Altered Expression, Localization, and Phosphorylation of Epithelial Junctional Proteins in Celiac Disease

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Abstract

We aimed to study the expression and localization of the molecular components of enterocyte junctions in celiac disease together with the level of tyrosine phosphorylation, a phenomenon known to affect their cellular distribution and function, and to explore the influence of proinflammatory cytokines. Duodenal biopsy specimens from patients with celiac disease and control subjects were used for immunoprecipitation, immunoblotting, and immunolocalization by using antioccludin, anti–zonula occludens (ZO)-1, anti–E-cadherin, anti–β-catenin, and antiphosphotyrosine antibodies. The same procedures were carried out on filter-grown Caco-2 cells incubated in the absence or presence of interferon γ and tumor necrosis factor α. In active celiac disease, the absence of a phosphorylated ZO-1 and the extensive phosphorylation of β-catenin might be responsible for the absence of membranous localization of occludin and E-cadherin, respectively. The in vitro system showed an influence of the cytokines on the assembly of these complexes that proved the opposite to celiac samples as far as tight junctions were concerned because the presence of a phosphorylated ZO-1 enables occludin to localize in the membrane.

Maintenance of the small intestine architecture and function requires close coordination between enterocyte proliferation and apoptosis,[1] which both are highly dependent on cell-cell and cell-matrix interactions.[2] Enterocytes are joined to each other by tight junctions (TJs) and adherens junctions (AJs), 2 specialized multimolecular complexes working as an integrated functional unit termed the *apical junctional complex* (AJC), which controls the cellular polarity and the paracellular permeability.[3]

TJs represent the most apical cell-cell contact formed by integral membrane proteins, mainly occludin and claudins, which have 4 transmembrane domains generating 2 extracellular loops that provide the sites for the intercellular interaction.[4] On the other hand, their N- and C-terminal ends are located in the cytoplasm where the latter serves as a binding site for a complex set of plaque proteins called zonula occludens (ZO)-1, ZO-2, and ZO-3, which are linked to the underlying actin cytoskeleton.[5,6]

Invariably close to the TJs are the AJs, which form a continuous circumferential zone of contact at the lateral membrane and whose integrity is essential for cell-cell recognition and cell sorting, such as for the development of TJs because only after AJ assembly does ZO-1 become able to migrate apically to join occludin.[7,8] The main molecular component of the AJ is E-cadherin, a transmembrane protein with 5 extracellular domains that interdigitate with those of an adjacent cell in a calcium-dependent, homophilic manner to form a continuous linear "zipper" structure.[3]

To exhibit functional adhesion activity, E-cadherin needs to be connected to the actin cytoskeleton by a cytoplasmic complex that includes α-, β-, or γ-catenin in a mutually exclusive manner.[9] The assembly of the molecular components of

the AJC is dependent on the level of their phosphorylation on specific residues of tyrosine.[10,11] In the TJs, the greater the degree of phosphorylation of ZO-1, the higher its ability to join occludin, localizing it in the apical part of the membrane and binding it to the cytoplasmic catenins; in contrast, in the AJs, the greater the degree of phosphorylation of β-catenin, the lower its ability to bind E-cadherin. Furthermore, extracellular signals such as epidermal growth factor, transforming growth factors, trefoil peptides,[12] T-helper (Th)-1 cytokines, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α[13,14] are able to regulate intercellular permeability.

In inflamed gut, these junctional complexes undergo a disruption leading to a breaking of the intestinal barrier.[15] This is the case in celiac disease (CD),[16,17] an autoimmune enteropathy caused by the activation of innate and adaptive immune responses as a consequence of the ingestion of dietary gluten and related prolamines in genetically susceptible people,[18,19] which is characterized by an increased production of Th-1 cytokines,[20] hyperactivation of matrix metalloproteinases,[21] and exaggerated enterocyte apoptosis.[22-24]

To our knowledge, complete and simultaneous evaluation of TJ and AJ molecular components in CD has not been performed, nor is information available about the level of their phosphorylation. The aim of the present study, therefore, was to analyze the expression and localization of the occludin–ZO-1 and E-cadherin–β-catenin complexes together with the level of their tyrosine phosphorylation directly in CD mucosa in comparison with healthy mucosa. In addition, an in vitro model of intestinal epithelium, represented by filter-grown Caco-2 cells, was used to study the effect of the 2 main proinflammatory cytokines, IFN-γ and TNF-α, on the integrity of intercellular junctions. Our findings strongly support the idea that an alteration of the tyrosine phosphorylated status of the cytoplasmic components of TJs and AJs is responsible for the disruption of the AJC observed in CD and that probably, proinflammatory cytokines are not the only culprits in such an alteration.

Materials and Methods

Biopsy Specimens

Multiple endoscopic biopsy specimens were obtained from the second part of the duodenum in 11 patients with untreated CD (6 women; mean age, 37.3 years; range, 21-65 years), 12 patients with CD who had been following a gluten-free diet for at least 12 months (7 women; mean age, 33.9 years; range, 19-66 years), and 12 consenting subjects (7 women; mean age, 37.1 years; range, 23-57 years) undergoing upper gastrointestinal endoscopy for functional dyspepsia. All cases of CD were diagnosed on the basis of commonly accepted histologic and serologic criteria.[25] In all untreated patients, a subsequent biopsy specimen, obtained after at least 12 months of gluten-free diet, showed a significant improvement of the lesions. The study was approved by the local ethics committee, and each patient gave informed consent for participation in the study. For each patient and control subject, 2 well-oriented biopsy specimens were processed according to standard methods for conventional histologic examination, 2 biopsy specimens were frozen at –80°C, and 2 additional biopsy specimens were included in OCT compound Tissue-Tek (Sakura Finetek, Torrance, CA).

Cell Culture

Human intestinal Caco-2 cells were supplied by Alain Zweibaum, MD (INSERM, Villejuif, Paris, France), and were cultured (90th to 100th passages) in a complete medium on polyethylene terephthalate membrane transwell supports (Becton Dickinson, San Jose, CA; 1.13 cm^2 area, 0.4 μm pore diameter). This procedure makes it possible to separate the apical medium in the upper chamber from the basolateral medium in the lower chamber and mimics the barrier function of the intestinal epithelium. It is remarkable that this experimental condition allows the formation of a monolayer of highly polarized cells that, after confluence, progressively manifest all of the phenotypic characteristics of small bowel enterocytes.[26] At the 18th postconfluence day, cells were incubated for 48 hours in the presence or absence, in the lower chamber, of 1,000 U/mL of IFN-γ, 50 ng/mL of TNF-α, or IFN-γ plus TNF-α (R&D Systems, Minneapolis, MN). At the end of the incubation, Caco-2 cells from each filter were evaluated morphologically by using light microscopy for the presence of apoptotic features such as nuclear fragmentation and cellular swelling. Quantification was performed at a constant magnification (×1,000) by a differential count of at least 400 cells and the result expressed as a percentage of cells with features of programmed cell death. Thereafter, at least 8 samples from each group were frozen at –80°C, and 4 additional samples were used for immunofluorescence study.

