Inflammatory bowel disease is associated with changes of enterocytic junctions

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1Pathologisches Institut, Universität Heidelberg, 69120 Heidelberg; 2BASF-LYNX Bioscience AG, 69120 Heidelberg; 3Deutsches Krebsforschungszentrum, 69120 Heidelberg; 4Universität Heidelberg, ZMF, Klinikum Mannheim, 68167 Mannheim; and 5Universität Frankfurt, Abteilung Nephrologie, IV. Medizinische Klinik, 60590 Frankfurt, Germany

Am J Physiol Gastrointest Liver Physiol 281: G216–G228, 2001.—Changes of the intestinal mucosal barrier are considered to play a role in the pathogenesis of inflammatory bowel disease (IBD). Our experiments were designed to identify dysregulation of epithelial junctional molecules in the IBD intestine and to address whether altered expression of these molecules is a primary event in IBD or a phenomenon secondary to the inflammatory process. Noninflamed and actively and actively inflamed mucosal tissues from patients with ulcerative colitis or Crohn’s disease as well as tissues from control subjects were analyzed for the expression of junctional molecules by different methods. Marked downregulation of junctional proteins and their respective mRNAs was observed in actively inflamed IBD tissues. In IBD tissues with inactive inflammation, only a few junctional molecules such as E-cadherin and α-catenin were affected, whereas expression of desmosomal or tight junction-associated proteins appeared almost unchanged. In noninflamed IBD tissues, junctional protein expression was not different from that seen in normal control subjects. In IBD, downregulation of junctional molecule expression is apparently associated with the inflammatory process and does not likely represent a primary phenomenon.

At least two forms of inflammatory bowel disease (IBD) can be distinguished: Crohn’s disease (CD) and ulcerative colitis (UC). Histologically, UC is associated with continuous mucosal inflammation, including crypt abscesses as well as ulcers, that typically extends from the most caudal part of the rectum for a variable distance in the proximal direction, whereas CD is characterized by segmental and transmural inflammation. The intestinal segment most commonly involved in CD is the terminal ileum, but all sites of the gastrointestinal tract may be affected discontinuously; in addition, fistulas, edema, and granulomas can be observed.

It has been suggested that IBD represents a dysregulated mucosal immune response to antigens, in a genetically susceptible host, that is subject to modification by a variety of environmental factors (3). Importantly, the epithelial intestinal barrier, representing the first cellular network to come in contact with luminal events, must be considered as one important structure in understanding IBD pathophysiology.

A series of studies (9, 12, 13, 15, 21, 43) has provided evidence for the perturbation of the main adhesive (AJs) and occluding junctions (TJs) in IBD, in which altered expression of E-cadherin, α-catenin, and p120 has been observed. Experiments with a chimeric/transgenic murine model indicate the important role of E-cadherin in the maintenance of the intestinal barrier, showing that dysregulation of this molecule leads to IBD (16). Jankowski and co-workers (19) have shown a strong correlation between the deregulation of two classic cadherin molecules, E- and P-cadherin, and the progression of human colitis (20). Furthermore, changes of intestinal permeability have been reported in patients with IBD (39, 52). In UC, perturbations in permeability seem to be limited to the inflamed intestinal segment, whereas in CD, discrete intestinal permeability alterations have been observed not only in gut tissues with evident intestinal lesions but also in...

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areas lacking any sign of macroscopic injury (10, 27, 38). It has been hypothesized that permeability defects might represent a primary disorder in CD, because in healthy first-degree relatives of CD patients similar permeability defects are already detectable (10, 17, 26, 33).

We hypothesized that the expression of various junctional molecules might be affected in IBD. Our experiments were therefore designed to identify candidate molecules and to clarify the question of whether any altered expression of junctional molecules is a primary event in IBD, which might already be detectable in noninflamed IBD mucosa, or a phenomenon secondary to the inflammatory process. Human mucosal samples of noninflamed and inactively and actively inflamed intestine from patients with IBD and normal control subjects were investigated with Western blot analysis, immunofluorescence, and quantitative PCR with a LightCycler approach.

