Immunolocalization and expression of kinin B₁R and B₂R receptors in human inflammatory bowel disease

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ical UC activity was assessed using a modification of the Truelove and Witts (23a) UC activity index. Patients with a score less than or equal to two were classified as mild colitis, those with a score from three to six were regarded as moderate colitis, and those with a score from seven to nine were considered as severe colitis (20). Disease activity of patients with CD was calculated according to the index of Best et al. (3). Patients with a score of 150 or greater were classified as having active CD. Exclusion criteria included systemic infections and other serious cardiopulmonary, liver, or renal diseases and patients with impaired fulminant colitis.

Blood was obtained from all patients and controls by venipuncture from the antecubital vein and anticoagulated in 3.8% sodium citrate [9:1 (vol/vol)]. All samples were centrifuged at 3,000 g for 15 min. Plasma samples were stored at −70°C until tested. Patients were studied in accordance with the protocol approved by the Institutional Committee on Human Subjects of the Medical University Hospitals. The patients gave written, informed consent.

**Immunolocalization of kinin receptors in human colonic tissue.** The specificity of rabbit anti-human polyclonal antibody reactivity to B2 (mix AS287–83) and B1 (mix AS434) used by us in all experiments has been reported. Antibodies were kindly donated by Dr. Werner Muller-Esterl, Institute of Biochemistry II, The University of Mainz (Mainz, Germany). Eight samples of normal colonic tissue were obtained from patients undergoing partial colectomy for colon cancer, and inflammatory intestinal tissue samples were obtained after surgery from UC or CD patients. Tissue samples were fixed overnight in 10% neutral-buffered formalin (phosphate buffer). The samples were subsequently passed through graded alcohol solutions, processed, and embedded in paraffin blocks. Slices of 5 μm thickness were placed on Apes-coated slides, deparaffinized, and rehydrated. To unmask the antigen, sections were boiled in 0.01 M citrate buffer (pH 6.0) in a microwave oven for 10 min at 800 watts. Thereafter, sections were allowed to stand undisturbed for 20 min and washed two times (5 min each) in Tris-buffered saline (TBS, 0.05 M, pH 7.4, and 0.85% NaCl). For quenching of endogenous peroxidase activity, tissue sections were blocked with 3% (vol/vol) H2O2 for 10 min. Before incubation with the primary antibody, the sections were washed two times (5 min each) in TBS and were pretreated with normal goat serum for 30 min. The primary rabbit polyclonal antihuman antibodies to B1-R and B2-R were added, and the sections were incubated overnight (4°C). This step was followed by incubation with biotinylated secondary antibody, and final visualization was achieved by using the avidin-biotin complex peroxidase kit (Vector Laboratories, Burlingame, CA), freshly prepared 3.3'-diaminobenzidine tetrahydrochloride, and hydrogen peroxide according to the protocol provided by the manufacturer. Normal rabbit serum instead of anti-B1-R or anti-B2-R antibodies was used as a control (9, 21).

**Expression of B1R mRNA and B2R mRNA kinin receptors.** In the first series of the experiment, specimens of rectal biopsies were obtained from patients with UC (n = 28) and inflamed controls (n = 27) during colonoscopy. There were 12 UC men and 16 women, with a median age of 44 yr. The control subjects (healthy subjects and individuals with gastrointestinal disturbances) were compatible with both subgroups (active and inactive UC patients) and matched for age and sex. Mucosal disease activity of UC patients was assessed as active (n = 17 UC patients) or inactive (n = 11 UC patients) using the modification of endoscopic index of Baron et al. (2), taking into consideration the mucosal vascular pattern, erythema, friability, granularity, erosion, and ulceration. The patients were given conventional medical treatment. All UC patients received sulfasalazine or its derivatives. Five UC active patients and two UC inactive patients were taking moderate doses of corticosteroids, and three of the UC patients (two in the active stage and one in the inactive stage) were on azathioprine. The UC colonic extent of disease was variable; however, the specimens were taken from the rectum in all cases. The diagnosis of UC was confirmed histologically; rectal biopsy specimens were stained with hematoxylin and eosin to determine the grade of histological inflammation.

