidermal growth factor receptor; PAGE, polyacrylamide gel electrophoresis; PBS-T, PBS/Tween 20.
and nitrotyrosination can serve as markers of oxidative protein damage (Haddad et al., 1993; Ischiropoulos et al., 1995; McKenzie et al., 1996; Singer et al., 1996; Ferro et al., 1997). Others have shown concomitant increases in protein nitration and in inducible nitric oxide synthase (iNOS) in the inflamed intestinal mucosa (Salzman et al., 1996; Singer et al., 1996; Kimura et al., 1998). However, the precise pathogenetic role of oxidative stress in the development of mucosal abnormalities, especially after EtOH exposure, remains unknown. There also is no effective means of preventing such abnormalities.

Based on our previous studies, we surmised that EtOH-induced barrier dysfunction may involve the oxidative disruption of microtubule component of the cytoskeleton. Microtubules are part of a complex array of cytoskeletal protein filaments in the eukaryotic cytosol that are critical to the preservation of normal homeostasis of cells (Allen, 1985; Bershadsky and Vasiliev, 1991; MacRae, 1992; Banan et al., 1998c). As such, they play a central role in maintaining cellular integrity, structure, and transport functions (MacRae, 1992; Rodinov and Gelfand, 1993). This structural element also provides a system for directing intracellular vesicular transport and secretion, as well as movement of cytosolic organelle. Furthermore, microtubules govern cell morphology, cell migration and cell polarity and maintain the plane of cell division (Bershadsky and Vasiliev, 1991; MacRae, 1992).

With an in vitro model of human colonic epithelial cells, we previously demonstrated the extensive disruption of microtubules after exposure to damaging agents and the key role of this critical structure in maintaining mucosal barrier function (Banan et al., 1998c, 1999). In other studies, we have shown the importance of microtubules in mucosal healing in rats (Banan et al., 1998a). Thus, an objective of the current study was to determine whether one potential mechanism of EtOH-induced abnormal barrier function involves the oxidation, nitration, and disassembly of the microtubule cytoskeleton.

Restoration of barrier function by protective agents after insults to the mucosa such as EtOH is essential for reestablishing normal intestinal homeostasis. In recent years, polypeptide growth factors have gained increasing importance in our understanding of the GI tract, especially their role in regulating proliferation, differentiation, and repair processes throughout the GI mucosa (Konturek et al., 1988; Podolsky, 1994). The epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) in particular have been implicated as peptides central to the maintenance of GI mucosal barrier integrity. Numerous findings show that GI mucosal disruption induced by a wide variety of insults such as oxidants, EtOH, and toxins is prevented by EGF or TGF-α pretreatment, independent of their known antiseecretory properties (Konturek et al., 1988; Ishikawa et al., 1992; Riegler et al., 1997; Banan et al., 1999). However, the mechanism through which EGF or TGF-α elicits such protection is not well established. One potential mechanism by which these growth factors provide protection may involve the prevention of oxidative disruption to the microtubule cytoskeleton and the promotion of its assembly and stability.

In the current study, we explored the interrelationships among tubulin-based nitration and oxidation, microtubule disassembly, barrier dysfunction, and growth factor protection after exposure to EtOH. To this end, we used a human colonic cell monolayer (Caco-2) under in vitro conditions. Because these cell preparations are devoid of neural and vascular connections and of input from circulating hormones, we hoped to focus on more fundamental mechanisms of intestinal disruption and protection.

Materials and Methods

Cell Culture. The Caco-2 (a human colonic) cell line used in our studies was obtained from American Type Culture Collection (Rockville, MD) at passage 15. Although of colonic origin, these cells resemble small intestinal cells in that they have defined apical brush borders, form tight junctions, and exhibit a highly organized microtubule network on differentiation (Dix et al., 1990; Gilbert et al., 1991; Peterson and Mooseker, 1993). Caco-2 cells were maintained at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) in an atmosphere of 5% CO₂ and 100% relative humidity. Cells were split at a ratio of 1:6 on reaching confluency every 6 days and set up in either 6-, 24-, or 48-well plates for experiments or T-175 flasks for the maintenance of stocks. Cells grown for barrier integrity work were split at a ratio of 1:2 and seeded at a density of 200,000 cells/cm² onto 0.4-μm Biocollagen I Cell Culture Inserts (0.3-cm² growth surface; Becton Dickinson Labware, Bedford, MA), and experiments were performed at least 7 days postconfluence. The utility, maintenance, and characterization of this cell line has been previously published (Dix et al., 1990; Gilbert et al., 1991; Peterson and Mooseker, 1993).