Immunoprecipitation and Immunoblotting

For immunoprecipitation, frozen duodenal tissue samples or Caco-2 cells were solubilized and homogenized, and the protein concentration was assayed by the bicinchoninic acid procedure (Pierce, Rockford, IL). Mucosal and cellular lysates, each containing 2.5 mg of protein for the detection of the occludin–ZO-1 complex or 1 mg of protein for the detection of the E-cadherin–β-catenin complex, then were precleared for 2 hours at 4°C with 25 μL of protein G–sepharose "4 fast flow" (Pharmacia Biotech, Uppsala, Sweden) and centrifuged. The lysates were precipitated overnight at 4°C with 6 μg of polyclonal rabbit anti–ZO-1 or 4 μg of monoclonal mouse anti–β-catenin antibodies (Zymed Laboratories, San Francisco, CA) and 25 μL of protein G–sepharose.

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After collection, the immune complexes were resuspended and submitted to 5% or 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis for ZO-1 or for β-catenin, E-cadherin, occludin, and phosphotyrosine, respectively. Proteins were transferred electrophoretically to nitrocellulose sheets according to the procedure described by Towbin et al.[27] The membranes were incubated (90 minutes at room temperature) with anti–ZO-1, anti–β-catenin, monoclonal mouse anti–E-cadherin, monoclonal mouse antioccludin, or monoclonal mouse antiphosphotyrosine antibodies (dilution 1:100, 1:300, 1:250, 1:150, or 1:300, respectively; Zymed Laboratories) in tris(hydroxymethyl)amino-methane-buffered saline (TBS) containing 1% nonfat milk powder. Nonspecific binding of the membranes previously was blocked with 5% nonfat milk powder in TBS, 0.1%, and polysorbate-20 at 4°C overnight.

Following incubation with an antimouse IgG-HRP (dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA) or antirabbit IgG-HRP (dilution 1:4,000; Santa Cruz) secondary antibodies, the protein-antibody complexes were visualized by using an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL). Negative control blots were obtained by omitting the primary antibody. Bands were quantified by scanning densitometry using an LKB Ultrascan XL Laser Densitometer (Kodak, Hemel Hempstead, England) and measured in terms of area of each band or as the sum of the areas when more than 1 band was present.

Confocal Immunofluorescence Microscopy

Cryostat serial sections (4 μm) of OCT-embedded endoscopic specimens from patients with CD, biopsy specimens from control subjects, and samples from cell cultures were mounted on poly-L-lysine–coated slides. Sections first were fixed in 3.7% paraformaldehyde for 10 minutes at 4°C and, after rinsing in phosphate-buffered saline (PBS) containing 0.2% glycine, were permeabilized with 0.2% Triton X-100 (Sigma Chemical, St Louis, MO) for 15 minutes and incubated for 1 hour at 37°C with the anti–E-cadherin antibody (dilution 1:50 in PBS 1% bovine serum albumin).

To study occludin localization, serial sections first were fixed in ethanol for 30 minutes at 4°C and then in cold acetone for an additional 3 minutes at room temperature. They then were incubated with the antioccludin antibody (dilution 1:50 in PBS 1% bovine serum albumin).

To study ZO-1 and β-catenin localization, samples were fixed in methanol for 10 minutes at –20°C and incubated for 1 hour at 37°C with anti–ZO-1 or anti–β-catenin (both, dilution 1:50 in PBS 1% bovine serum albumin). After washing with PBS, the slides were incubated with a fluorescein isothiocyanate (FITC)-conjugated goat antimouse or Cy3-conjugated goat antirabbit IgG (both, dilution 1:50 in PBS containing 10% normal goat serum; Zymed).

To study occludin–ZO-1 colocalization, tissue samples first were fixed in ethanol and then in acetone and incubated with the antioccludin antibody and subsequently with the FITC-conjugated secondary antibody; after extensive washing, the slides were incubated with the anti-ZO-1 antibody and the Cy3-conjugated secondary antibody. Double staining for the colocalization of AJ molecular components had not been performed owing to the same source of the primary antibodies.

After appropriate rinsing, the coverslips were mounted on slides in an aqueous medium, and the slides were examined under a confocal scanning laser microscope (SARASTRO 2000, Molecular Dynamics, Sunnyvale, CA). The light source was an argon ion laser (25 mW) giving excitation wavelengths in the region of 458 to 584 nm. FITC was excited at 495 nm and Cy3 at 550 nm. Digital images of single optical sections were acquired using a 63× Nikon Plan Apo objective (Nikon, Tokyo, Japan); image sizes were 512 × 512 (pixel size, 0.17 μm). For vertical sections, voxel dimensions were 0.17 μm lateral and 0.19 μm axial. Negative control cover slides were set by exposing the serial sections under similar conditions but without the primary antibody.

Statistics

Statistical analysis by the Student *t* test was performed using the StatPac Computerized Program (StatPac, Bloomington, MN), and a *P* value of less than .05 was used as the significance criterion. The correlations were examined by using the Spearman rank correlation test.

Results

Tight Junctions

Figure 1A shows that a 220-kd band corresponding to ZO-1 was present in the mucosal samples of control subjects and untreated and treated patients with CD, without quantitative differences as shown by densitometric analysis of all samples. The blotting of the ZO-1-immunoprecipitates with the antioccludin and antiphosphotyrosine antibodies revealed the almost total absence of the corresponding bands (65 and 220 kd, respectively) in untreated CD ($P < .0001$ vs treated patients with CD and control subjects for both proteins). Furthermore, in active CD, the expression of occludin directly correlated with that of tyrosine-phosphorylated ZO-1 ($r_s = 0.72$). However, when considering the total (bound and unbound) level of occludin in the same mucosal samples, no differences were observed among the 3 groups **Figure 1B**.

These results are consistent with those obtained by confocal microscopy **Figure 1C** showing that in normal mucosa, ZO-1 fluorescence (red) stained the lateral membrane of enterocytes and occludin (green) was evident only at the apical level of the lateral membrane, where the double

