Dynamics of enterocyte tight junctions: effect of experimental colitis and two different anti-TNF strategies

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Am J Physiol Gastrointest Liver Physiol 294: G938–G947, 2008. First published February 7, 2008; doi:10.1152/ajpgi.00469.2007.—An alteration of the intestinal barrier is considered to be an early step in the pathogenesis of Crohn’s disease. The integrity of intestinal barrier function is guaranteed among other factors by enterocyte tight junction (TJ) proteins. Clinical and experimental data indicate the TNF-α to be the major responsible factor for these defects. In the present study we investigated the very early effects of DNBS-ethanol colitis on ileal enterocyte TJ proteins [occludin, zonula occludens-1 (ZO-1), claudin-2] in controls, mice treated with infliximab (IFX) or with etanercept (ETC), and in knockout mice for the TNF-α receptor 1 (TNFR-1). Circulating TNF-α levels were effectively reduced by IFX and ETC (P < 0.01, both) at 3 and at 6 h. DNBS colitis induced disappearance of occludin and ZO-1 from enterocyte cell-cell contact, whereas claudin-2, absent under control conditions, appeared in the ileal epithelium. These alterations were prevented equally by both treatments, IFX and ETC, and in TNFR-1−/− animals. DNBS colitis induced a very rapid loss of occludin and ZO-1 from ileal TJ together with an upregulation of claudin-2. Our data are consistent with the hypothesis that TNF-α is involved in early TJ rearrangement and that its effects are mediated through TNF-1. Despite clinical differences, both anti-TNF treatments were equally effective in the present setting.

infliximab; etanercept; occludin; zonula occludens-1; claudin-2

INTESTINAL BARRIER FUNCTION is guaranteed by an intact epithelial cell lining linked together at their apical portion through junctional complexes represented by the tight junctions (TJ) and adherens junctions (AJ). The junctional complexes are highly dynamic structures being involved under physiological conditions in the regulation of absorption, secretion of water and electrolytes, and transmigration of inflammatory cells (3, 18, 36). A defective intestinal barrier on the other hand may lead to water, electrolyte, and protein losses together with the potential entry of gut-derived antigens and bacteria. This is actually thought to represent an important step in the pathogenesis of Crohn’s disease (CD).

An altered intestinal permeability and the resulting abnormal passage of different marker substances has been shown in CD patients and at least in part of their healthy relatives (13, 19, 32, 35); moreover, it has been demonstrated also in the absence of intestinal inflammation (22). However, the molecular basis of this defect is still unclear.

By now, one of the most persuasive hypotheses to explain the barrier loss associated with CD is that mediators of inflammation such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and the TNF superfamily member LIGHT (lymphotxin-like inducible protein that competes with glycoprotein D for herpesvirus entry mediator on T cells) interacting with specific membrane receptors (e.g., TNF-α receptors 1 and 2, lymphotoxin β receptor) on enterocytes activate intracytoplasmic proteins that regulate transporters involved in transcellular electrolyte and water movements (e.g., Na+/glucose cotransporter, Na+/H+ exchanger isomor 3). Moreover, these mediators may alter also the paracellular route through the activation of the myosin light chain kinase (MLCK) and the resulting effects on the actin cytoskeleton that lead to the dislocation of specific TJ proteins such as occludin and zonula occludens-1 (ZO-1) (4, 27, 30). Although the majority of data leading to the formulation of this model were obtained from in vitro experiments (28) or from in vivo mouse models of experimental colitis (4, 27), several observations in patients with inflammatory bowel disease are consistent with this hypothesis, like the expression and activity of the MLCK in both CD and ulcerative colitis (UC) patients (2) or the dislocation of occludin and ZO-1 from the site of cell-cell contact in patients with CD (21). Finally, the clinical observation that the anti-TNF treatment infliximab (IFX), introduced recently in the therapy of inflammatory bowel disease, restores the intestinal barrier function (14, 33) points to TNF-α, the key mediator of the T helper-1 immune response, as a major responsible factor for barrier alterations.

On this basis, the present study was thought to investigate the actions of two anti-TNF strategies, IFX and etanercept (ETC), on the alterations of the major TJ proteins with barrier functions (occludin and ZO-1) and on the ion channel inducing claudin-2. Whereas IFX is currently used in CD and UC (5, 34, 35), ETC failed to show clinical efficacy (26). This difference was explained by the proapoptotic effect of IFX, but not of ETC, on inflammatory T cells because IFX, a complement-binding G1 immunoglobulin, acts also on membrane-bound TNF; some doubt about this explanation was raised after the positive studies with certolizumab, a pegylated humanized Fab’ fragment of an IgG4 (40).