Experimental Design. The first series of experiments evaluated the effect of graded concentrations of EtOH (v/v) or vehicle (isotonic saline/DMEM) on cell viability, barrier integrity, oxidative stress, and microtubule stability/instability as described later. For these studies, clinically relevant serial dilutions in the range of 1 to 15% EtOH (Bjorkman and Jessop, 1994; Dinka et al., 1996; Banan et al., 1998c) or isotonic saline/DMEM were added to monolayers of Caco-2 cells for 30 min. In the second series of experiments, we assessed the protective effects of EGF (10 ng/ml) or TGF-α (10 ng/ml; Sigma Chemical Co., St. Louis, MO) after a 10-min pretreatment on EtOH-induced cell injury, barrier integrity, and microtubule stability. The concentrations of EGF or TGF-α that were used in these studies have previously been shown to have protective properties in GI epithelial cultures (Banan et al., 1998b, 1999). To determine the specificity of the protective actions of the growth factors, monoclonal anti-EGF receptor antibody (anti-EGFR, 1 μg/ml) was coadministered with the growth factors.

In a third series of experiments, the potential protective effects of pretreatment with antioxidants on cell oxidative state, barrier function, and microtubule stability in monolayers exposed to EtOH were determined. For these studies, before incubation with EtOH, Caco-2 monolayers were preincubated for 30 min with either 1) an NO scavenger, L-cysteine (1 mM) (or as control, the inactive analog D-cysteine, 1 mM), 2) a superoxide scavenger, superoxide dismutase (SOD; 300 U/ml) (or its inactive analog, iSOD, 300 U/ml; Ferro et al., 1997), or 3) isoform-selective iNOS inhibitor, L-NAME-1-iminoethyl-lysine (L-NIL, 1 mM; Salzman et al., 1996). Effects on iNOS activity, barrier function, and microtubule integrity were then determined.

In a fourth series of experiments, we determined the effects of colchicine (a microtubule disruptive drug) and paclitaxel (a drug known to stabilize microtubules; Sigma Chemical Co.), agents known to influence the microtubules in a “nonoxidative manner” on EtOH-induced injury, barrier function, and microtubule stability. Colchicine or paclitaxel (50 μM) in DMEM or saline was added to cells at 37°C (Parczyk et al., 1989; Banan et al., 1998c). For colchicine studies, monolayers were preincubated with colchicine (4 h) or vehicle and then incubated in medium containing growth factor (10 ng/ml, 10 min) or vehicle, followed by exposure to EtOH (1–15%) or vehicle for 30 min. For paclitaxel studies, cells were incubated with paclitaxel (1 h) or vehicle and then exposed to EtOH or vehicle (in the absence of growth factors).
In a fifth series of experiments, to determine the role of microtubule alterations (i.e., nitration, oxidation, and assembly/disassembly) in mucosal barrier function/dysfunction, monomeric and polymerized fractions of tubulin (backbone of the microtubules) were isolated following the aforementioned protocols (series 1, 2, and 4 above) and subsequently analyzed using two immunoblotting procedures.

In all experiments, microtubule stability/integrity was assessed by using at least two of the following three procedures: 1) immunofluorescent labeling to determine the percentage of cells with normal microtubules by fluorescence microscopy; 2) detailed analysis by high-resolution laser scanning fluorescent microscopy; and 3) quantitative immunoblotting analysis of monomeric and polymerized fractions of tubulin.