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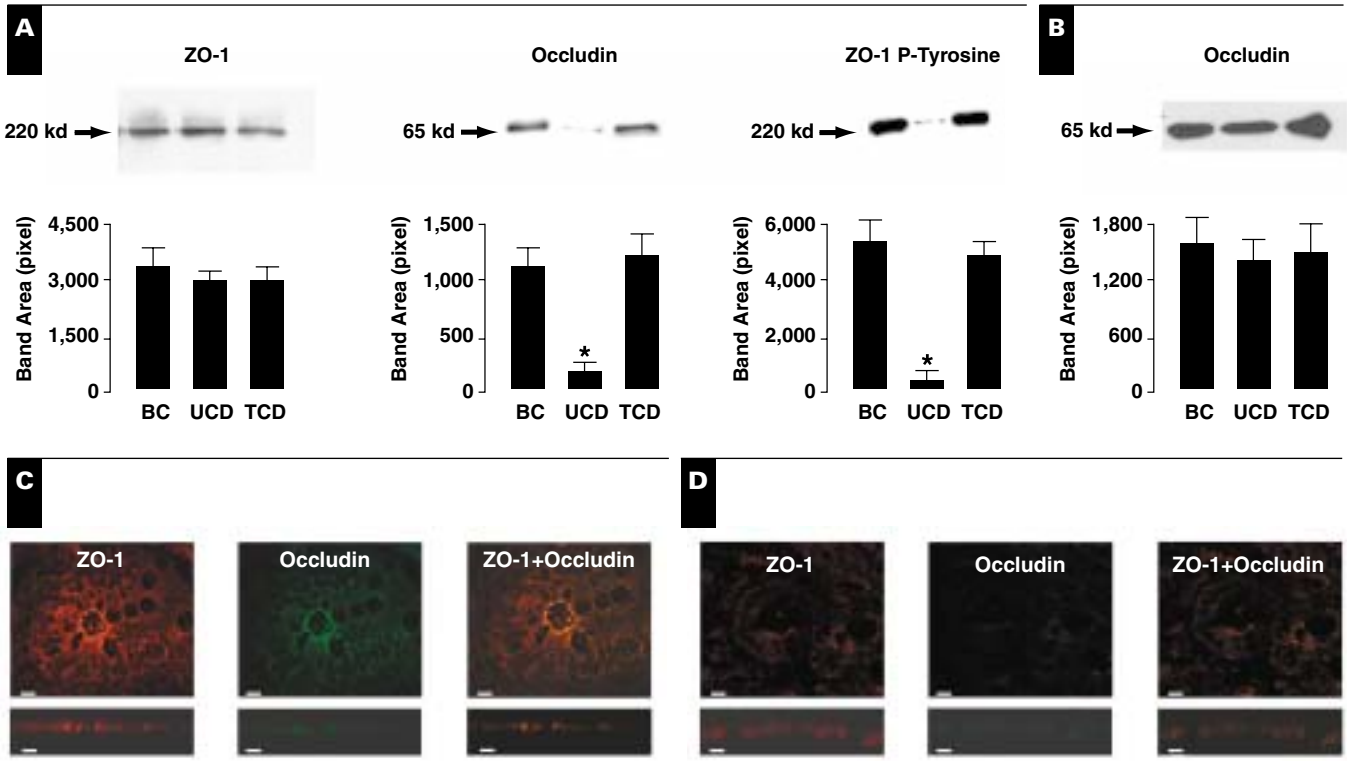

Figure 1 **A**, Western blot and densitometric analysis of immunoprecipitates obtained by the anti–zonula occludens (ZO)-1 antibody on duodenal biopsy specimens from patients with untreated celiac disease (UCD), treated celiac disease (TCD), and biopsied control specimens (BC). A 220-kd band (arrow) was evident in all cases, and the quantitation demonstrated the presence of similar levels in the 3 groups examined. Blotting of these immunoprecipitates with the antioccludin and antiphosphotyrosine antibodies revealed the absence of the corresponding bands (65 and 220 kd, respectively, as indicated by the arrows) in UCD. **B**, When considering the total (bound and unbound) level of occludin in the same mucosal samples, no differences among the 3 groups were evident. Values are given as mean ± SD. * $P < .0001$ vs BC and TCD. **C**, Confocal images of the crypt region of a mucosal biopsy specimen of one representative BC case incubated with the anti–ZO-1 antibody, the antioccludin antibody, and the corresponding double fluorescence, demonstrating colocalization of these 2 molecules at the apical portion of the lateral enterocyte membrane. The marker corresponds to a length of 10 μm. At the bottom, the relative vertical sections are shown. **D**, Confocal images of the crypt region of a mucosal biopsy specimen of one representative case of UCD incubated with the anti–ZO-1 antibody, the antioccludin antibody, and the corresponding double fluorescence, demonstrating the almost total absence of occludin and of a colocalization of the 2 molecules. The marker corresponds to a length of 10 μm. At the bottom, the relative vertical sections are shown. P-tyrosine, phosphotyrosine.

staining (yellow) was indicative of the colocalization of the 2 molecules. In untreated CD **Figure 1D**, the ZO-1 fluorescence was weaker and more diffuse than in normal mucosa, whereas occludin was almost completely absent and, consequently, double staining showed only minimal yellow fluorescence. The pattern of colocalization observed in treated CD did not differ from that in control subjects (data not shown).

To determine whether Th-1 proinflammatory cytokines were able to modulate the expression and localization of the molecular components of TJ, cultures of Caco-2 cells were incubated in the absence or presence of IFN-γ, TNF-α, or both. Immunoprecipitation and blotting of cellular lysates with the anti–ZO-1 antibody **Figure 2A** revealed a significant decrease of the relative band area in all cytokine-treated samples in comparison with the untreated, paralleled by a concomitant significant increase of its tyrosine phosphorylation ($P < .001$ vs cytokine-treated samples for both proteins). In fact, the levels of the 2 coimmunoprecipitated proteins showed a significant inverse correlation ($r_s = –0.70$). In contrast, no difference was found in occludin expression after incubation with cytokines as concerns the portion bound to ZO-1 (Figure 2A) and the total (bound and unbound) amount of protein **Figure 2B**.

In **Figure 2C**, confocal microscope analysis of Caco-2 cells showed that in cytokine-free conditions, occludin and ZO-1 gave membranous staining, particularly evident at vertical sections. On the other hand, in cytokine-treated samples, a

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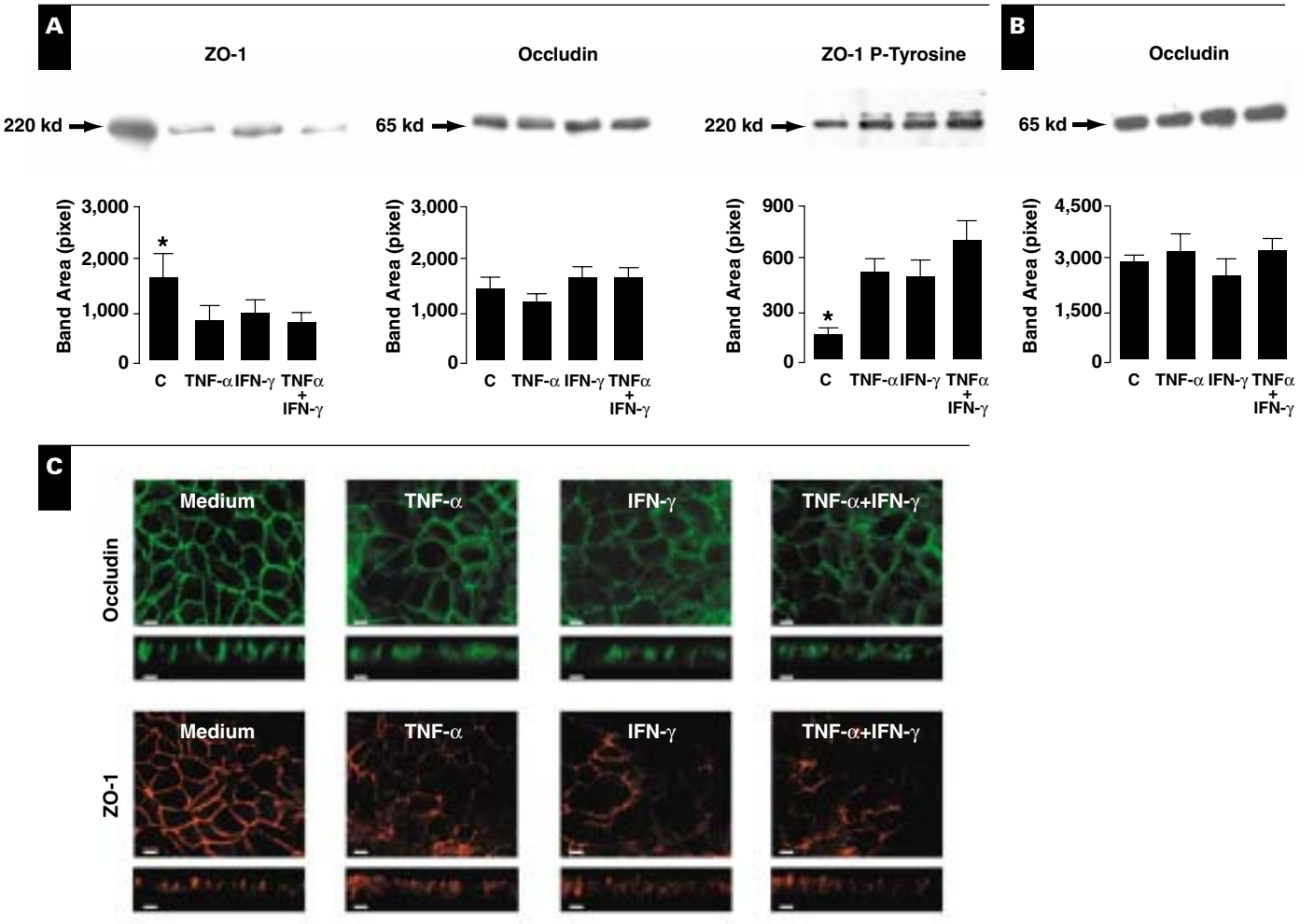