For molecular evaluation, rectal biopsy specimens of ~4 × 4 mm were removed by endoscopic forceps. The tissue samples were immediately frozen at −70°C until use. The expression of B1 and B2 kinin receptor genes in biopsy specimens was evaluated as a number of mRNA copies by QRT-PCR using Sequence Detector ABI PRISM 7700 by Perkin-Elmer. The single-step method of mRNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction was employed.

The numbers of mRNA molecules for B1R and B2R in 1 μg total RNA were detected with an ABI PRISM 7700 Sequence Detector and with the primers and probe designed with the use of Primer Express 10 software. This was done on the basis of mRNA sequences obtained from the internet databases of http://www.ebi.ac.uk and http://www.ncbi.nlm.nih.gov/PubMed in relation to the RT-PCR for β-actin, a marker of the number of copies for all genes studied in each reaction. We used the following complementary oligonucleotides: B2-R, FBR2: 5'-CAG GGT GCT AGT CCT GGT GAA-3'/B2-R: 5'-TGC AGG TCC GTA GTC CGC-3'; B1-R, FBR1: 5'-CGT CAC AGA GTG CTG CCG ACA TT-3'/BRB1: 5'-ACA CCA GAT CAG GTG CCA GG-3'/ and SRB1: 5'-CCG GCA ACT GAC GTG GCA GAA-3'. Probes used for the quantitative RT-PCR analysis were coupled with the following fluorescent aducts: 5'-end with 6-carboxyfluorescein, 3'-end with 6-carboxy tetramethylrosamine. The number of mRNA molecules for B1-R and B2-R in 1 μg total RNA were quantified using real-time PCR (TaqMan). First, a standard curve was constructed after amplification of the five concentrations of β-actin standards (260, 2,600, 5,200, and 26,000 copies) using a β-actin Control Reagent Kit (Applied Biosystems). A standard curve was created for each separate experiment. As constructed, a 10-fold increase in the number of copies for β-actin mRNA contributes to the increase of twofold of the threshold cycle (Ct). The Ct is the number of cycles before the fluorescence emitted reaches a fixed limit. The log10 of the number of targets initially present in the sample is proportional to the Ct value and can be measured using the standard curve. In addition, for each sample, a number of mRNA molecules (constitutive gene) copies was evaluated as an endogenous control of real-time PCR reaction. Finally, the number of B1-R and B2-R mRNA molecules present in each examined sample was calculated for 1 μg total RNA. Total RNA level was measured spectrophotometrically using the program GeneQuant DNA/RNA Calculator (Pharmacia).

**Kinin receptor levels in intestinal tissue samples.** In the second series of the investigation, colonic tissue specimens were taken from 15 UC active patients (10 men and 5 women, 50 yr age average), 15 UC inactive patients (8 men and 7 women, 44 yr age average), and 12 normal control subjects (6 men and 6 women, 49 yr age average). These biopsy samples for immunohistochemical analysis were transferred directly to tubes containing 10% buffered formalin, fixed for 4 h, and prepared as described above.

In the third series of the study, the colonic tissue samples were obtained from 10 active CD patients undergoing partial intestinal resection (4 men and 6 women, 39 yr age average) and 10 controls (normal colonic samples) matched according to sex and age. All UC patients were treated with sulfasalazine, five active and two inactive patients with corticosteroids, four active and four inactive patients with azathioprine. Six CD patients had corticosteroids, with three of these additionally having azathioprine and two azathioprine alone.

Tissue samples were prepared and stained with rabbit anti-human polyclonal antibodies for B1-R and B2-R, as described above. The number of positively stained epithelial cells for B1-R and B2-R in eight different areas (173 × 130 μm) of each tissue sample was determined using computer-assisted image analysis. In each enterocyte, the intensity of staining was measured as the optical density of the reaction product, as described previously (13), with the program KS 100.
VIDAS video image analyzer served by IBAS 2.5 system and a Panasonic WV-CL700 digital camera (Kontron Elektronic, Warsaw, Poland) at a magnification of 25-fold (10, 11, 21). For each analyzed area, 173 × 130 μm average optical density per unit area was calculated. Finally, the arithmetic mean was calculated from 160 microscopic images for controls (20 tissue samples × 8 areas), 120 for UC, and 120 for CD.

