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Pathogenetic mechanisms of IBD: participation of intestinal microflora and immunological factors

Summary of PhD Thesis

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Contents

Introduction …… 4

Aims …… 5

Materials and methods …… 6

Results …… 8

Discussion and conclusion …… 10

References …… 13

Bibliography …… 16

Introduction

IBD such as Crohn's disease (CD) and ulcerative colitis (UC) are severe chronic disorders with periods of remission and relapse which affect the gastrointestinal tract. The etiology and pathogenesis of IBD remain unclear despite intense study. IBD appear to involve interactions between immune, enviromental and genetic factors; the combination of these factors results in induction of inflammation, subsequent mucosal lesions and their repair, an altered host response to the intestinal microflora assumed to contribute to disease manifestation (Shanahan, 2001; Hibi et al., 2006; Scaldaferri et al., 2007).

It was suggested that crucial role in the pathogenesis of IBD play intestinal microbiota (Duchmann et al., 1995; Hudcovic et al., 2001). Intestinal macrophages and dendritic cells located below the epithelium in the lamina propria are the first phagocytic cells of the innate immune system which interact with micro-organisms and their products through for example Toll-like receptors (TLRs) (Tlaskalova et al., 2005). Recognition of microbes activates NF-κB signaling pathway, triggering production of cytokines and other inflammatory mediators (Aderem et al., 2000; Medzhitov et al., 2000; Akira et al., 2004). $CD4^{+}$ T-lymphocytes also participate in the pathogenesis of CD and UC. In Crohn's disease there is predominantly a T-helper cell type 1 (Th1) response and in ulcerative colitis there is Th2 response develop in the genetically susceptible host (Hanauer et al., 2006; Young et al., 2006).

Aims

1. To characterize the expression of Toll-like receptor 2 (TLR2), TLR4 and their transmembrane coreceptor CD14 in the intestinal mucosa obtained from different parts of the intestine from patients with ulcerative colitis (UC) and Crohn` s disease (CD), and compare it with controls.

2. To determine whether galectin-3 (Gal-3) as an indicator of the association with disease manifestation can be detected in serum of patients. Moreover, to introduce reverse lectin histochemistry to visualize binding sites for this lectin on *E. coli* O83 in inflamed intestinal mucosa (biopsies) from patients with Inflammatory Bowel Diseases (IBD). These lines of our study are aimed at defining clinical relevance of this tissue lectin for the disease.

3. To analyze whether manipulation of intestine microflora by probiotics could affect experimentaly induced intestinal inflammation by examining whether repeated preventive oral administration of live probiotic strains *Escherichia coli* O83: K24: H31, *E. coli Nissle* O6: K24: H31 and *Lactobacillus casei* DN 114001 could protect mice against DSS-induced colitis.

Materials and methods

Study objects and ethics

All blood and tissue samples for furthes studies were collected from IBD patients (active and remission stage) and non-IBD controls. Patients were under thorough medical supervision at the Teaching Thomayer Hospital, Prague, the Institute of Clinical and Experimental Medicine, Prague and IBD Clinical and Research Center Iscare IVF. In each individual case the diagnosis (UC or CD) was confirmed by standard endoscopy, histological criteria and disease activity were graded according to the CD activity index or Mayo score (Best, 2006). Control samples were taken from patients who underwent colonoscopy because of colon cancer screening examination; all of them had normal endoscopic findings without macroscopic and microscopic evidence of inflammatory or neoplastic disease. Blood samples were also obtained from healthy volunteers. Informed consent was obtained from all patients and the protocols was approved by the Human Studies Committees.

The experiments with mice were approved by the Commission for Experimental Animal Studies of the Academy of Science of the Czech Republic.

Imunohistochemistry

Fresh tissue samples were frozen in liquid nitrogen.
Immunohistochemistry was used to characterize the expression of TLR2, TLR4 and their transmembrane coreceptor CD14 in the intestinal mucosa from patients with UC and CD and controls. The sections were analyzed under an Olympus BX 40 microscope equipped with Olympus DP 70 camera.

To determine presence of Gal-3 on inflamed tissue samples and its potencial role in binding (adhesion) of bacteria, multiple labeling at the single-cell level was performed. The specimens were inspected and analyzed using an Eclipse 90i fluorescence microscope (Nikon,) equipped with suitable filterblocks, a high resolution Vosskühler Cool-1300Q CCD camera and a computer-assisted image analyzer (LUCIA 5.10, Laboratory Imaging, Czech Republic).

SDS-PAGE and Western blot

Western blot analysis was carried out to determine whether Gal-3 is present in serum samples of IBD patients and controls.

ELISA

Concentrations of Gal-3 were measured by a commercial ELISA following the manufacturer`s instructions (BMS 279; Bender Medsystems GmbH, Austria) in patients with IBD and controls.

Concentration of specific antibodies against probiotic bacteria was evaluated by ELISA using bacterial sonicates similarly to Lodinova-Zadnikova (1991).

Conjugation of *E.coli* O83 with FITC

Fluorescent surface labeling of *E. coli* O83 with FITC was performed as described previously (Kwapinski, 1972).