Since nearly all studies have been carried out on epithelial monolayers or in patients with established disease, the present experiments have been designed to investigate the very early effects of experimental colitis, alone and under treatment with IFX and ETC, on the ileal epithelium in an intact organism and...
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the mediation of these alterations using knockout mice for the receptor I of TNF-α (TNFR-1−/−).

MATERIALS AND METHODS

All experiments were carried out in accordance with the national law on animal protection. The experimental protocols were approved by the Ethics Committee of the University of Messina. Male CD mice (Harlan) were housed for 2 wk under standard conditions with free access to tap water and standard laboratory chow. Colitis was induced by intrarectal administration of 5 mg of dinitrobenzenesulfonic acid (DNBS) dissolved in 50% ethanol under light ether anesthesia. Control colitis was induced by intrarectal instillation of 50% ethanol only. Twenty-five animals per group were euthanized at 3 and at 6 h after colitis induction. Blood was drawn by intracardiac puncture under ether anesthesia, the abdomen was opened by a midline incision, the entire colon was removed and opened, and the macroscopic damage score was determined (37). Subsequently the distal colon and the terminal ileum were cut in longitudinal slices and fixed in buffered formalin.

In a second experiment mice were treated 1 h before colitis induction with IFX (Schering-Plough, Milan, Italy, 5 mg/kg ip) or with ETC (Wyeth, Milan, Italy, 5 mg/kg sc). In parallel, mice subjected to anti-TNF treatment in sham colitis and colitis with sham treatment (saline ip) served as controls with an identical protocol as above. Both treatments have been shown to effectively antagonize TNF effects in vivo in mice (10, 31).

In a third experimental setting, in mice lacking the TNF membrane receptor I (TNFR-1−/−, Jackson Laboratory, Bar Harbor, MN) colitis was induced as above and animals were euthanized at 3 and at 6 h.

Serum TNF-α determination. The blood was spun and serum was stored at −80°C until analysis. Serum concentrations of TNF-α were determined by ELISA (Euroclone, Devon, UK).

Histological evaluation. After fixation for 1 wk at room temperature in buffered formaldehyde solution (10% wt/vol in PBS), samples were dehydrated in graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ). Thereafter, 7-μm sections were deparaffinized with xylene, stained with hematoxylin-eosin, and observed with a Axostar Plus equipped with AxioCam MRC (Zeiss, Milan, Italy) and studied via an Imaging computer program (AxioVision, Zeiss).

Immunohistochemical localization of TJ and AJ proteins. After deparaffinization, for ZO-1 and occludin detection, slices were treated with protease (type XIV, Sigma) (2 mg/ml) for 10 min at 37°C. Detection of claudin-2 and E-cadherin was carried out after boiling in 0.01 M citrate buffer for 4 min. Endogenous peroxidase was quenched with 0.3% (vol/vol) hydrogen peroxide in 60% (vol/vol) methanol for 30 min. Nonspecific adsorption was minimized by incubating the section in 2% (vol/vol) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with biotin and avidin (DBA, Milan, Italy), respectively. Sections were incubated overnight with 1) polyclonal rabbit anti-occludin antibody (1:100 in PBS, wt/vol), 2) anti-ZO-1 (1:100 in PBS, wt/vol), 3) monoclonal mouse anti-claudin-2 antibody (1:100 in PBS, wt/vol), and 4) anti-cadherin (1:100 in PBS, wt/vol) (all antibodies from Zymed, San Francisco, CA). Sections were washed with PBS and incubated with the secondary antibody. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA). The counterstain was carried out with nuclear fast red (red background). All sections were obtained using light microscopy (Axostar Plus equipped with AxioCam MRC, Zeiss) and studied via an Imaging computer program (AxioVision, Zeiss).

Total protein extraction and Western blot analysis. Tissue samples from the terminal ileum were homogenized with an Ultra-turrax T8 homogenizer in a buffer containing 20 mM HEPES pH 7.9, 1.5 mM MgCl₂, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 1.5 μg/ml trypsin inhibitor, 3 μg/ml pepstatin, 2 μg/ml leupeptin, 40 μM benzamidine, 1% NP-40, 20% glycerol. The homogenates were centrifuged (13,000 rpm, 15 min, at 4°C), the supernatant was collected to evaluate contents.