Statistical analysis. The significance of differences between the means of each group was determined by the Student’s t-test. In the immunochemical data, the means were compared using the Kolmogorov-Smirnov test and the Mann-Whitney U-test. Because the mRNA concentration was distributed logarithmically, we evaluated the significance of the differences between the controls, active UC patients, and inactive UC patients for the measurement for B2R mRNA and B1R mRNA expression using the logarithmic values of each parameter. The means were compared using the Mann-Whitney U-test. All values were expressed as means ± SE.

RESULTS

Immunohistochemical localization of kinin B2R and B1R. The specificity of rabbit polyclonal antibodies to human B2 and B1 kinin receptors used by us has been reported previously (1). We visualized both B1 and B2 kinin receptors in normal and inflammatory human colon and ileum. Figure 1A shows local-

Fig. 1. Immunolocalization of kinin B1 and B2 receptors in human normal and inflammatory bowel disease colonic tissue. A: B2 receptor in normal colon, with more intense staining in the apical part of epithelial cells; magnification ×280. B: B2 receptor in enterocytes of ulcerative colitis (UC) colon. Specific staining mainly in the basal part of enterocytes; magnification ×360. C: B2 receptor in vessel endothelium of Crohn’s disease colon; magnification ×280. D: B1 receptor in epithelial cells of normal colon; magnification ×280. E: B1 receptors in enterocytes of UC colon; magnification ×360. F: B1 receptor in enterocytes of Crohn’s disease colon. Positive reaction mainly in the apical part of the cells; magnification ×560. G: specific staining for B1 in nerves localized in UC colon; magnification ×280. H: B1 receptor in macrophages within granuloma; magnification ×200.
Zonation of immunoreactive B₂R in the epithelial cells, mostly in the apices of enterocytes of the normal human colon.

Figure 1B demonstrates B₁R mainly intracellularly and in the basal part of enterocytes in inflammatory UC tissue. In Fig. 1C, staining for B₂R is shown in the endothelial cells of blood vessel walls in the colonic submucosa. Figure 1D shows B₁R localization in the epithelial cells of normal colon, mainly immunostained in the basal part of enterocytes (in striking contrast to B₂R localized mainly in the apical part). Figure 1E presents the positive reaction for B₁R in the gladaris mucosa of UC-inflamed colon, with positive staining in the apical part of enterocytes and intracellularly. Figure 1F shows B₂R in the apaxes of enterocytes in CD inflammatory tissue and in plasmocytes. Figure 1G presents the positive staining for B₁R in the nerve of the colonic submucosa, whereas Fig. 1H presents the immunoreactive staining for B₂R in macrophages inside granulomas of CD intestine. However, specific antibodies against B₂R did not show a positive reaction in granulomas (data not shown). Intestinal sections treated with nonimmune rabbit serum instead of B₁Ro rB₂R antibodies were always (data not shown). Intestinal sections treated with nonimmune rabbit serum instead of B₁Ro rB₂R antibodies were always (data not shown).

Quantitation of kinin receptors in intestinal samples. Positive immunohistochemical reaction for both B₁R and B₂R was observed in a vast majority of enterocytes in both normal and inflamed colon. To compare the level of both B₁R and B₂R in normal and inflamed intestinal tissue, we quantified the intensity of staining in mucosal tissue samples using image analysis. The units are optical density per unit area (see Kinin receptor levels in intestinal tissue samples). The mean level of B₁R in the apical part of enterocytes in active UC (124.5 ± 5.8) and inactive UC (107.9 ± 7.7) was significantly (P < 0.0001) higher compared with noninflamed control (50.5 ± 1.9; Table 1). However, there was no statistical difference in the mean level of B₂R in the apaxes of enterocytes between the UC active and UC inactive patients. In contrast, the mean level of B₂R was found to be significantly higher (P < 0.001) in the apical part (130.6) and basal part (101.3) of enterocytes in CD patients compared with controls; (107.4 and 101.3, respectively). In contrast, the mean level of B₂R significantly decreased (P < 0.001) in the apical part of CD enterocytes (143.9 ± 25.0) compared with controls (165.4 ± 22), whereas in the basal part of CD enterocytes, the B₂R level was higher (128.2 ± 20, P < 0.001) than in controls (109.2 ± 18.6).