Figure 2 **A**, Western blot and densitometric analysis of immunoprecipitates obtained by the anti–ZO-1 antibody of Caco-2 cellular samples incubated in the absence or presence of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, or both. A 220-kd band (arrow) was evident in all cases, even though the quantitation analysis demonstrated that in all cytokine-treated samples, there was a significant decrease of protein expression. Blotting of these immunoprecipitates with the antiphosphotyrosine antibody revealed a statistically significant increase of the corresponding band (220 kd as indicated by the arrow) in all treated samples. Conversely, blotting with the antioccludin antibody (65-kd band as indicated by the arrow) showed no modification within the 4 groups examined within the immunoprecipitates (**A**) or the total cellular lysates (**B**). Values are given as mean ± SD. * $P < .001$ vs cytokine-treated samples. **C**, Confocal images of Caco-2 cells cultured on suitable filters for 3 weeks, a period that allows the formation of a highly polarized cell monolayer, and incubated for 48 hours in the absence or presence of TNF-α, IFN-γ, or both. In cytokine-free samples, ZO-1 and occludin showed membranous staining, whereas the cytokine-treated samples showed a reduction of membranous staining, even though staining was less intense for occludin, together with a relative increase of their cytoplasmic pool. The marker corresponds to a length of 10 μm. At the bottom, the relative vertical sections are shown. P-tyrosine, phosphotyrosine.

reduction of their membrane positivity was observed, even though it was less intense for occludin, together with a relative increase of their cytoplasmic pool, as clearly evident in the vertical sections.

Adherens Junctions

Figure 3A shows that the expression of β-catenin in duodenal mucosal biopsy samples from control subjects and untreated and treated patients with CD was similar, as confirmed by densitometric analysis of all samples. However, β-catenin turned out to be extensively tyrosine phosphorylated in untreated CD ($P < .0001$ vs patients with treated CD and control subjects), and the blotting of β-catenin immunoprecipitates with the anti–E-cadherin antibody showed a statistically significant reduction of E-cadherin expression in untreated CD in comparison with the other 2 groups ($P < .001$ vs patients with treated CD and control subjects), even though its total amount did not differ among the 3 groups **Figure 3B**.

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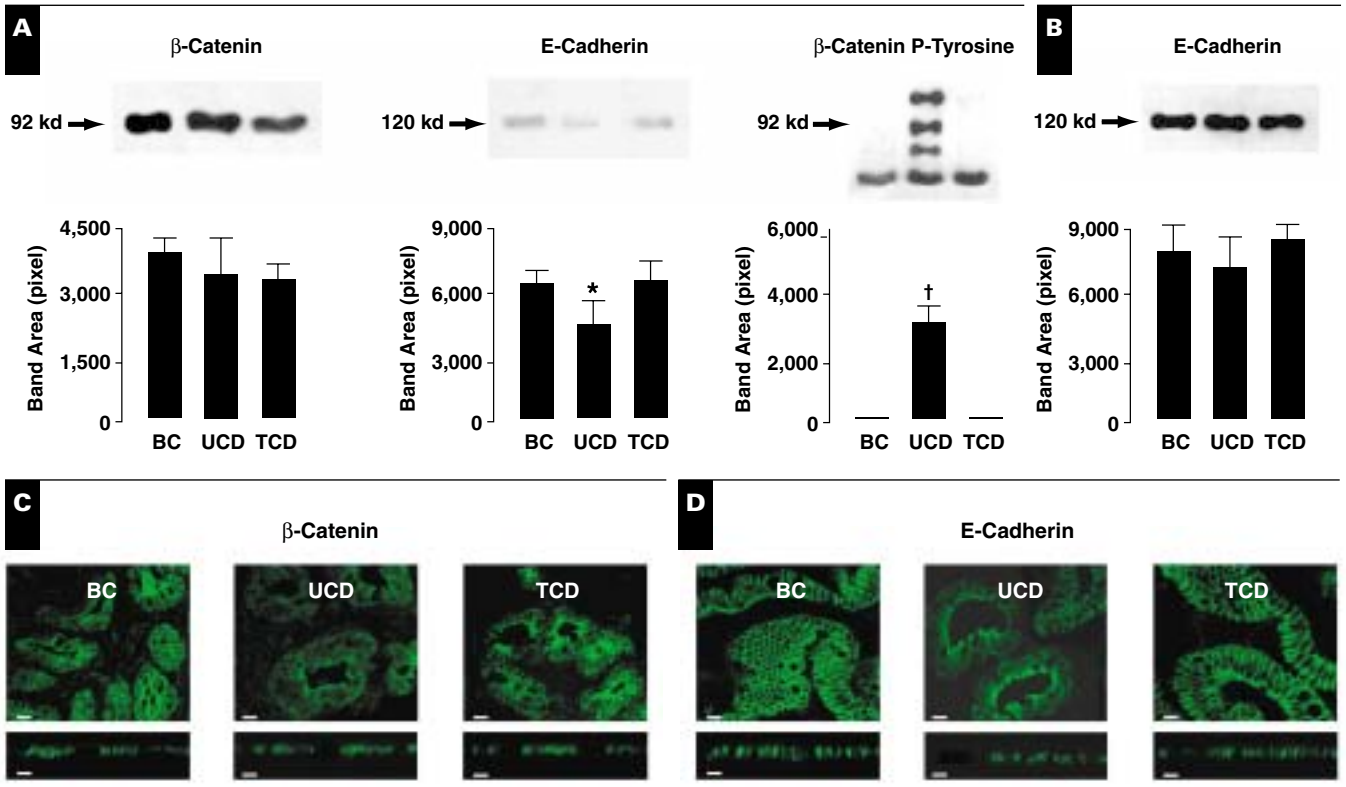