Protein concentration was determined with the Bio-Rad protein assay kit. Proteins were mixed with gel loading buffer (0.5 mM Tris, 10% wt/vol SDS, 10% wt/vol glycerol, 10% vol/vol 2-mercaptoethanol, 2 mg/ml bromophenol), boiled for 5 min, and centrifuged at 10,000 rpm for a few seconds. Protein concentration was determined and equivalent amounts (50 μg) of each sample were electrophoresed in a 12% (wt/vol) discontinuous polyacrylamide minigel. Proteins were separated electrophoretically and transferred to nitrocellulose membranes. For immunoblotting, membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) for 1 h and then incubated with primary antibodies against occludin (1:1,000), ZO-1 (1:500), claudin-2 (1:500) (all from Zymed Laboratories, Milan, Italy), and β-actin (1:5,000) (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The membranes were washed three times for 10 min in TBS with 0.1% Tween 20 and incubated with AffiniPure goat anti-rabbit IgG coupled to peroxidase (1:5,000). The immune complexes were visualized by using the SuperSignal West Pico chemiluminescence substrate (Pierce). The secondary antibody was obtained from Jackson Immuno Research Laboratories (San Francisco, CA).

Data presentation and statistics. Data of circulating TNF concentrations, the macroscopic damage score, and the densitometric units of Western blots are given as mean values ± SE; comparison was made with the Mann-Whitney test and Bonferroni’s correction; a P value <0.025 was considered significant.

RESULTS

Macroscopic damage score and serum TNF-α are effectively reduced by anti-TNF treatment. DNBS colitis led to high circulating TNF-α levels at 3 and 6 h postinduction (3 h: 149 pg/ml ± 8; 6 h: 100 pg/ml ± 2) that were effectively reduced by IFX (3 h: 42 pg/ml ± 2, P < 0.01; 6 h: 67 pg/ml ± 6, P < 0.01 vs. DNBS alone) and at 6 h by ETC (3 h: 110 pg/ml ± 9, n.s.; 6 h: 39 ± 9, P < 0.01 vs. DNBS alone). The macroscopic damage score was similar in all groups at 3 h (DNBS: 1.4 ± 0.2; IFX: 1.2 ± 0.2; ETC: 1.9 ± 0.4; TNFR-1−/−: 0.8 ± 0.1). At 6 h, the damage score was not significantly different in knockout mice for TNFR-1 compared with untreated colitis but reduced in both treatment groups (DNBS: 2.2 ± 0.3; IFX: 1.1 ± 0.4 and ETC: 0.9 ± 0.4, P < 0.05 both; TNFR-1−/−: 1.4 ± 0.4).

Fate of functional proteins of the terminal ileum in experimental colitis and effects of TNF-α inhibition and the functional absence of TNFR-1. In control animals occludin (Fig. 1, A and B) was localized at both levels, villus tips and crypts, in the upper part of the enterocytes at the sites of cell–cell contact corresponding to the TJ complexes. Basal membrane staining intensively positive for occludin. Sham colitis with or without treatment with IFX or ETC did not induce any change at any time point. Three hours after administration of DNBS-ethanol, occludin was virtually lost from its localization to ileal TJ complexes. However, the cytoplasm of ileal enterocytes showed a diffused speckled positivity for occludin. Moreover, enterocyte nuclei stained positive for occludin. Occludin localization at the basal membrane was not affected by colitis (Fig. 1C).

At 6 h, the signal of occludin was present within the nuclei of the enterocytes together with a weaker but still visible presence within the cytoplasm (Fig. 1D). ETC and IFX abolished completely the dislocation of occludin from the TJs at 3 h and at 6 h with a normal honeycomb distribution at the apical part of enterocytes (Fig. 1, E–H). Similarly, in TNFR-1−/− mice the normal distribution of occludin was conserved at 3 h and at 6 h after colitis induction (Fig. 1, I and J).
On Western blot analysis, ileal protein concentrations of occludin (Fig. 2, A and B) were significantly reduced in mice with colitis at 3 h and at 6 h ($P < 0.001$). Both treatments, ETC and IFX, as well as the absence of the receptor 1 of TNF-α, prevented the reduction of protein expression at 3 and at 6 h.

The TJ protein ZO-1, normally distributed at the sites of cell-cell contact under basal conditions (Fig. 3, A and B), behaved in an identical manner compared with occludin at 3 h and at 6 h (virtual absence from TJ) in animals with colitis; at 3 h and at 6 h (Fig. 3, C and D). ETC treatment (Fig. 3, E and

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but not IFX (Fig. 3, G and H), did prevent dislocation of ZO-1. Sham colitis with or without treatment with IFX or ETC did not induce any change at any time point for ZO-1. ZO-1 was not altered in TNFR-1/−/− mice with colitis at both time points (Fig. 3, I and J).