**Determination of Cell Viability.** The Live/Dead Viability Kits (Molecular Oribases, Eugene, OR) were used. This assay measures two parameters of cell viability: intracellular esterase activity and plasma membrane integrity. After treatments, cells were loaded with two fluorescent probes (2 mM calcine AM and 4 mM ethidium homodimer-1) for 20 min at 37°C and subsequently examined using a Dophot inverted fluorescent microscope with the appropriate filter cubes. All experiments were performed by counting 100 to 200 cells in four different fields from each slide. Cell viability was expressed as follows: percentage viability = [live cells/(live cells + dead cells)] × 100.

**Determination of Barrier Integrity/Function.** Barrier integrity was determined by measuring apical-to-basolateral flux of a fluorescent marker [fluorescein sulfonic acid (FSA; 200 μg/ml; 478 Da)] as described previously (Unno et al., 1996). After treatments, fluorescent signals from samples were quantified with the use of a fluorescence multiplate reader. The excitation and emission spectra of FSA were excitation of 485 nm and emission of 530 nm. Clearance was calculated using the following formula: Clearance (nl/h/cm²) = Fab/[FSA]a × S, where Fab is the apical-to-basolateral flux of FSA (light units/h), [FSA]a is the concentration at baseline (light units/ml), and S is the surface area (0.3 cm²; Unno et al., 1996). Simultaneous controls were performed with each experiment.

**Determination of Cell Oxidative Stress.** Oxidative stress was assessed by measuring the conversion of a nonfluorescent compound, 2′,7′-dichlorofluorescein diacetate (DCFH-DA) as described previously (Unno et al., 1996). After treatments, fluorescent signals from samples were quantified with the use of a fluorescence multiplate reader. The excitation and emission spectra for DCF are excitation of 485 nm and emission of 520 nm. Clearance for DCF was calculated using the following formula: Clearance (nl/h/cm²) = Fab/[CF]a × S, where Fab is the apical-to-basolateral flux of DCF (light units/h), [CF]a is the concentration at baseline (light units/ml), and S is the surface area (0.3 cm²; Unno et al., 1996). Simultaneous controls were performed with each experiment.

**Nitric Oxide Synthase (NOS) Assay.** Cells grown to confluence were preincubated with the membrane-permeable dichlorofluorescein diacetate (10 μg/ml for 30 min) before the subsequent treatment (see series 1 and 3). After treatments, fluorescent signals (i.e., DCF fluorescence) from samples were quantified with a fluorescence multiplate reader set at excitation of 485 nm and emission of 530 nm.

**Oxidation and Nitration.** Oxidation or nitration of tubulin was assessed by measuring protein carbonyl and nitrotyrosine formation, respectively (Haddad et al., 1993; Ischiropoulos et al., 1995; Ferro et al., 1997). Carboxylic form dinitrophenylhydrazone (DNPH) adducts via the Schiff reaction (Ferro et al., 1997). Nitrotyrosination is a
tion with primary and secondary antibodies followed by mounting in aquamount. Samples were stored in the dark at −20°C and were examined by standard or high-resolution laser fluorescence microscopy (below) within 48 h.

**High-Resolution Laser Scanning Fluorescence Microscopy.** Cells on slides were observed in a blinded fashion with high-resolution laser scanning fluorescence microscopy using a 63× oil immersion plan-aphochromat objective, NA 1.4 (Zeiss). An argon laser (wavelength 488 nm) was used to examine fluorescein isothiocyanate-labeled cells, and the cytoskeletal elements were examined for their overall morphology, orientation, and disruption as described previously (Ban
nan et al., 1998c).

**Microtubules (Tubulin) Fractionation and Quantitative Immunoblotting of Tubulin.** Polymersized (S2) and monomeric (S1) fractions of tubulin were isolated as previously described from our laboratory (Ban
nan et al., 1998c). Fractionated S1 and S2 samples were flash frozen in liquid N₂ and then stored at −70°C until immunoblotting. For immunoblotting, samples (5 μg) were placed in SDS sample buffer (250 mM Tris·HCl, pH 6.8, 2% glycerol, 5% mercaptoethanol), boiled for 5 min, and then subjected to electrophoresis on 7.5% polyacrylamide gels. Procedures for Western blotting were performed at room temperature (Ban
nan et al., 1998c). To quantify the relative levels of tubulin, the absorbance of the bands corresponding to immunoreadiolabeled tubulin were measured with a laser densitometer.