Figure 3 **A**, Western blot and densitometric analysis of immunoprecipitates obtained by the anti–β-catenin antibody on duodenal biopsy specimens of untreated celiac disease (UCD), treated celiac disease (TCD), and biopsied control samples (BC). A 92-kd band (arrow) was evident in all cases without significant differences among the 3 groups. Blotting of these immunoprecipitates with the antiphosphotyrosine antibody revealed that only in UCD samples was there an extensive tyrosine-phosphorylation of β-catenin (92-kd band as indicated by the arrow), which was completely absent in the other 2 groups. Blotting of the β-catenin immunoprecipitates with the anti–E-cadherin antibody demonstrated the presence of a decreased amount of protein in the UCD group. * $P < .001$. † $P < .0001$ vs BC and TCD. **B**, In contrast, when considering the total (bound and unbound) level of E-cadherin, similar amounts were found in the 3 groups in the same mucosal samples in all samples as indicated by densitometric analysis. Values are given as mean ± SD. **C**, Confocal images of the crypt region of mucosal biopsy specimens of 1 representative case each of BC, UCD, and TCD incubated with the anti–β-catenin antibody. At the bottom, the corresponding vertical sections are shown. **D**, Confocal images of serial sections of the precedent cases incubated with the anti–E-cadherin antibody are shown. The marker corresponds to a length of 10 μm. At the bottom, the corresponding vertical sections are shown, in which the differences in the enterocyte height are evident. P-tyrosine, phosphotyrosine.

Confocal microscopic analysis demonstrated that in normal mucosa, β-catenin–related **Figure 3C** and E-cadherin–related **Figure 3D** fluorescence was intense and localized at the enterocyte lateral membrane, as better demonstrated in vertical sections. In untreated CD, the membranous staining of β-catenin and E-cadherin was reduced with a redistribution over the entire cell surface and into the cytoplasm, as clearly evident in the vertical sections. In treated CD, the localization and the intensity of fluorescence of β-catenin and E-cadherin were similar to those of control mucosa.

The experiments performed by using the Caco-2 cell line, as summarized in **Figure 4**, showed that immunoprecipitation and blotting of lysates with the anti–β-catenin antibody revealed 2 bands, the second probably corresponding to a degraded form of the protein, without any quantitative difference between the cytokine-treated and cytokine-untreated samples (Figure 4A). The subsequent blotting of these immunoprecipitates with the anti–E-cadherin antibody demonstrated a significant reduction of E-cadherin expression in cytokine-treated samples ($P < .001$), whereas their blotting with the antiphosphotyrosine antibody revealed that cytokines increased the tyrosine-phosphorylated status of β-catenin in a statistically significant manner ($P < .001$) (Figure 4A). Furthermore, in active CD mucosal biopsy specimens and in cytokine-treated cellular samples, a statistically significant inverse correlation between the degree of tyrosine phosphorylation of β-catenin and the level of E-cadherin ($r_s = –0.74$ and $r_s = –0.69$, respectively) was found. No difference between groups was found in the E-cadherin total amount (Figure 4B).

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Confocal microscopic analysis (Figure 4C) showed that in Caco-2 cells cultured in cytokine-free conditions, β-catenin and E-cadherin were localized at the membrane level, whereas cytokines caused a strong reduction of their membrane staining paralleled by the appearance of diffuse and granular cytoplasmic positivity.

A negligible percentage of apoptotic cells (<5%) was observed both in the cytokine-treated and cytokine-untreated samples (data not shown).

Discussion

The present study focused on the molecular arrangement of TJs and AJs in CD and on the level of tyrosine phosphorylation of their cytoplasmic components, a phenomenon known to regulate their assembly and functionality.[10,11] Until now, only a few studies have provided evidence for a perturbation of intercellular junctions in active CD. Indeed, a reduction in depth and number of strands of TJ in active disease first was demonstrated by using