MATERIALS AND METHODS

Patients. Surgical specimens from 10 patients with UC (mean age, 42 yr; range, 28–62 yr) and 10 patients with CD (mean age, 37 yr; range, 22–59 yr) and unaffected bowel tissues from 10 patients with sporadic colonic cancer (mean age, 58 yr; range, 39–69 yr) were investigated (Tables 1 and 2). The use of human tissue was approved by Heidelberg University. Diagnoses were established by conventional clinical and histological criteria. In UC, colectomy was performed because of resistance to medical therapy. In CD, surgical resection was indicated by stenosis of the intestinal segment and/or resistance to medical therapy. Anti-inflammatory medication, including corticosteroids and/or nonsteroidal anti-inflammatory drugs, was used in all patients with IBD. All patients with UC underwent colectomy with pouch-anal anastomosis, whereas 8 of 10 patients with CD were treated by ileocecal resection and a further 2 patients with CD underwent other segmental resection of the intestine (Table 2). Surgical specimens were opened longitudinally along the antimesenteric border. Samples of ~1 cm² were taken from each 10-cm length of bowel wall, half of which was snap-frozen in isopentane cooled by liquid nitrogen for the preparation of cryosections. The other half was used for molecular experiments; mucosal tissue layers were mechanically dissected from the underlying submucosa, immediately cooled in liquid nitrogen, and stored at −80°C until use. In parallel, tissues immediately adjacent to the experimental samples were paraffin embedded and processed for both histomorphological grading and routine histopathology.

Table 1. Anamnestic and experimental data from 10 patients with ulcerative colitis

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age, yr</th>
<th>Intestinal Segment</th>
<th>Histomorphological Grading of Inflammation</th>
<th>Alteration of Junctional Molecule Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>34</td>
<td>Ascending colon</td>
<td>N</td>
<td>+/−</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>28</td>
<td>Transverse colon</td>
<td>I</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>34</td>
<td>Descending colon</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>41</td>
<td>Ascending colon</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>39</td>
<td>Descending colon</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>56</td>
<td>Sigmoid colon</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>44</td>
<td>Cecum</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>62</td>
<td>Transverse colon</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>37</td>
<td>Descending colon</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>42</td>
<td>Rectum</td>
<td>A</td>
<td></td>
</tr>
</tbody>
</table>

Summary of data from intestinal segments of 10 patients with ulcerative colitis (UC). N, noninflamed; I, inactively inflamed; A, actively inflamed. Expression of various junctional proteins was highly affected (+) in A tissues, remained unchanged in N tissues (−), and was occasionally changed in a small number of junctional proteins in I tissues (+/−).
normal intestinal tissue (control) and actively inflamed (CDA) mucosa were compared with intestinal tissue (control). In cases of CD, noninflamed (CDN) and UC, respectively) were compared with normal inflamed tissue. "Active inflammation" designates tissues in which granulocytes or any epithelial damage. The term "inactive inflammation" represents a condition with an increase in lymphocytes and plasma cells without granulocytes or any epithelial damage. The term "active inflammation" designates tissues in which granulocytes, cryptitis, or cystic abscesses as well as erosive defects or ulcers were seen. Further subdivision of active inflammation was not performed. In conclusion, areas of noninflamed, inactively inflamed, and actively inflamed UC mucosa (UCN, UCI, and UCA, respectively) were compared with normal intestinal tissue (control). In cases of CD, noninflamed (CDN) and actively inflamed (CDA) mucosa were compared with normal intestinal tissue (control).

Preparation of tissues. For molecular experiments, mucosal samples were homogenized in TRI Reagent (Molecular Research Center, Cincinnati, OH) with Ultra Turrax equipment (IKA Labortechnik, Staufen, Germany). Protein and RNA were extracted according to the method of Chomczynski (7). The RNA pellet was dissolved in diethyl pyrocarbonate water, and the concentration of total RNA was assessed by spectrophotometric measurements (260- to 280-nm ratio = 1.6–1.9; LKB Ultraspec III; APBiotech, Uppsala, Sweden). The final preparation was supplemented with 1 μl of RNasin (Promega, Madison, WI).

The protein pellet was washed three times in a solution containing 0.3 M guanidine hydrochloride in 95% ethanol (50). Protein was solubilized in a solution of 9 M urea-50 mM dithiothreitol. Protein measurements were performed with the Bio-Rad assay reagent according to the manufacturer’s suggested protocol (Bio-Rad, Munich, Germany). Concentrated Laemmli buffer was added to the sample volumes to obtain onefold final concentrations. Samples were stored at −20°C until use.