On Western blot analysis, ileal protein concentrations of ZO-1 (Fig. 4, A and B) were significantly reduced in mice with colitis at 3 h and at 6 h (P < 0.001) compared with controls. Both treatments, ETC and IFX, prevented the reduction of protein expression at 3 and at 6 h.

Claudin-2 (Fig. 5, A1 and B) was not expressed under basal conditions. As indicated in the manufacturer’s data sheet, a control colon served as positive control for claudin-2 (as shown in Fig. 3 A2). At 3 and 6 h after colitis induction, the lower half of the mucosal compartment stained positive for claudin-2. This positivity was completely absent at 3 h in colitis treated with ETC (Fig. 5E) but was only incompletely prevented by IFX treatment (Fig. 5G) where claudin-2 was detectable in the lower third of the mucosa. At 6 h claudin-2 was not found with either treatment, ETC and IFX (Fig. 5, F and H). Sham colitis with or without treatment with IFX or ETC did not induce any change at any time point for claudin-2. In TNFR-1/−/− mice with colitis claudin-2 was not detectable at 3 and at 6 h (Fig. 5, I and J).

On Western blot analysis, protein concentration of claudin-2 (Fig. 6, A and B), normally not present under basal conditions, was expressed only in mice with colitis compared with treated or untreated sham colitis. In mice with colitis treated with IFX or ETC or in TNFR-1/−/− mice with colitis, claudin-2 was virtually absent at 3 and at 6 h.

The AJ-associated E-cadherin was not influenced by colitis at any time point (data not shown) nor by any type of treatment and in TNFR-1/−/− mice.

DISCUSSION

In the present experiment we showed for the first time in a whole organism the short-term effects of DNBS colitis on different proteins of the junctional complexes of the ileal enterocytes, underlining the reactivity and the plasticity of...
these structures. Additionally, to define the mediation of these alterations, we studied the effects of two commercially available anti-TNF treatment strategies and the lack of the receptor 1 for TNF.

**Fate of TJ proteins occludin and ZO-1.** As early as 3 h after colitis induction, junctional occludin and ZO-1 disappeared from their localization at cell-cell contacts together with their appearance within the cytoplasm and the enterocyte nuclei. These changes were somewhat more pronounced after 6 h. Both treatment regimens, as well as the lack of TNFR-1, prevented completely the observed alterations of occludin, whereas ZO-1 was preserved only by ETC.

From the available literature it is unclear which intracytoplasmic pathway leads to the activation of MCLK and finally to disruption of these TJ proteins. TNF-α acts on nuclear factor kappa B (NF-κB) and on activator protein-1 and the preferential pathway seems to be differentiation dependent (11). In experiments employing TNF-α alone, the inhibition of NF-κB or the silencing of NF-κB p65 prevented the TNF-α-induced increase of MLCK promoter activity in Caco-2 cells (41), whereas, in another setting using both TNF-α and IFN-γ added basolaterally, inhibition of NF-κB did not prevent barrier dysfunction (38). In Caco-2 cells transfected with a constitutively active MLCK, a separation of ZO-1 and occludin from the perijunctional F-actin has been reported (28). Such dislocation has been also described in the sigmoid mucosa from patients with inactive CD (21). Both MLCK protein expression and the phosphorylation of the myosin light chain have been investigated recently in intestinal epithelia of patients with CD and UC (2), and it has been shown that MLCK expression was...
increased in the ileal epithelium in active as well as in inactive CD paralleling disease activity, whereas in UC colonic MLCK expression increased only with moderate to severe activity of disease.

In several cell lines the effect of TNF-α, either alone or in conjunction with or sequentially to IFN-γ, has been investigated, and it has been shown that TNF-α alone induces a decrease of transepithelial resistance of Caco-2 cells at 24 h (17) or at 4 h when administered (to the basal compartment) together with IFN-γ (38). The reduction of barrier function was accompanied by a reduction of ZO-1 (17) protein expression, or without this reduction (38), but always with a loss of ZO-1 localization at the TJ. In a very elegant setting Wang et al. (39) showed that incubation of CaCo-2 cells with IFN-γ upregulates the expression of both receptors for TNF, namely TNFR-1 and TNFR-2, and their localization to the basolateral membrane. In this experiment barrier dysfunction was prevented by blocking TNFR-2, whereas blocking TNFR-1 did not confer any protection to TNF-induced barrier dysfunction. These data are in contrast to our findings obtained in TNFR-1−/− mice that colitis-induced effects are absent in mice lacking the type I receptor of TNF-α. However, in our experiments the total absence of TNFR-1 may have influenced the local amplification of the signal by the absence of other contributing factors (e.g., LIGHT).