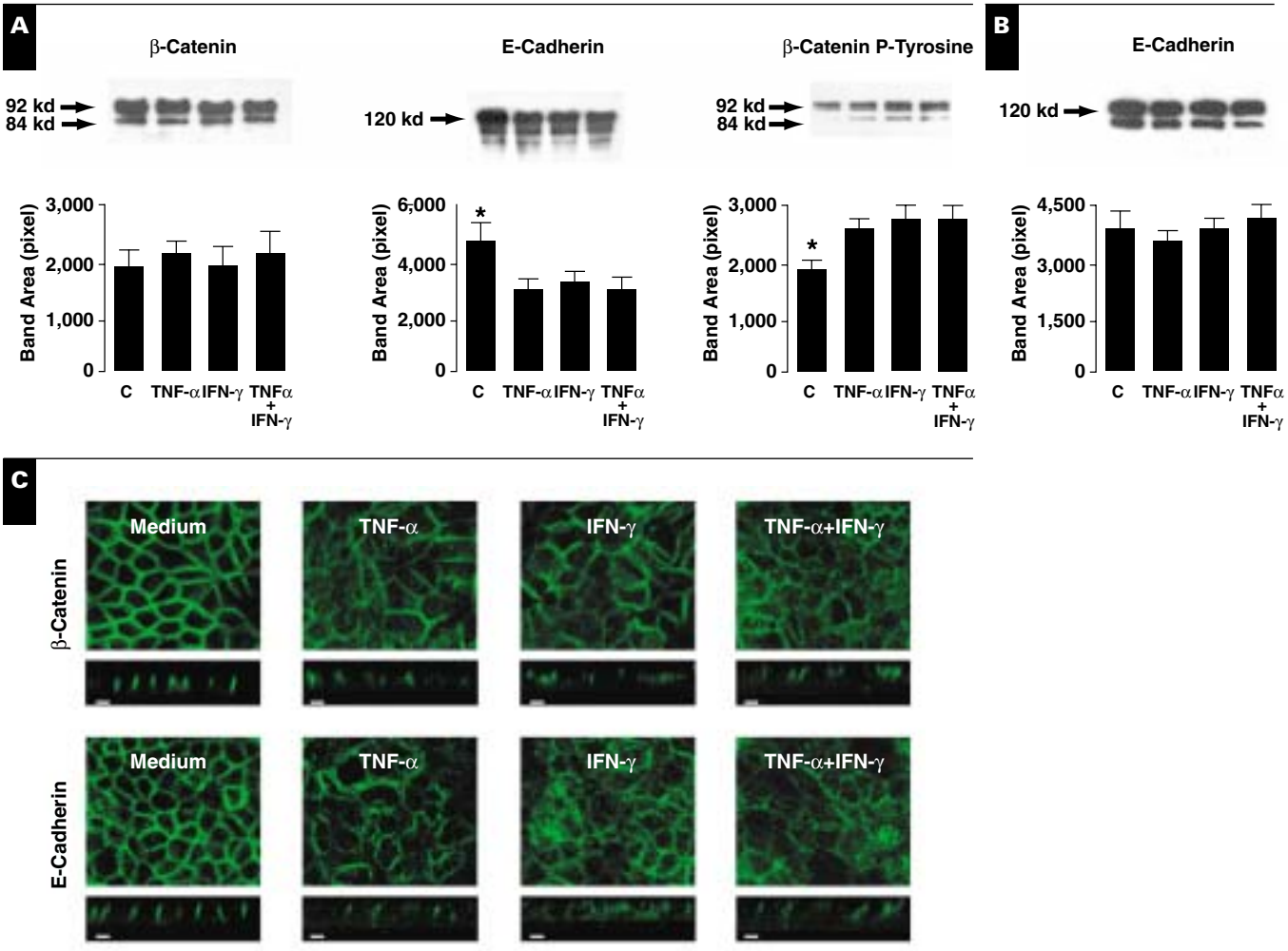

Figure 4 **A**, Western blot and densitometric analysis of anti–β-catenin antibody immunoprecipitates of Caco-2 cellular samples treated with tumor necrosis factor (TNF)-α, interferon (IFN)-γ , or both, in comparison with the untreated condition. Two bands of 92 and 84 kd (arrows) were evident in all cases without significant differences between the groups. However, blotting of these immunoprecipitates with the antiphosphotyrosine antibody revealed an increase of the band areas (92 and 84 kd as indicated by the arrows) in all treated samples with a statistically significant difference in comparison with the control sample. Blotting with the anti–E-cadherin antibody (120-kd band as indicated by the arrow) showed a significant decrease of the bound protein in all 3 cytokine-treated groups (**A**), whereas no modification of the total amount of this protein in cellular lysates was evident among the 4 groups (**B**). Values are given as mean ± SD. * $P < .001$ vs cytokine-treated samples. **C**, Confocal images of Caco-2 cells incubated for 48 hours in the absence or presence of TNF-α, IFN-γ, or both. In cytokine-free samples, β-catenin and E-cadherin showed membranous staining, whereas the cytokine-treated samples showed a reduction of the membranous staining together with a relative increase of their cytoplasmic pool. The marker corresponds to a length of 10 μm. At the bottom, the corresponding vertical sections are shown. P-tyrosine, phosphotyrosine.

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the freeze-fracture electron microscopy technique in the late 1970s[28] and subsequently was confirmed,[29,30] together with the suggestion of down-regulation of ZO-1 expression.[31,32] As for the AJs, a significant reduction of E-cadherin and β-catenin expression, which returns to normal levels after following a gluten-free diet, was reported in children[33] and adults with untreated CD.[34]

The present study, the only one that used coimmunoprecipitation and colocalization techniques, clearly demonstrated that in active disease, the absence of tyrosine-phosphorylated ZO-1 makes it unable to join occludin and to localize at the apical end of the lateral membrane, as appeared evident in confocal microscopy in which the TJ complex proved to be disrupted. Similarly, the extensive phosphorylation of β-catenin found in untreated CD mucosa leads to a reduction of the level of coimmunoprecipitated E-cadherin with a disassembly of the AJ complex and a consequent increase of the β-catenin cytoplasmic pool, as confirmed by confocal microscopy. In this scenario, it is likely that ZO-1 remains trapped in AJ molecules, as happens in immature epithelial cells during the initial phase of polarization.[35]

Our data also fit with the notion that crypt enterocytes have a higher level of tyrosine phosphorylation capacity in comparison with differentiated enterocytes, an event responsible for the high mobility characteristic of the immature cells.[36] However, in both junctional complexes, the total protein level of both transmembrane molecules remained unchanged, whereas their localization was altered. Since evidence has emerged suggesting a heterophilic interaction of E-cadherin with $\alpha_E\beta7$ integrin on the surface of intraepithelial lymphocytes, which is important in mediating the retention of lymphocytes within the epithelium in a tissue-specific manner,[37] the presence of a normal level of E-cadherin mainly unbound to β-catenin in active CD allows us to speculate that in this condition, it might be responsible for the well-known increase of intraepithelial lymphocytes. The consequent shift of β-catenin toward the participation in the Wingless/Wnt signaling pathway[38] or the formation of complexes with adenomatous polyposis coli proteins[39] leads to a perturbation of cell-cell contact and the initiation of cell migration.[40] Because involvement in signaling pathways also has been recognized for the TJs,[41] it can be argued that the molecular components of the AJC are more than a simple mechanical barrier separating different compartments, participating in many key events responsible for cellular function and fate.

On the other hand, CD is characterized by increased enterocyte proliferation, migration, and apoptosis due to the activation of the 2 main lymphocytic mechanisms, Fas/Fas ligand and perforin/granzymes,[23,24] and of a peculiar matrix metalloproteinase pattern[21] as well **Figure 5**. In this regard, a recent study carried out on several cell lines demonstrated that matrix