Reverse transcription. Reverse transcription was performed with the SuperScript amplification system for first-strand cDNA synthesis (Life Technologies, Eggenstein, Germany). In accordance with the conditions recommended by the supplier, 5 μg of DNase-digested total RNA were used for oligo(dT)-primed first-strand cDNA synthesis. Reverse transcription was terminated by heating to 70°C for 15 min and was followed by RNase H digestion (20 min at 37°C). Control experiments included substitution of a commercially provided control RNA (50 ng) and substitution of distilled water for the enzyme reverse transcriptase.

Quantitative PCR and sequencing reaction. For PCR analysis with the LightCycler system (Roche-Diagnostics, Mannheim, Germany), 25 different cDNA probes were used (5 different cDNA probes/group (UCN, UCI, CDA, UCN, and control)). Quantitative analysis of the fluorescence data was performed with the designated LightCycler software package. Fresh threefold dilution series of respective cDNAs were prepared (1:3, 1:9, 1:27, 1:81, 1:243, and 1:729). The dilution series of normal control cDNAs served to provide a standard curve and allowed the quantification of the PCR-produced yield for all samples. A well-adapted set of primers was used that correspond to the coding sequences of human β-catenin (X87838; sense: 5’-GC-CGGCTATTTGAGAAGCTG-3’; antisense: 5’-TGATGCTCTCCCTGTGTCACA-3’), human cytokeratin 19 (NM002276; sense: 5’-TTTGAGACGGAACAGGCTCT-3’; antisense: 5’-TCCTCCAAGGCAGCTTTCAT-3’), human desmoglein-2 (Z26317; sense: 5’-GAAGGT-GC-TCCTTCCAAGCCAATGCACAT-3’; antisense: 5’-GAAGGT-CAGCAGCTGTCACAACCA-3’), and human occludin (NM002538; sense: 5’-TGTTTCTGTGTGCCTCCACAAA-3’; antisense: 5’-TGTTTCTGTGTGCCTCCACAAA-3’). All primers were synthesized by Life Technologies. PCR analysis was quantitatively performed as described previously (51). Briefly, 20-μl aliquots of the PCR mixtures were loaded into LightCycler capillary tubes (3 mM MgCl2, each primer at 0.5 μM, 2 μl of
SYBR green mix (Roche Diagnostics), and 2 μl of cDNA]. The tubes were sealed and loaded into the LightCycler. Forty PCR cycles were performed consisting of a denaturation step to 94°C followed by a 62°C primer annealing step for 15 s, ramping to 72°C for amplicon extension for 10 s. At each cycle, a fluorescence reading was conducted at 82°C. At the end of the whole run, a melting step was performed to identify amplicons by their appropriate melting temperature. The melting step consisted of denaturation, cooling, and ramping to 95°C with continuous monitoring of fluorescence. To verify amplicon purity and integrity, PCR amplicons were loaded onto a 1.5% agarose ethidium bromide gel in Tris-EDTA buffer and visualized on an ultraviolet transilluminator. The original data of PCR product quantifications for each group were transferred to Excel 7.0 (Microsoft, Redmond, WA) and normalized to the respective mean mRNA expression of the housekeeping gene cytokeratin 19 in the same cDNA pool. For each experimental group, the mean values ± SD were determined from the normalized PCR product concentration data of every dilution step. The resulting quantitative differences represent relative changes in mRNA expression between the experimental groups. The data were then further analyzed (see Statistical analyses).

Sequencing reactions were performed with 100 ng of each purified PCR product and 10 pmol of the appropriate primer (see Quantitative PCR and sequencing reaction) with the BigDye Terminator kit (ABI PRISM, Weiterstadt, Germany). Reactions were run on an ABI 3700 capillary sequencer according to the manufacturer’s recommendations. Sequences were assembled with the program SeqMan (LaserGene, Madison, WI).