Preparation of bacterial strains

Strains were cultivated to the stationary growth phase. *E.coli* O83 and *E. coli* Nissle were cultured on tryptan-soya agar (Oxoid), Lactobacillus casei on MRS agar (Oxoid).

Mice

Balb/c mice aged 17 weeks (n=60) were fed for 14 d with live bacterial suspension (n=20 in one group for each bacterial strain). Mice in the control group (n=15) were fed with PBS. DSS colitis was induced by administering 5% DSS (MP Biomedicals) in drinking water for 7 d as described Hudcovic et al. (2001) to 10 mice in every bacterial group and to 10 mice in the control group.

Results

Expression of Toll-like Receptor 2 (TLR2), TLR4, and CD14 in Biopsy Samples of Patients With Inflammatory Bowel Diseases: Upregulated Expression of TLR2 in Terminal Ileum of Patients With Ulcerative Colitis.

Frolova L, Drastich P, Rossmann P, Klimesova K, Tlaskalova-Hogenova H. J Histochem Cytochem 56: 267-274, 2008.

Immunohistochemistry showed statistically significant upregulation of TLR2 protein expression was observed in the ileal epithelium from UC patients with inactive (0.88 ± 0.35) and active disease (0.94 ± 0.30) as compared to the normal intestine (0.41 ± 0.46). Moreover, significant difference of TLR4 expression were found in the terminal ileum (1.06 ± 0.68) and rectum (1.75 ± 0.60) of UC patients in remission and in the terminal ileum (1.05 ± 0.60) of CD patients with active disease as compared to controls (0.38 ± 0.43; 0.94 ±0.63). Mononuclear cells in the lamina propria displayed strong membranous expression of CD14 in the terminal ileum of CD patients in remission (1.64 ± 0.74) and active stage (1.50 ± 0.35) as compared to controls (1.04 ± 0.37) and in the caecum of UC patients in remission (1.63 ± 0.44) and with active disease (1.95 ± 0.42) and in the rectum of UC patients with active disease (1.85 ± 0.58) as compared to controls (1.11 ± 0.40; 1.08 ±0.56).

Detection of galectin-3 in patients with Inflammatory Bowel Diseases: New serum marker of active forms of IBD?

Froľová L, Smetana K Jr, Borovská D, Malíčková K, Janatková I, Lukáš M, Drastich P, Beneš Z, Kitanovičová A, Klimešová K, Tučková L, André S, Gabius H-J, Tlaskalová-Hogenová H.

Western blot analysis showed that Gal-3 is present in serum and its concentration in sera from IBD patients appeared to be increased relative to controls.

For quantitative measurement of Gal-3 Elisa kit was used. UC patients with active disease (mean, 6.13 ng/ml) and in remission (mean, 4.40 ng/ml) presented a significantly higher concentration of Gal-3 in the serum than seen in control samples (mean, 2.38 ng/ml). Moreover, in the group of CD patients, a statistically significant difference was

found in patients with active disease (mean, 5.65 ng/ml) and in patients in remission (mean, 3.42 ng/ml) as compared to controls (mean, 2.38 ng/ml).

By immunohistochemistry, strong expression of Gal-3 was determined on $CD14^+$ cells in biopsies from IBD patients, while positive enterocytes were minimal. Moreover, colocalization between cells interacting with FITC-labeled bacteria and expressing Gal-3 was detected. Tissue samples from non-IBD controls revealed no Gal-3 presence on $CD14^+$ cells but on enterocytes. Binding sites for bacteria were seen on $CD14^+$ cells in IBD patients. On the other hand, no $CD14^+$ cells were found in non-IBD controls. Furthermore, biopsy samples from non-IBD controls failed to be reactive with FITC-labeled bacteria.

<u>Oral administration of probiotic bacteria (E. coli Nissle, E. coli O83, Lactobacillus casei) influences the severity of dextran-sodium-sulfate-induced colitis.</u>

Kokesova A, <u>Frolova L</u>, Kverka M, Sokol D, Rossmann P, Bartova J, Tlaskalova-Hogenova H

Relative to animals from PBS and DSS groups, colitic mice fed with probiotic strains exhibited a decrease in the symptom score (was constructed attributing 1 point to each of the following events: rectal prolapse, rectal bleeding, colonic bleeding and death) Ec O83 (1.44 ± 0.33), Ec Nis (0.85 ± 0.54), Lc (0.65 ± 0.32) as compared to PBS control (2.20 ± 0.18) and had significantly longer colon at the end of the experiment. There was significant decrease in mass loss of colitic Lc mice. While severe inflammation, crypt destruction and ulceration of the mucosa were observed in the PBS-fed group by histology evaluation (1.23 ± 0.17), significantly less inflammation was seen in the Lc-fed group (0.86 ± 0.23). Mice exposed to both strain of *E. coli* had the inflammation score comparable to PBS control group (1.23 ± 0.17). Mice drinking water instead of DSS had no colonic inflammation.