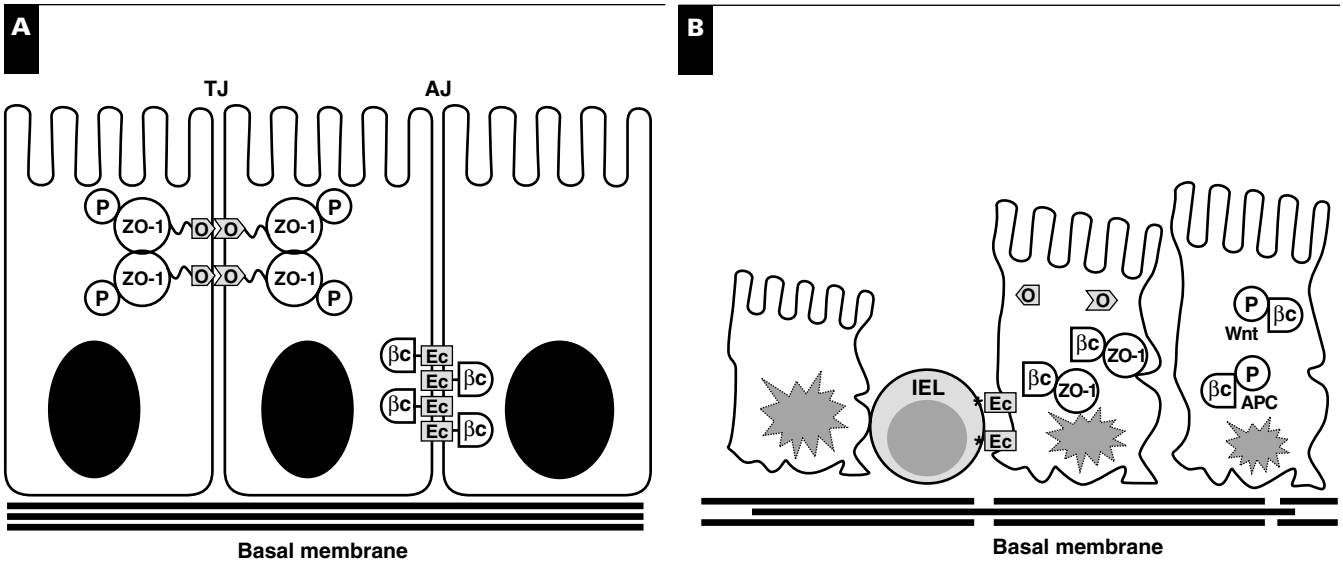

Figure 5 Small intestinal epithelium in healthy (**A**) and in celiac (**B**) mucosa. **A**, Enterocytes are joined to each other by tight junctions (TJ) and adherens junctions (AJ), which are formed by transmembrane (O, occludin; Ec, E-cadherin) and cytoplasmic (ZO-1, zonula occludens-1; βc, β-catenin) proteins. The degree of phosphorylation (P) of the latter proteins regulates the localization and the connection of the molecular components of the junctions with the actin cytoskeleton. **B**, In celiac disease, the junctional complexes undergo disruption. The lack of phosphorylation of ZO-1 makes it unable to detach from β-catenin and to join occludin. On the other hand, the higher degree of phosphorylation of β-catenin makes it unable to join E-cadherin, which, in turn, may bind $\alpha_E\beta7$-integrin (*) of intraepithelial lymphocytes (IELs). The phosphorylated β-catenin participates in the Wingless/Wnt signaling pathway and in the formation of complexes with adenomatous polyposis coli (APC) proteins. The disruption of junctional complexes together with increased production of T-helper-1 cytokines, matrix metalloproteinases, and the activation of Fas/Fas ligand and perforin/granzyme pathways lead to exaggerated enterocyte apoptosis.

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metalloproteinases and caspases, a family of intracellular enzymes leading to programmed cell death, are able to cleave occludin and its cytoplasmic counterparts ZO-1 and ZO-2 during apoptosis induced by TNF-α and anti-Fas antibody.[42]

If comprehensively considered, this evidence suggests that the disruption of the mucosal barrier observed in CD[16,17] might be the end result of all of the proapoptotic stimuli acting on enterocytes and of the action of Th-1 proinflammatory cytokines.[43,44] The results obtained by the in vitro model of intestinal epithelium seem to confirm this hypothesis because, by increasing the level of tyrosine phosphorylation of β-catenin, the cytokines used, IFN-γ and TNF-α, caused a significant decrease of the E-cadherin engaged in forming the junctional complexes, similar to active CD. This is not the case for TJs, however, where these cytokines did not affect the level of occludin expression, probably as a consequence of the increased tyrosine phosphorylation of ZO-1 that might counterbalance its decrease. On the other hand, the uncoupling between AJs and TJs in Caco-2 cells and their independent regulation by tyrosine phosphorylation has been well recognized for a long time.[45] In addition, the lack of an increased percentage of cells with apoptotic features after cytokine treatment may be due to the inability of this cell line to express the Fas receptor and then to undergo immune-mediated apoptosis.[46]

Taken together, our results extend our knowledge on epithelial modification in CD and suggest that the disruption of AJs might be a direct consequence of cytokine action, whereas that of TJs probably also is influenced by apoptotic stimuli. The explanation for the discrepancy with the conclusions of other authors[31-34] lies mainly in the use of different techniques or antibodies with different epitope targets, as in the original design of our study targeting the coimmunoprecipitation and colocalization of the junctional components. With this aim, we considered the expression of the specific proteins after an immunoprecipitation step performed by using the cytoplasmic components of TJs and AJs, and, in accordance with our results, other authors did not find changes in E-cadherin expression in childhood CD.[47] Furthermore, the strong influence of cytokines on AJs in Caco-2 cells found by Perry and coworkers[34] probably is due to the use of this cell line after a few days postconfluence, a situation that did not correspond completely to that of the small intestine phenotype.[26]

Finally, although it is difficult to answer the question of whether the altered expression, distribution, and phosphorylation of junctional molecules that we have shown is a primary or a secondary event in active CD, preliminary results on mucosal samples of 2 potential[48] CD cases (unpublished results), which failed to detect any modification of AJC components (unpublished results), support the hypothesis that they are merely a consequence of inflammatory-apoptotic phenomena.

In CD, a posttranslational modification, ie, tyrosine phosphorylation, of the molecular components of junctional complexes seems to have a major role in determining their abnormal expression and distribution. Furthermore, not only proinflammatory cytokines might be considered the culprits because growth factors, such as transforming growth factor β,[49] epidermal growth factor,[50] and hepatocyte growth factor,[51] or trefoil peptides[52] strongly influence the tyrosine phosphorylation of TJs and AJs. Further studies are required to understand how many and which signals, extrinsic and locally generated, are coordinated to regulate cell adhesion, migration, and apoptosis in CD.

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References

1. Hall PA, Coates PJ, Ansari R, et al. Regulation of cell number in the mammalian gastrointestinal tract: the importance of apoptosis. *J Cell Sci*. 1994;107:3569-3577.
2. Efstathiou JA, Pignatelli M. Modulation of epithelial cell adhesion in gastrointestinal homeostasis. *Am J Pathol*. 1998;153:341-347.
3. Gumbiner BM. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell*. 1996;84:345-357.
4. Schneeberger EE, Lynch RD. The tight junction: a multifunctional complex. *Am J Physiol Cell Physiol*. 2004;286:C1213-C1228.
5. Anderson JM, Van Itallie CM. Tight junctions and the molecular basis for regulation of paracellular permeability. *Am J Physiol*. 1995;269:G467-G475.
6. Fanning AS, Jameson BJ, Jesaitis LA, et al. The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. *J Biol Chem*. 1998;273:29745-29753.
7. Takeichi M. Cadherin cell adhesion receptors as a morphogenetic regulator. *Science*. 1991;251:1451-1455.
8. Tunggal JA, Helfrich I, Schmitz A, et al. E-cadherin is essential for in vivo epidermal barrier function by regulating tight junctions. *EMBO J*. 2005;24:1146-1156.
9. Wijnhoven BPL, Dinjens WNM, Pignatelli M. E-cadherin–catenin cell-cell adhesion complex and human cancer. *Br J Surg*. 2000;87:992-1005.
10. Van Itallie CM, Balda MS, Anderson JM. Epidermal growth factor induces tyrosine phosphorylation and reorganization of the tight junction protein ZO-1 in A431 cells. *J Cell Sci*. 1995;108:1735-1742.
11. Roura S, Miravet S, Piedra J, et al. Regulation of E-cadherin/catenin association by tyrosine phosphorylation. *J Biol Chem*. 1999;274:36734-36740.
12. Nusrat A, Turner JR, Madara JL. Molecular physiology and pathophysiology of tight junctions, IV: regulation of tight junctions by extracellular stimuli: nutrients, cytokines, and immune cells. *Am J Physiol Gastrointest Liver Physiol*. 2000;279:G851-G857.