**Immunoblotting Determination of Microtubule (Tubulin) Oxidation and Nitration.** Oxidation or nitration of tubulin was assessed by measuring protein carbonyl and nitrotyrosine formation, respectively (Haddad et al., 1993; Ischiropoulos et al., 1995; Ferro et al., 1997). Carboxylic form dinitrophenylhydrazone (DNPH) adducts via the Schiff reaction (Ferro et al., 1997). Nitrotyrosination is a
marker of oxidative damage associated with tyrosine residues (Haddad et al., 1993; Ischiropoulos et al., 1995). Determination of protein carbonyl and nitrotyrosine groups was accomplished in a similar manner to the quantitative blotting of tubulin (Ferro et al., 1997; Banan et al., 1998c) except for differences in primary antibodies and buffers. To avoid unwanted oxidation of tubulin samples, all buffers contained 0.5 mM dithiothreitol and 20 mM 4,5-dihydroxy-1,3-benzene sulfonic acid. To determine the carbonyl content, samples were blotted to a polyvinylidene difluoride membrane followed by successive incubations in 2 N HCl and 2,4-dinitro-phenyl-hydrazine (100 μg/ml in 2 N HCl) for 5 min each. Membranes were then washed three times in 2 N HCl and subsequently washed seven times in 100% methanol for 5 min each, followed by blocking for 1 h in 5% BSA in 10× PBS/Tween 20 (PBS-T). Immunologic evaluation of carbonyl formation was performed for 1 h in 1% BSA/PBS-T buffer containing anti-2,4-dinitro-phenyl-hydrazine (1:25,000 dilution; Molecular Probes) that was conjugated to peroxidase by protein cross-linking with 0.2% glutaraldehyde. To determine nitrotyrosine content, after the blocking step above (i.e., BSA/PBS-T buffer), membranes were probed for nitrotyrosine by incubation with 2 μg/ml monoclonal anti-nitrotyrosine antibody for 1 h (Upstate Biotechnology, Lake Placid, NY) followed by conjugation to peroxidase by protein cross-linking with glutaraldehyde. Wash steps, film exposure, and quantification were as described previously (Banan et al., 1998c).

Statistical Analysis. Data are presented as mean ± S.E. All experiments were carried out with a sample size of at least six observations per group. Statistical analysis was carried out using ANOVA followed by Dunnett’s multiple range test (Harter, 1960). A value of p < .05 was deemed to represent statistical significance.

Results

Deleterious Effects of EtOH on Cell Viability and on Barrier Integrity and Protective Actions of Growth Factors. Exposure of Caco-2 cells to a range of concentrations of EtOH (2.5–15%) for 30 min caused a dose-dependent decrease in viability as determined with the Live/Dead assay (Fig. 1). The lowest concentration of EtOH that significantly reduced cell viability was 2.5%. Preincubation with EGF or TGF-α (Fig. 1) at 10 ng/ml before subsequent exposure to damaging 2.5 to 15% EtOH significantly blunted the deleterious effects of EtOH on cell viability.

Exposure of intestinal monolayers to EtOH (2.5–15%) for 30 min in a concentration-dependent manner disrupted epithelial barrier function as demonstrated by increased clearance of FSA (Fig. 2). Pretreatment with either EGF or TGF-α (10 ng/ml) significantly prevented the loss of barrier function induced by EtOH (Fig. 2).

To determine whether the protective effects of EGF or TGF-α on mucosal barrier function are specific to these growth factors, monolayers were pretreated with monoclonal anti-EGFR antibody before subsequent exposure to growth...
factor. Anti-EGFR completely abolished the protective effects of EGF or TGF-α on the restoration of barrier integrity after EtOH insult (Fig. 3).