Antibodies. For immunofluorescence (IF) staining experiments and immunoblotting (IB) analyses, the following primary mouse monoclonal antibodies, diluted as indicated in parentheses, were used. Anti-desmoglein-2 (IF, 5 μg/ml; IB, 1 μg/ml), anti-desmoplakin-1 and -2 (IF, 5 μg/ml; IB, 1 μg/ml), anti-desmocollin-2 (IF, 5 μg/ml; IB, 1 μg/ml), anti-plakoglo-
Table 3. Summary of junctional protein expression in inflammatory bowel disease

<table>
<thead>
<tr>
<th></th>
<th>UCN</th>
<th>UCI</th>
<th>UCA</th>
<th>CDN</th>
<th>CDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desmosome</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desmocollin-2</td>
<td>74.0±22.74</td>
<td>68.8±6.46*</td>
<td>44.2±6.98*</td>
<td>84.4±5.32</td>
<td>74.4±4.98*</td>
</tr>
<tr>
<td>Desmoglein-2</td>
<td>81.4±9.13</td>
<td>65.4±14.7*</td>
<td>46.4±9.45*</td>
<td>86.6±6.95</td>
<td>70.4±5.41*</td>
</tr>
<tr>
<td>Desmoplakin-1</td>
<td>88.5±5.54</td>
<td>76.8±8.29*</td>
<td>40.0±9.77</td>
<td>85.5±4.44</td>
<td>44.4±9.34*</td>
</tr>
<tr>
<td>Tight junction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occludin</td>
<td>89.2±7.89</td>
<td>74.6±4.77*</td>
<td>4.4±6.19*</td>
<td>84.3±8.1</td>
<td>0.2±0.61*</td>
</tr>
<tr>
<td>ZO-1</td>
<td>79.5±9.68</td>
<td>47.6±15.7*</td>
<td>3.4±6.54*</td>
<td>79.2±9.42</td>
<td>25.8±14.6*</td>
</tr>
<tr>
<td>Adherens junction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E-cadherin</td>
<td>84.6±15.18</td>
<td>71.2±9.09*</td>
<td>0.2±0.45*</td>
<td>91.6±6.07</td>
<td>1.2±1.71*</td>
</tr>
<tr>
<td>Armadillo repeat molecule β-catenin</td>
<td>95.4±1.16</td>
<td>75.8±6.69*</td>
<td>16.2±6.75*</td>
<td>94.4±3.29</td>
<td>91.0±2.35</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 30 for each protein, 5 different probes/group. ZO, zona occludens. Junctional protein expression was quantified by densitometric analysis of Western blots, and statistical evaluation was performed in SPSS/PC+. ONEWAY RANGES procedure (analysis of variance, Scheffe’s test). All measurements in tissues of noninflamed (UCN), actively inflamed (UCI), and actively inflamed ulcerative colitis (UCA) as well as noninflamed (CDN) and actively inflamed Crohn’s disease (CDA) represent relative values, whereby 100 designates the expression in control tissues (accordingly, respective values do not appear in the table). *Significant suppression of expression of various junctional proteins in inflamed IBD tissue compared with normal controls, P < 0.05.
desmoglein-2, claudin-1, and claudin-2 was additionally visualized by using the routine anti-biotin complex (ABC) technique and diaminobenzidine as a chromogen (DAKO, Hamburg, Germany). Negative controls included similarly processed sections in which the primary antibody had been totally omitted as well as sections in which the appropriate normal serum was used (Dianova).

For double-labeling experiments, sections were incubated for 60 min with the first primary antibody and detected with the appropriate secondary antibody (30 min) before the second primary antibody was applied to the sections (60 min) and visualized with the suitable secondary antibody (30 min). Each incubation time was followed by three 3-min washes with PBS. Histosafe-mounted sections were viewed with a laser scanning confocal microscope (TCS NT, Leitz, Wetzlar, Germany). Computer images were collected on optical memory disks and arranged with the Corel Draw program.