DOI: 10.1309/DTYRA91G8R0KTM8M

13. McKay DM, Baird AW. Cytokine regulation of epithelial permeability and ion transport. *Gut*. 1999;44:283-289.
14. Mankertz J, Tavalali S, Schmitz H, et al. Expression from the human occludin promoter is affected by tumor necrosis factor α and interferon-γ. *J Cell Sci*. 2000;113:2085-2090.
15. DeMeo MT, Mutlu EA, Keshavarzian A, et al. Intestinal permeation and gastrointestinal disease. *J Clin Gastroenterol*. 2002;34:385-396.
16. Hamilton I, Cobden I, Rothwell J, et al. Intestinal permeability in celiac disease: the response to gluten withdrawal and single-dose gluten challenge. *Gut*. 1982;23:202-210.
17. Bjarason I, Peters TJ, Veall N. A persistent defect in intestinal permeability in celiac disease demonstrated by a ^{51}Cr-labelled EDTA absorption test. *Lancet*. 1983;1:323-325.
18. Londei M, Ciacci C, Ricciardelli I, et al. Gliadin as a stimulator of innate responses in celiac disease. *Mol Immunol*. 2005;42:913-918.
19. Alaedini A, Green PH. Celiac disease: understanding a complex autoimmune disorder. *Ann Intern Med*. 2005;142:289-298.
20. Forsberg G, Hernell O, Melgar S, et al. Paradoxical coexpression of proinflammatory and down-regulatory cytokines in intestinal T cells in childhood celiac disease. *Gastroenterology*. 2002;123:667-678.
21. Ciccocioppo R, Di Sabatino A, Bauer M, et al. Matrix metalloproteinase pattern in celiac duodenal mucosa. *Lab Invest*. 2005;85:397-407.
22. Moss SF, Attia L, Scholes JV, et al. Increased small intestinal apoptosis in celiac disease. *Gut*. 1996;39:811-817.
23. Ciccocioppo R, Di Sabatino A, Parroni R, et al. Increased enterocyte apoptosis and Fas/FasL system in celiac disease. *Am J Clin Pathol*. 2001;115:494-503.
24. Di Sabatino A, Ciccocioppo R, D'Alò S, et al. Intraepithelial and lamina propria lymphocytes show distinct patterns of apoptosis, whereas both the populations are active in Fas-based cytotoxicity in coeliac disease. *Gut*. 2001;49:380-386.
25. James SP. This month at the NIH: final statement of NIH Consensus Conference on celiac disease. *Gastroenterology*. 2005;128:6.
26. Engle MJ, Goetz GS, Alpers DH. Caco-2 cells express a combination of colonocyte and enterocyte phenotypes. *J Cell Physiol*. 1998;174:362-369.
27. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A*. 1979;76:4350-4354.
28. Madara JL, Trier JS. Structural abnormalities of jejunal epithelial cell membrane in celiac sprue. *Lab Invest*. 1980;43:254-256.
29. Kohl D, Ashkenazi A, Ben-Shaul Y, et al. Tight junctions of jejunal surface and crypt cells in celiac disease: a freeze-fracture study. *J Pediatr Gastroenterol Nutr*. 1987;6:57-65.
30. Schulzke JD, Bentzel CJ, Schulzke I, et al. Epithelial tight junction structure in the jejunum of children with acute and treated celiac sprue. *Pediatr Res*. 1998;43:435-441.
31. Montalto M, Cuoco L, Ricci R, et al. Immunohistochemical analysis of ZO-1 in the duodenal mucosa of patients with untreated and treated celiac disease. *Digestion*. 2002;65:227-233.
32. Pizzuti D, Bortolami M, Mazzon E, et al. Transcriptional downregulation of tight junction protein in active coeliac disease is reversed after a gluten-free diet. *Dig Liver Dis*. 2004;36:337-341.
33. Barshack I, Goldberg I, Chowers Y, et al. Immunohistochemical analysis of candidate gene product expression in the duodenal epithelium of children with celiac sprue. *J Clin Pathol*. 2001;54:684-688.
34. Perry I, Tselepis C, Hoyland J, et al. Reduced cadherin/catenin complex expression in celiac disease can be reproduced in vitro by cytokine stimulation. *Lab Invest*. 1999;79:1489-1499.
35. Itoh M, Nagafuchi A, Yonemura S, et al. The 220-kd protein colocalizing with cadherins in non-epithelial cells is identical to ZO-1, a tight junction–associated protein in epithelial cells: cDNA cloning and immunoelectron microscopy. *J Cell Biol*. 1993;121:491-502.
36. Burgess DR, Jiang W, Mamajiwalla S, et al. Intestinal crypt stem cells possess high levels of cytoskeletal-associated phosphotyrosine-containing proteins and tyrosine kinase activity relative to differentiated enterocytes. *J Cell Biol*. 1989;109:2139-2144.
37. Cepek KL, Shaw SK, Parker CM, et al. Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and $\alpha_E\beta7$ integrin. *Nature*. 1994;372:190-193.
38. Hagen T, Sethi JK, Foxwell N, et al. Signalling activity of beta-catenin targeted to different subcellular compartments. *Biochem J*. 2004;379:471-477.
39. Bienz M, Hamada F. Adenomatous polyposis coli proteins and cell adhesion. *Curr Opin Cell Biol*. 2004;16:528-535.
40. Müller T, Choidas A, Reichmann E, et al. Phosphorylation and free pool of β-catenin are regulated by tyrosine kinases and tyrosine phosphatases during epithelial cell migration. *J Biol Chem*. 1999;274:10173-10183.
41. Matter K, Balda MS. Signalling to and from tight junctions. *Nat Rev Mol Cell Biol*. 2003;4:225-236.
42. Bojarski C, Weiske J, Schöneberg T, et al. The specific fates of tight junction proteins in apoptotic epithelial cells. *J Cell Sci*. 2004;117:2097-2107.
43. Youakim A, Ahdieh M. Interferon-γ decreases barrier function in T84 cells by reducing ZO-1 levels and disrupting apical actin. *Am J Physiol*. 1999;276:G1279-G1288.
44. Poritz LS, Garver KI, Tilberg AF, et al. Tumor necrosis factor alpha disrupts tight junction assembly. *J Surg Res*. 2004;116:14-18.
45. Gómez S, del Mont Llosas M, Verdù J, et al. Independent regulation of adherens and tight junctions by tyrosine phosphorylation in Caco-2 cells. *Biochim Biophys Acta*. 1999;1452:121-132.
46. Tillman DM, Petak I, Houghton JA. A Fas-dependent component in 5-fluorouracil/leucovorin–induced cytotoxicity in colon carcinoma cells. *Clin Cancer Res*. 1999;5:425-430.
47. Patey N, Scoazec J, Cuendo-Jabri BG, et al. Distribution of cell adhesion molecules in infants with intestinal epithelial dysplasia (tufting enteropathy). *Gastroenterology*. 1997;113:832-843.
48. Corazza GR, Andreani ML, Biagi F, et al. Clinical, pathological, and antibody pattern of latent celiac disease: report of three adult cases. *Am J Gastroenterol*. 1996;91:2203-2207.
49. Tian YC, Phillips AO. Interaction between the transforming growth factor-beta type II receptor/Smad pathway and beta-catenin during transforming growth factor-beta1–mediated adherens junction disassembly. *Am J Pathol*. 2002;160:1619-1628.
50. Lorch JH, Klessner J, Park JK, et al. Epidermal growth factor receptor inhibition promotes desmosome assembly and strengthens intercellular adhesion in squamous cell carcinoma cells. *J Biol Chem*. 2004;279:37191-37200.
51. Hollande F, Blanc EM, Bali JP, et al. HGF regulates tight junctions in new nontumorigenic gastric epithelial cell line. *Am J Physiol Gastrointest Liver Physiol*. 2001;280:G910-G921.
52. Liu D, el-Hariry I, Karayiannakis AJ, et al. Phosphorylation of beta-catenin and epidermal growth factor receptor by intestinal trefoil factor. *Lab Invest*. 1997;77:557-563.

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