Very little is known of the further fate of TJ proteins after MLCK activations. Some recent data suggest that these proteins are endocytosed in distinct caveolae-mediated vesicles to be reutilized or degraded (15, 29, 30). In the present study we showed a very early disappearance of ileal occludin and ZO-1 from the TJs, together with their appearance within the cytoplasm and within the nuclei of enterocytes. Cytoplasmic localization most likely represents the removal of TJ-associated proteins through the aforementioned endocytosis pathways, whereas nuclear expression of occludin most likely represents some form of repair. This endocytosplasmic pathway has been shown for occludin and ZO-1 in IFN-γ-primed Caco-2 monolayers incubated with TNF-α (38); thus the cytoplasmic appearance of occludin and ZO-1 in our study seems to fit with this hypothesis.
Fig. 5. Claudin-2 was not expressed under basal conditions in the ileal epithelium at 3 and at 6 h after induction of sham colitis (A1 and B). Staining of the colon epithelium served as positive control (A2). At 3 and 6 h after colitis induction with DNBS-ethanol the lower half of the mucosal compartment of the ileum stained positive for claudin-2 (C and D). This positivity was completely absent at 3 h in colitis treated with ETC (E) but only incompletely prevented by IFX treatment (G) where claudin-2 was detectable in lower third of the mucosa. At 6 h claudin-2 was not found with either treatment, ETC (F) or IFX (H). In TNFR-1−/− mice with colitis claudin-2 was not expressed at any time point (I and J).
Ileal occludin and ZO-1 protein concentrations were reduced in our experiment, which is consistent with the findings by immunohistochemistry. From former studies in rats we know that at least ZO-1 protein content returns to normal within 12 h after colitis induction (20).

The only paper published on effects induced by IFX on human colon TJ proteins in CD patients did not show differences in occludin, claudin-1, and claudin-4 expression induced by treatment, but morphological data were not shown (42). Comparison with our data, however, is difficult because treatment was carried out in a state of established chronic inflammation whereas in our setting an acute effect on TJ proteins was investigated.

Appearance of the TJ protein claudin-2. Claudin-2 was first described in 1998 (7). It localizes to TJs and a “pore-forming” property by inducing cation-selective channels has been attributed to it as it has been shown in MDCK cells (1, 8). An enhanced expression of claudin-2 in DNBS colitis has never been shown before. Available data report on a colonic claudin-2 expression in human ulcerative colitis, in Crohn’s colitis, and in cell lines treated with IL-13 (12, 23, 43) or IL-17 (24), whereas normal human colon epithelium does not express claudin-2. Incubation of T84 cells with 1 ng/ml TNF-α and 100 ng/ml IFN-γ has been shown to reduce claudin-2 expression, whereas IL-13 increases its expression (23). On the other hand, treatment with only TNF-α (100 ng/ml) added to the basolateral compartment stimulates its synthesis in HT29/B6 cells (43). This apparent contradiction may be due to the different cell lines or to the different experimental setting (apical vs. basolateral). In our in vivo model the increase of claudin-2 was inhibited completely by ETC and at 6 h also by IFX, emphasizing the importance of TNF-α. With respect to the other TJ proteins investigated in the present setting, occludin and ZO-1, activation of MLCK seems not to be involved in claudin-2 expression (2). In the present experiment where we used a Th-1 model, claudin-2 was expressed as early as 3 h after colitis induction especially in the lower half of the ileal mucosa. Ileal expression of claudin-2 as well as its localization to TJs was absent in control animals whereas it was expressed in crypts of colon epithelium (positive control). In the intestinal epithelium
of healthy rats claudin-2 has been found in the epithelium of the jejunum and ascending colon with a decreasing gradient from crypts to the villus surface cells (24). This gradient from dividing cells who express claudin-2 to differentiated cells at the epithelial surface was also reported in fetal human colon (6). In our experiment the colitis-associated increase of ileal claudin-2 expression was prevented by ETC at both time points with no sequence similarity to occluding. J Cell Biol 14: 1539–1550, 1998.