SDS-PAGE, Western blot analysis, and densitometric analysis. One-dimensional SDS-PAGE (7.5%) was performed according to the method of Laemmli (25). Molecular weight markers were purchased from Amersham (Amersham International, Little Chalfont, UK). SDS-PAGE-resolved mucosal-derived proteins (equivalent total protein of ~15 μg/sample) were transferred to polyvinylidene difluoride (PVDF) Immobilon-P membranes (Millipore, Bedford, MA) with a semi-dry blot system followed by incubation of the membrane in 5% skim milk powder and Tris-buffered saline-Tween 20 for 60 min. For immunoreaction, primary antibodies and horseradish peroxidase-conjugated secondary antibodies were applied as detailed in Antibodies. The ECL substrate system (Amersham International) was used for subsequent detection. The incubation time for each antibody was 1 h at room temperature. The amount of each sample analyzed was adjusted to the intensity of cytokeratin 19 and THF staining on the PVDF membrane. Therefore, blots were stripped of antibodies and reprobed with an anti-cytokeratin 19 antibody or an anti-THF antibody as positive epithelial loading controls, as well as with β-actin as a global tissue-loading control. For antibody stripping, blots were immersed in methanol, washed in water, and incubated in stripping buffer consisting of 2% SDS, 100 mM β-mercaptoethanol, and 62.5 mM Tris-HCl (pH 7.5) for 30 min at 50°C. Subsequently, blots were processed as described above. Negative controls included similarly processed blots in which the primary antibody was totally omitted. Lysates of A431, Caco-2, CCL-6, and HeLa cells were used as positive controls.

To evaluate differences in protein expression, blots were analyzed with the BioImage Intelligent Quantifier system (BioImage, Ann Arbor, MI). For each protein quantified, five different blots were analyzed \( n = 30; 5 \) different probes/group (UCN, UCI, UCA, CDN, CDA, and control). In accordance with the conditions recommended by the supplier, scanning images of blots were given a computer-assisted evaluation. The signaling intensity of each protein of interest in the control tissues served as the standard value. For each experimental group, the mean values \( \pm \) SD were determined. The data were further analyzed and represent differences in protein expression between the experimental groups (see below).

Statistical analyses. All statistical analyses were performed with the Statistical Package for Social Sciences analysis program (SPSS/PC+, Chicago, IL). In SPSS/PC+, the ONEWAY RANGES procedure (analysis of variance, Scheffe’s test) was used to calculate relative changes of mRNA as well as differences in protein expression between the groups.

RESULTS

Histomorphological grading of 34 different UC tissue regions revealed that 21 samples (62%) were actively inflamed, 6 samples (18%) showed inflammation that was chronic inactive, and 7 tissues (20%) were nonin-
flamed (Table 1). Of 18 CD tissues examined, 9 (50%) were actively inflamed, 2 (11%) showed inactive inflammation, and 7 (39%) were noninflamed (Table 2). Severe alterations of junctional protein expression, detailed below, were mainly seen in actively inflamed IBD tissues. In chronic inactively inflamed tissues, expression of AJ-associated proteins (E-cadherin, α-catenin) was frequently affected, whereas expression of various proteins of desmosomes or TJs appeared almost unchanged. The expression of junctional proteins in noninflamed samples from IBD patients receiving immunosuppressive/anti-inflammatory therapy was not different from that seen in normal control subjects (no medication) (see Fig. 1, C–E, Fig. 4, G–I, and Fig. 6, G–H). Abnormal epithelial differentiation (dysplasia) was observed in 3 of 10 UC cases, in which expression of junctional proteins was severely disturbed.