**Involvement of Microtubule Cytoskeleton in Deleterious Effects of EtOH and in Protective Effects of Growth Factors.** EtOH in the range of 2.5 to 15% dose-dependently caused an extensive disruption of the microtubules as assessed by laser fluorescent microscopy (Fig. 4A). The lowest EtOH concentration that induced disruption of microtubules was 2.5%. In contrast, preincubation with EGF or TGF-α completely abolished the disruption of the microtubule cytoskeleton in Caco-2 cells that otherwise elicited damage when exposed to damaging EtOH (Fig. 4A, EGF data shown). Figure 4B shows the fragmentation, disorganization, and collapse of the microtubule cytoskeleton after exposure to damaging EtOH levels (2.5% shown) as indicated by immunofluorescent staining (Fig. 4B, b). Pretreatment with protective EGF before exposure to injurious EtOH stabilized the microtubules as shown by their intact radial distribution, which is similar in appearance to that of the controls (Fig. 4, c). Controls exhibited a normal, stellate, and radial distribution of the microtubules (Fig. 4B, a).

To determine whether the deleterious effects of EtOH on microtubules is secondary to the disassembly of this cytoskeletal element, quantitative Western immunoblotting of the polymerized pool of tubulin (S2 fraction, an index of stability) and the monomeric pool of tubulin (S1 fraction, an index of disruption) in response to various treatments were performed (Fig. 5A). EtOH dose-dependently caused a significant reduction in the stable S2 tubulin and an increase in the S1 monomeric tubulin compared with controls. On the other hand, pretreatment with EGF or TGF-α (Fig. 5A) increased the stable S2 tubulin and decreased the monomeric S1 tubulin in monolayers exposed to damaging concentrations of EtOH. Figure 5B shows a Western blot gel [polyacrylamide gel electrophoresis (PAGE), followed by autoradiography] demonstrating that EtOH exposure decreases the S2 tubulin band density well below the control level. Conversely, growth factors (EGF shown) in combination with damaging EtOH concentrations enhanced the S2 tubulin band density to a comparable level as the controls, suggesting enhanced microtubule assembly (and stabilization).

We then further evaluated the role of microtubule stability in mucosal barrier function utilizing a microtubule-disrup-

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**Fig. 4.** A, percent Caco-2 cells displaying normal microtubule cytoskeleton as determined by immunofluorescent labeling. Cells were exposed to graded EtOH concentrations (2.5–15%) or pretreated with EGF (10 ng/ml) followed immediately by a 30-min exposure to EtOH. *p < .05 versus control, EGF alone, or pretreatment. +p < .05 versus 2.5 to 15% EtOH alone. B, representative immunofluorescent photomicrograph of the microtubule cytoskeleton in intestinal cells as stained by fluorescein-conjugated anti-β-tubulin antibodies. a, microtubule cytoskeleton in control, isotonic saline-treated, intestinal cell. The microtubules can be seen as filamentous network of proteins that course in a radial fashion throughout the cytosol. b, in cell treated with 2.5% EtOH, the microtubules appear to be collapsed, fragmented, and disorganized. c, in cell pretreated with EGF before incubation with 2.5% EtOH, microtubule morphology is highly preserved and resembles the control. Bar, 25 μm.
tive agent (colchicine) and a microtubule-stabilizing agent (paclitaxel). Colchicine increased FSA clearance, indicating that microtubule disruption per se could induce loss of barrier function in mucosal monolayers (Fig. 6A). Colchicine also abolished the protective effects of either growth factor, further suggesting the importance of microtubules in the mechanism of growth factor protection against EtOH-induced barrier dysfunction. Colchicine in combination with subsequent incubation with EtOH potentiated the deleterious effects of EtOH on mucosal barrier function (data not shown). On the other hand, paclitaxel completely prevented EtOH-induced barrier dysfunction, indicating a key role for microtubules in maintaining normal barrier integrity.