B₁R and B₂R gene expression. In patients with the active phase of UC, mRNA expression for B₁R (887 ± 292 no. of mRNA copies/μg RNA) was significantly higher (P < 0.02) compared with the mRNA level for B₂R (221 ± 52). However, in UC inactive patients, B₁R and B₂R expression levels did not differ significantly. The mean level of copies for B₁R in UC active patients (887 ± 292) and in UC inactive patients (1,660 ± 298) was significantly lower compared with controls (5,420 ± 1,316). Similarly, B₂ mRNA levels were significantly lower (P < 0.01) in active UC (221 ± 54 mRNA copies/μg RNA) and inactive UC (934 ± 431) patients compared with noninflammatory controls (5,500 ± 1,795). Finally, the B₁-to-B₂ expression ratio was calculated in normal and UC inflammatory tissue (Table 4). The B₁-to-B₂ ratio was 0.9 in normal tissue, was increased in the UC active phase (1.8), and was found to be the highest in the UC active phase (4.03).

DISCUSSION

Recently, we have focused our attention on the TK-kinin system in IBD. To study the localization of TK and its

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Values are means ± SE; units are optical density (OD). B₁R, bradykinin receptor 1; B₂R, bradykinin receptor 2; UC, ulcerative colitis. P < 0.05, statistical significance, active UC and *inactive UC vs. controls and †inactive UC vs. active UC.

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Values are means ± SE; units are OD. *Statistical significance between active UC and controls, P < 0.0271.

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<th>Table 3. Level of B₁R and B₂R proteins in Crohn’s intestinal tissue and controls</th>
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Values are means ± SE; units are OD. *P < 0.001. †P < 0.01.
In inflamed active IBD tissue, we documented a significantly higher B1R protein level compared with the controls, in all probability stimulated by endotoxin, which is detectable in almost all active IBD patients and, in turn, may mediate endothelial perturbation that results in leukocyte stimulation with cytokine release. We also observed a colocalization of TK and kallistatin as well as kinin receptor proteins in the endothelium of intestinal vessels. In macrophages forming granulomas, we found positive staining again for TK kallistatin and the B1R, but not the B2R, indicating the significance of the B1R-related pathway in Crohn’s inflammation.

Despite a moderate increase of B1R protein level in active inflammatory tissue, the mRNA level for both B1R and B2R was found to be reduced in inflamed colon compared with the normal control. The discrepancy between the receptor protein concentration and its mRNA level is difficult to explain. In UC, local homeostasis is disrupted, leading to active inflammation to epithelial cell loss and ulcer formation. However, in the present study, we calculated gene expression for kinin receptors in relation to β-actin gene expression. The β-actin gene is unchanged in inflammation and thus serves as an endogenous denominator of gene expression from the same cell. Thus the decrease of both B1 and B2 kinin receptor encoding genes is the result of an alteration of transcription rather than an effect of cell loss. It is well recognized that exposure to endotoxins may cause, in vitro and in vivo, a refractory state to the synthesis of cytokines and nitric oxide. Alternatively, an abundance of protein for both kinin receptors in the vast majority of enterocytes may feed back to inhibit new kinin receptor synthesis and lower mRNA level in inflamed tissue. In addition, the B1R may be recycled several times in the same enterocytes after internalization (1a). This process is supported by the appearance of B1R intracellularly in some enterocytes in UC intestine (17). In contrast, the B2R is thought to not be internalized, but rather redistributed, to caveolae-related lipid rafts in the cell membrane (14). Regardless of transcriptional and/or translational modification, the most striking of our results is the increase in the ratio of B1R to B2R gene expression in relation to the degree of intestinal inflammation. Thus the increase of B1R gene expression (relative to B2R gene expression) is concomitant with an increase of B1R protein concentration in UC active intestine, indicating that B1R is a major structural background for kinin function in IBD. These observations provide insight in the pathogenesis of IBD and suggest that selective B1R inhibitors may have potential in a therapeutic trial. However, we have found (24) that B1R antagonists in rat enterocolitis appear to aggravate the inflammation, suggesting that further studies are needed.

What is the role of kinins in the inflamed colon? BK not only has many proinflammatory actions, as indicated earlier, but also can stimulate release of mediators for endothelial, epithelial, and white blood cells, such as thromboxanes (12), nitric oxide (8), and cytokines, known to be important in IBD. Receptors for these reactions are present in the human intestinal epithelium; thus, BK can initiate these inflammatory reactions in the inflamed ileum and colon.

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REFERENCES