*Enterocytic DMs in IBD.* In actively inflamed UC and CD tissues, desmosomal staining of epithelial cells located at the luminal surface and the upper part of crypts was progressively decreased in accordance with the grade of inflammation (Fig. 1, A and B), whereas expression of cytokeratin 19 and THF synthase was still preserved. These changes did not correlate with the occurrence of areas displaying cryptitis (Fig. 1A). In histomorphologically noninflamed UC and CD tissues, the distribution and intensity of desmosomal staining was not strikingly different from that seen in control tissue (shown for desmoglein-2 in Fig. 1, C–E). The staining was seen especially at the upper part of the lateral cellular membrane of enterocytes in a dis-
Enterocytic TJ-associated proteins in IBD. In all actively inflamed IBD tissues, the continuous string of TJ staining was disturbed, paralleling the degree of inflammation (shown in Fig. 4, A–F for ZO-1 by immunofluorescence). Expression of ZO-1 and occludin was apparently more severely affected in epithelial cells of the luminal surface than in crypts, whereas expression of immunoreactive ZO-2, cytokeratin 19, and THF synthase was preserved. The distribution of immunoreactive claudin-1 and claudin-2 was essentially similar to that seen for the integral membrane protein occludin with regard to inflamed and noninflamed tissues (data not shown). Translocation of any TJ-associated protein into the nuclei of enterocytes, as shown previously for ZO-1 (14), was not seen. Endothelial expression of the TJ-associated proteins investigated was not affected in actively inflamed IBD tissues (Fig. 4, A–F). In normal control and histomorphologically noninflamed IBD tissues, immunofluorescent labeling of occludin, ZO-1, and ZO-2 was predominantly seen at the terminal web of enterocytes in a distinct reticular and linear pattern without disruptions (shown for ZO-1 in Fig. 4, G–I). In Western blot analysis, expression of occludin protein (corresponding to bands of 65 kDa) was quantitatively more decreased in mucosal tissues with an active inflammation than was the case with ZO-1 (225 kDa) (Fig. 5; Table 3). In inactively inflamed mucosal tissues, expression of occludin was slightly decreased, whereas ZO-2 expression was nearly unchanged. As with the immunofluorescence results, expression of the ZO-2 protein (160 kDa), THF synthase (115 kDa), and cytokeratin 19 (45 kDa) was not changed. The reduced number of occludin transcripts paralleled the decrease of the immunoreactive protein in actively inflamed mucosal IBD tissues (Fig. 3). Especially in actively inflamed mucosal CD probes, the expression of occludin mRNA was apparently downregulated.

Enterocytic AJs in IBD. As previously shown, immunoreactive E-cadherin and α-catenin were expressed at the basolateral cellular membrane of enterocytes (20, 21). In actively inflamed IBD tissue, the expression of E-cadherin and α-catenin protein was highly reduced, as shown by both immunofluorescence and Western blot analyses (120-kDa bands correspond to E-cadherin; 102-kDa bands correspond to α-catenin), whereas expression of THF synthase (115 kDa) and cytokeratin 19 (45 kDa) was preserved (data not shown). In actively inflamed mucosal IBD samples, E-cadherin immunoreactive protein could not be detected; however, the mRNA transcripts were always detected; however, the mRNA transcripts were always found to be expressed (Fig. 3; Table 3). The expression and localization of E-cadherin and α-catenin in histomorphologically noninflamed UC and CD tissues were not different from those seen in normal control tissue. Junction-associated proteins of the armadillo gene family in IBD. Junction-associated proteins of the armadillo gene family are components of different types of junctions, i.e., AJs and DMs (53). APC, p120, plakophilin-1, plakophilin-2, β-catenin, and plakoglobin, as members of the armadillo gene family, were analyzed by immunofluorescence studies and Western blot analysis. In actively inflamed tissues, protein expression in epithelial cells located at the luminal surface as well as in the upper part of crypts was affected [e.g., β-catenin (Fig. 6, A–F), p120, plakophilin-2, and plakoglobin], whereas immunofluorescence of cytokeratin 19 and...
THF synthase was not altered. In general, the changes observed did not correlate with the occurrence of areas displaying cryptitis or crypt abscesses (Fig. 6F). Immunofluorescence of APC, β-catenin, and plakoglobin was apparently more reduced in actively inflamed UC than in CD tissue. In histomorphologically noninflamed IBD tissues, immunofluorescent labeling of all these proteins was not different from that seen in normal controls (shown for β-catenin in Fig. 6, G and H). Immunofluorescence results were confirmed by Western blot analysis with the use of mucosal samples; in UC, APC (corresponding to bands of 300 kDa), p120 (115 kDa), plakophilin-2 (100 kDa), β-catenin (92 kDa), and plakoglobin (82 kDa) were progressively decreased, correlating well with the degree of inflammation (Fig. 7). The reduced expression of the β-catenin protein in inflamed UC tissues was statistically significant (P < 0.05; Table 3). In actively inflamed CD tissue, the amount of immunoreactive β-catenin and APC protein was apparently not suppressed, whereas the expression of plakophilin-2 and plakoglobin protein was reduced. In addition, β-catenin mRNA transcript levels were significantly decreased in mucosal probes of actively inflamed UC but not in CD samples (P < 0.05; Fig. 3). In all mucosal specimens investigated, expression of plakophilin-1 was neither detectable by Western blot analysis nor by immunofluorescence.