Colchicine decreased polymerized tubulin to almost undetectable levels (≤0.8% of total tubulin pool; Fig. 6B) as determined by immunoblotting. In addition, colchicine in combination with growth factors and EtOH disassembled the tubulin-based cytoskeleton, suggesting the key role of microtubule assembly (stability) in the process of protection. Paclitaxel alone or in combination with EtOH (in the absence of growth factor) stabilized the microtubules as demonstrated by the presence of a large polymerized (S2) tubulin pool comparable to controls (Fig. 6B). These effects of colchicine and paclitaxel are consistent with the possibility that microtubule integrity is important in the mechanisms of EtOH injury and growth factor protection.

**Involvement of Oxidative Mechanisms in Deleterious Effects of EtOH and in Protective Effects of Growth Factors on Microtubules and on Barrier Function.** To further evaluate the molecular mechanism of EtOH-induced microtubule disruption and barrier dysfunction, we determined whether the disruptive effects of EtOH are due to the oxidation and/or nitration (i.e., oxidative stress) of the structural protein of microtubules, namely tubulin. To this end, we measured tubulin carbonylation and tubulin nitration (indices of protein oxidation) by Western immunoblotting. Figure 7A shows the “fraction” of polymerized tubulin (S2) oxidized (i.e., carbonylated) as determined by anti-DNP immunoreactivity. EtOH increased tubulin oxidation compared with controls, paralleling findings for tubulin disassembly. In contrast, EGF or TGF-α pretreatment caused a significant reduction in the “fraction” of tubulin oxidized in cells exposed to EtOH. These findings paralleled the protective actions of growth factors on enhancement of tubulin polymerization. Figure 7B shows a Western immunoblot of anti-DNP immunoreactivity associated with polymerized tubulin (S2). EtOH increased the autoradiographic band density (i.e., oxidation) associated with the S2 tubulin well in excess of the controls. Growth factor pretreatment caused the band density to return to a level similar to that of controls.

Figure 8A shows the “fraction” of cytoskeletal tubulin (S2) nitratas as quantified by immunoblotting. Exposure to EtOH caused a significant increase in the anti-nitrotyrosine immunoreactivity associated with tubulin. Preincubation with EGF or TGF-α, in contrast, prevented the nitration of polymerized tubulin in monolayers exposed to EtOH. A blot of anti-nitrotyrosine immunoreactivity (Fig. 8B) demonstrates the presence of nitration band after exposure to EtOH compared with the controls exhibiting no nitration. Pretreatment with EGF or TGF-α prevented the formation of nitration bands.

To further determine whether oxidative stress is involved in the mechanism of EtOH-induced microtubule disruption/barrier dysfunction and whether protection involves the prevention of oxidative damage, we measured the oxidative stress state of Caco-2 monolayers using a fluorometric assay (DCF). We demonstrated significant and dose-dependent increases in DCF fluorescence after exposure to EtOH (Fig. 9). The oxidative stress induced by EtOH was completely abolished in monolayers pretreated with oxidant scavengers such as L-cysteine and superoxide dismutase (SOD) but not by their inactive analogs (D-cysteine and iSOD, respectively; Fig. 9, cysteine data shown).

Preincubation with antioxidants, L-cysteine (but not the inactive D-cysteine), and SOD (but not the inactive iSOD) prevented the disruption of barrier function induced by EtOH as shown by reduced FSA clearance (Fig. 10). The same doses of L-cysteine or SOD (but not their inactive forms) increased the percentage of colonocytes displaying intact mi-
crotubules to a level comparable to the controls in monolayers exposed to EtOH (Fig. 11).

Studies with L-NIL, a selective inhibitor of enzyme iNOS, led to similar results linking EtOH injury to oxidative stress and cytoskeletal disruption and suggest that NO synthesis may be involved in the oxidative process. Indeed, EtOH significantly increased iNOS activity of Caco-2 cells; these increases were abolished by pretreatment with L-NIL (Fig. 12) at a dose that maintained a high percentage of cells with normal microtubules and prevented barrier dysfunction induced by EtOH (Fig. 10). Overall, these findings are consistent with the hypothesis that EtOH injury involves oxidative stress to the microtubule cytoskeleton and that protection involves prevention of these alterations.