**GSK-3 in IBD.** Expression of the α- and β-subunits of GSK-3 was analyzed with the Western blot technique. Bands of ~48 kDa were expressed independently from the degree of inflammation, and no difference was found in their expression between CD and UC tissue.
when compared with normal control tissue (data not shown).

DISCUSSION

The present investigation relates to the molecular organization of junctional complexes in the intestinal epithelial barrier under pathological conditions. The barrier consists of the epithelial cells themselves, the mucus and soluble molecules secreted by them, the interepithelial junctions sealing the spaces between the cells (TJs), and the junctions responsible for interepithelial adherence (DMs and AJs). A well-balanced intestinal homeostasis is promoted by rapid resealing of this barrier after injury. The present study was designed to provide more insight into the structure and molecular composition of enterocytic TJs and AJs as well as DMs in situations of disturbed intestinal homeostasis such as UC and CD.

Our results yield experimental evidence that, in IBD, expression of AJs is generally more affected by active inflammation than that of TJs and DMs. In actively inflamed IBD tissues, immunofluorescence of E-cadherin and α-catenin was completely lost in epithelial cells, whereas several TJ- and DM-associated proteins were immunostained to a variable extent. The expression of further molecules (i.e., THF synthase and cytokeratin 19) was fully preserved, which indicates morphological integrity of the cells. Obviously, epithelial cells tolerate considerable change in the organization of AJs without the occurrence of extensive apoptosis or necrosis.

This study shows that various junctional molecules are affected by the actively inflamed status in IBD. Notably, the expression of transmembrane proteins as constituents of junctions (e.g., occludin, E-cadherin, desmoglein-2) was more suppressed than was the case for proteins of the cytoplasmic plaque. As demonstrated by immunofluorescence, expression of such transmembrane proteins was predominantly affected in the surface epithelium and by enterocytes lining the upper part of crypts. Integrity of these epithelia was ensured by hematoxylin and eosin-stained sections, the ABC technique, and double labeling experiments. Therefore, our findings provide further support for the view that this surface epithelia might represent a motile population of cells, with the motility facilitated by reduced cell-cell adhesion (15). Because changes in the organization of interepithelial junctions seem to be involved in promoting cell migration during epithelial restitution of the intestinal mucosa (21), we assume that the observed downregulated expression of junction-associated molecules may reflect a situation in which the epithelium is able to tolerate a milieu of chronic inflammation (20). Notably, the changes observed are obviously not due to anti-inflammatory medical treatment (e.g., corticosteroids and nonsteroidal anti-inflammatory drugs), because expression and localization of junctional molecules in noninflamed (normal) IBD tissues were not different from those seen in control tissues.

The assembly of TJs by various proteins is important for the development of intestinal barrier function (22, 30, 31). It has been shown that the impaired barrier function in IBD is associated with an altered TJ structure (27, 41, 52). In IBD, however, expression of the primary sealing and integral membrane components of TJs is not yet well characterized. At present, occludin and a family of proteins called claudins are identified as the primary sealing and integral membrane molecules (11, 22, 31). In this study, we concentrated on the junction-associated transmembrane protein occludin, which was found to be suppressed in the inflamed mucosa. The data fit to the observation that the permeability of the intestinal barrier is increased in inflamed IBD mucosa (27, 43, 52). It has been hypothesized that permeability defects might represent a primary disorder in CD, because intestinal permeability defects have been observed not only in mucosal tissues with evident intestinal alterations but also in areas lacking any macroscopic lesions (10, 27, 38).
Therefore, it is of interest that no significant differences in expression of the integral membrane proteins investigated were found between noninflamed IBD and control tissues. However, despite a sizeable and growing list of proteins that are components of TJs, further as yet undefined junctional molecules might be involved in the regulation of intestinal permeability.

Our study revealed that in enterocytes, abnormal expression of TJ-related transmembrane proteins (i.e., occludin) always coexisted with an altered expression of the peripheral membrane protein ZO-1. It is of note that ZO-1 expression was exclusively downregulated by enterocytes of the intestinal mucosal barrier but not in the endothelial cellular layer of adjoining mucosal capillaries and small vessels. Interestingly, despite changes in the expression of ZO-1 in inflamed mucosal tissues, expression of the ZO-2 protein was not affected. Both ZO proteins are members of the membrane-associated guanylate kinase (MAGUK) superfamily, characterized by different molecular domains (18, 20, 21, 41). Our findings argue for specialized and diverse functions of ZO-1 and ZO-2 in the establishment and rearrangement of the TJ molecular network in a chronic inflamed milieu.