**Discussion**

Many prior in vivo studies have demonstrated that alcohol consumption can cause both functional and structural alterations, including barrier dysfunction in the intestinal mucosa (Robinson et al., 1981; Talbot et al., 1984; Bode et al., 1987; Carter et al., 1987; Keshavarzian et al., 1994). For example, an abnormal intestinal barrier has been suggested as one of the underlying mechanisms of EtOH-induced endotoxemia in patients with alcoholics liver disease (Bode et al., 1987; Keshavarzian et al., 1994, 1999). Despite intensive investigation in the past decade, however, the mechanism for EtOH-induced loss of intestinal barrier integrity remains ill defined and merits further study.

Accordingly, in the present study, we investigated one possible mechanism for EtOH-induced disruption of intestinal barrier integrity: oxidative damage to the microtubule cytoskeletal network. A previous study from our laboratory showed that EtOH disrupted the cytoskeletal protein (microtubules) in Caco-2 cells (Banan et al., 1998c); however, the exact nature of this effect was unclear. Thus, we sought to confirm and extend our previous pilot finding by investigating whether in our monolayer model exposure of Caco-2 cells to EtOH causes oxidation of tubulin and thereby disrupts the microtubule cytoskeleton at EtOH concentrations that reduce barrier integrity. Finally, we investigated the possibility that growth factors (EGF and TGF-α), known GI-protective agents, could protect against injury to EtOH and whether this protection is mediated, at least in part, by prevention of oxidative injury to microtubules that is induced by EtOH.

Some evidence suggests that oxidative tissue damage may be a key mechanism in the deleterious effects of EtOH on the GI tract. Oxidative stress injury has been implicated in the
pathogenesis of various intestinal disorders involving an abnormal mucosal barrier, including EtOH-induced mucosal disruption (Kvietys et al., 1990; Dinka et al., 1996; Singer et al., 1996; Lih-Brody et al., 1996; McKenzie et al., 1996). Moreover, recent studies have shown increased levels of protein carbonylation and nitration, markers of oxidative damage to proteins, in several GI disorders such as inflammatory bowel disease (Haddad et al., 1993; Ischiropoulos et al., 1995; McKenzie et al., 1996; Singer et al., 1996; Ferro et al., 1997). Furthermore, it has been suggested that protein nitration and barrier dysfunction may exist in concert with the expression of an iNOS in the inflamed mucosa (McKenzie et al., 1996; Salzman et al., 1996; Singer et al., 1996; Kimura et al., 1998). Nevertheless, the roles of oxidation, nitration, and disruption of essential proteins in the molecular mechanism of EtOH-induced barrier disruption remain largely unexplored.

In the current study, we present evidence that EtOH-induced barrier disruption involves oxidative injury to specific cellular proteins, microtubules, that are critical for maintaining normal structural and functional integrity of enterocytes. In particular, we found not only that EtOH disrupted monolayer barrier function and damaged the microtubules but also that the underlying mechanism of this injurious effect of EtOH appears to involve the nitration, oxidation, and disassembly of the tubulin, backbone of microtubules. Parallel to these findings, EtOH decreased polymerized tubulin, increased monomeric tubulin, and reduced the percentage of cells displaying intact microtubules. Identical concentrations of EtOH also induced oxidative stress (DCF fluorescence) and up-regulated iNOS activity, parallel to the findings on tubulin nitration and oxidation. iNOS is
known to cause overproduction of nitrating and oxidizing agents, especially NO and derivative oxides (McKenzie et al., 1996; Singer et al., 1996; Kimura et al., 1998), and this could explain our observations regarding increased nitration and oxidation of tubulin. To our knowledge, this is the first demonstration that concentrations of EtOH that disrupted intestinal barrier function caused oxidation and nitration of a major cytoskeletal protein, tubulin, while simultaneously leading to its disassembly and disruption. This notion is also supported by our experiments with growth factors and antioxidants (see later).