A number of studies (8, 43) have shown that interepithelial junctions express a high degree of plasticity and may be greatly attenuated in disease states. Our observation that a great number of various junctional molecules are simultaneously affected by the inflamed status in IBD is surprising because molecules of cellular adhesion, cellular polarity, and paracellular pathways are assumed to be tightly controlled in their expression. Inappropriate intestinal inflammation could be one mechanism responsible for “uncontrolled” downregulation of several classes of junction-associated molecules in IBD (29, 48, 54). Most findings implicate cytokines, sex steroids, transforming growth factor, hepatocyte growth factor, tumor necrosis factor-α, and others as agents that regulate barrier integrity at the posttranscriptional level (5, 40). Our study gives further evidence for a posttranscriptional regulation of barrier integrity in IBD because E-cadherin mRNA transcripts were clearly expressed in actively inflamed mucosal tissues of UC and CD, whereas the immunoreactive mature E-cadherin protein could not be detected. Because erroneous and misfolded proteins are subject to rapid degradation, one can assume various somatic mutations of the E-cadherin gene as an explanation for this discrepancy. However, partial sequencing of the E-cadherin gene in different diseased individuals revealed no mutations (data not shown). In addition, our data also show that expression of occludin, β-catenin, and desmoglein-2 is apparently regulated at the transcriptional level because the amount of different mRNA species was in accordance with the levels of the respective immunoreactive proteins. To clarify the importance of exaggerated and inappropriate immune reactions for the expression of junction-associated molecules in IBD, characterization of intercellular junctions in types of colitis other than IBD is evidently necessary.

Our study revealed that disorganization of interepithelial junctions was apparently more pronounced in UC than in CD tissues. Generally, disorganization of interepithelial junctions is suggested to play a role in cellular dedifferentiation and carcinogenesis (19). Thus our results could have importance for two observations: 1) intestinal epithelial cells have a increased turnover in UC, with a marked increase in actively inflamed mucosa, whereas crypt cell proliferation is apparently normal in CD (13), and 2) development of malignancies is apparently more frequent in UC than in CD (37).

Experimental evidence points to an involvement of E-cadherin, β-catenin, APC, and GSK-3 in tumorigenesis (32, 34, 53). In sporadic colorectal carcinomas, loss of E-cadherin expression has been recorded and suggested as one important mechanism that determines invasive behavior (19). In colon cancer cell lines, formation of a complex including GSK-3, the tumor suppressor APC, and the signaling molecule β-catenin may imply that inactivation of the serine/threonine kinase GSK-3β as a downstream event of Wnt-1 signaling could promote tumorigenesis (32, 53). Our study reveals that expression of the molecules detailed above is changed to a greater extent in UC than in CD. In actively inflamed UC tissues, immunoreactive E-cadherin was totally absent and expression of β-catenin and APC was dramatically reduced, but GSK-3 expression was preserved. In addition, we observed that cellular junctions of the epithelium located at the luminal surface and the upper part of crypts were highly affected, a location in which development of dysplasia is frequently seen in UC. In conclusion, disturbances of junction-associated molecules are likely to be involved in carcinogenesis in IBD.

In inactively inflamed IBD tissues, the expression of junction-associated proteins that are structurally characterized as junctional integral membrane components, e.g., E-cadherin, occludin, and desmosomal cadherins, was variably affected. Altered or reduced expression of such proteins could favor the influx of luminal antigens and consequently the inflammatory damage as well as the chronic character of IBD.

In noninflamed IBD tissues, expression of all the junctional molecules investigated was not different from that seen in normal control tissues. Despite other reports (2, 17, 26, 33, 39, 44, 45, 52) that intestinal permeability is clearly increased in noninflamed IBD mucosa, especially in CD patients and their relatives, our data collectively do not indicate a primary defect in the expression of major junctional proteins in IBD. However, further as yet undefined junctional components or incompletely characterized molecules such as junction adhesion molecules (28) might be involved in the regulation of intestinal permeability.

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