The EGF and TGF-α are prototypic members of a family of several polypeptides that are key components in the maintenance and repair of the GI mucosa. Numerous studies have documented that EGF and TGF-α prevent GI damage in vivo and in vitro due to a variety of insults (Konturek et al., 1988; Ishikawa et al., 1992; Podolsky, 1994; Riegler et al., 1996). The mechanisms by which EGF and TGF-α elicit protection have remained elusive despite intensive investigation. Several mechanisms have been proposed in recent years to explain their protective action, but the majority are considered to be too slow. For instance, EGF-induced enhancement of mucosal blood circulation, stimulation of mucous and bicarbonate secretions, and epithelial restitution, although important in ensuring a healthy GI epithelium, cannot explain the rapid protective effects of EGF under acute in vitro conditions in cell cultures, a rapid phenomenon that occurs independent of intact blood flow, humoral agents, and neural connections (Ishikawa et al., 1992; Riegler et al., 1996; Lawrence et al., 1997; Banan et al., 1999). Such considerations point to a more basic cellular process as being directly responsible for EGF or TGF-α protection.

In the current investigation, we demonstrated that EGF or TGF-α protected intestinal monolayers against the injurious EtOH and restored normal barrier. It was our belief that EGF or TGF-α should prevent cytoskeletal disassembly and oxidation if the microtubule component of the cytoskeleton is indeed intimately involved in the mechanism of protection. In fact, we showed that EGF or TGF-α prevented the nitration and oxidation of tubulin while, in concert, increasing the polymerized stable tubulin and decreasing the unstable monomeric tubulin in Caco-2 monolayers exposed to EtOH. In parallel to such effects, growth factors also maintained a significantly high percentage of enterocytes displaying normal microtubules. In further support of our findings that protection involves prevention of oxidative damage to the microtubule cytoskeleton, we also found that antioxidants (L-cysteine, SOD, L-NIL) protected against EtOH-induced loss of barrier function and concomitantly maintained normal microtubules. Our data indicate, for the first time, the importance of EGF or TGF-α in promoting the organization
and stabilization of microtubules, in preventing oxidative damage to microtubules, and in maintaining normal barrier function.

Our studies with microtubule modulators paclitaxel and colchicine provide further evidence for the importance of microtubule integrity in the mechanism of barrier maintenance and of EGF and TGF-α protection. The facts that paclitaxel enhanced microtubule stability and maintained normal barrier function in monolayers exposed to injurious EtOH levels suggest that microtubules play an essential role in EtOH injury and in the protective effects of growth factors. Direct evidence that paclitaxel stabilized the tubulin-based cytoskeleton is provided by our immunoblotting data showing increased tubulin polymerization. Furthermore, colchicine not only abolished monolayer barrier function but also prevented protection by growth factors, indicating again that microtubules play an intimate role in barrier integrity and in the protective action of growth factors. In support of this interpretation, colchicine completely disassembled microtubules showing the presence of less than 1% of polymerized tubulin.

Protection and stabilization of the microtubule cytoskeleton by growth factors may not be limited to their antioxidative properties, and other mechanisms such as protein phosphorylation (direct or indirect) may be involved. For instance, one proposed mechanism is through direct phosphorylation of microtubule-associated proteins, which have been shown to play a key role in constructing and stabilizing microtubules (MacRae, 1992; Mandelkow and Mandelkow, 1995). Other key GI-protective agents such as prostaglandins are known to induce the phosphorylation of microtubule-associated proteins, which is thought to account, in part, for the mucosal protective actions of prostaglandins involving the stabilization of microtubules (Mandelkow and Mandelkow, 1995; Banan et al., 1998c). Another possible mechanism is indirect protein phosphorylation by EGF such as rapid phosphorylation of the cytoskeletal chaperone (stabilizing) proteins known as heat shock proteins (e.g., HSP-27). Phosphorylated HSP-27 is believed to protect against cytoskeletal disruption under oxidative conditions (Liang and MacRae, 1997). Future studies will be needed to further explore these mechanisms.

In conclusion, our results suggest that EtOH-induced barrier disruption is mediated, in part, by the nitration and oxidation of tubulin leading to the disassembly and disruption of the microtubule cytoskeleton and mucosal barrier dysfunction. The EGF or TGF-α protects the mucosa from EtOH by preventing the same abnormalities that EtOH induces.

References