ELISA showed a significant difference in IgA Ab content between Ec Nis (18 ± 3.4) and Lc (20 ± 2.0) pretreatment in DSS colitic mice as compared to healthy colonized mice (11.5 ± 3.4; 15 ± 1.5) while Ec O83 stimulated intestinal IgA production in healthy mice. No difference in specific IgM Ab production between mice with DSS-induced colitis and control animals was found. There was a significant difference in specific IgG Ab production in Lc group with DSS-induced colitis (50 ± 2.7) and healthy Lc-colonized mice (45 ± 3.7).

Discussion and Conclusion

In our study we are interested in the pathogenesis of IBD in terms of innate immunity. Controversial data of other authors (Cario et al., 2000; Cario et al., 2002; Hausmann et al., 2000; Melmed et al., 2003) about the expression of TLR2 and TLR4 on mRNA and protein level obtained from intestinal biopses (usually only colon) or intestinal epithelial cell (IEC) lines led us to characterize the expression of TLR2, TLR4 and their transmembrane coreceptor CD14 in the intestinal mucosa obtained from different parts of intestine including terminal ileum from patients with UC and CD. We focused our interest on three different parts of the bowel, which can play different roles in the development of the disease and expression of TLRs and CD14 in patients and control individuals. It was found that CD is associated with increased expression of CD14 in the terminal ileum and that UC is characterized by upregulation of CD14 in caecum and rectum (the areas of disease localization). Other authors in compliance with us showed that nearly a third of lamina propria macrophages in the inflamed mucosa of patients with IBD express CD14 (Rugtveit et al. 1994; Grimm et al. 1995; Rogler et al. 1997). Furthermore, CD displays upregulation of TLR4 in the terminal ileum, in UC upregulation of TLR4 is localized in rectum and TLR2 in the terminal ileum irrespective of the localization and activity of the disease. According to Duchmann (1995) and Lange (1996) increased TLR4 expression in IBD patients could be the consequence of impaired host tolerance towards luminal LPS antigens. On the other hand, large quantities of luminal LPS are usually well tolerated within the healthy intestine. The immunohistochemical findings have been confirmed by studies measuring TLRs expression on mRNA level (Hausmann et al. 2000; Abreu et al. 2002; Melmed et al. 2003; Furrie et al. 2005).

Based on the results of our study, we can suggest that epithelial cells are the predominant site of TLR2 and TLR4 expression in intestinal mucosa. Interestingly, in case of UC marker of increased immune activity (upregulation of TLR2) is distributed not only in the inflamed parts of the intestine but also in the non-inflamed part (terminal ileum).

At the beginig of our next report was a suggestion that Gal-3 is able to play an important role in modulation of chronic inflammatory disorders such as inflammatory bowel diseases (IBD) and other autoimmune diseases (Mey et al., 1996; Sano et al., 2000; Rabinovich et al., 2002; Sano et al., 2003; Almkvist et al., 2004; Nieminen et al., 2005) and evidence that enterocytes are the source of tissue Gal-3 and, in IBD, Gal-3 is downregulated in these cells (Jensen-Jarolim et al., 2002; Muller et al., 2006; Shiobara et al., 2007). We addressed

the question whether Gal-3 as an indicator of an association with disease manifestation can be detected in serum of patients. Moreover, we introduced reverse lectin histochemistry to visualize for example the binding sites for this lectin on *E. coli* O83 in inflamed intestine mucosa (biopsies) from patients with IBD. ELISA revealed that Gal-3 was significantly upregulated in the serum of patients with UC (active and remission) and CD (active and remission) as compared to healthy controls. Applying multiple labeling at the single-cell level binding sites for labeled bacteria were visualized on $CD14^{+}$ cells (monocytes/macrophages) in the lamina propria of IBD patients. No binding sites for bacteria were found in tissue samples from non-IBD patients. We observed CD14-positive cells with Gal-3 in the biopsy samples from IBD patients, while biopsies from healthy controls presented positivity in enterocytes. In consequence, intestinal macrophages and dendritic cells located below the epithelium in the lamina propria are the first phagocytic cells of the innate immune system that interact with microorganisms and their products (Tlaskalova et al., 2005). Human intestinal macrophages in normal mucosa do not express innate immunity receptors including CD14 (Smith et al., 2001). However, monocytes recruited to the inflamed mucosa do not undergo such stringent downregulation and retain their pr-inflammatory potential and functional profile (Rogler et al., 1997; Smith et al., 2005).

These results implicate Gal-3-positive cells as being active in bacterial adhesion and Gal-3 then could be involved in the pathogenesis of IBD. Food-derived antigens and changes in microbiota composition could be initial signals for the onset of development of a chronic inflammatory process involving CD14-positive cells (McDonald et al., 2005; Muller et al., 2006; McDermott et al., 2007).

The involvement of gut microbiota in pathogenetic mechanisms led us to examine if preventive oral administration of live bacterial strains *E. coli* O83, *E. coli* Nissle and *Lactobacillus casei* can protect mice against DSS-induced colitis. Our experiments were supported by previous studies where authors suggested a potential role of probiotic preparation in the treatment of IBD (Schultz et al., 2003,) or demonstrated beneficial effect of *E. coli* Nissle treatment in the maintenance of remission of UC (Rembacken et al., 1999; Kruis et al., 2001). We shoved that manipulation of intestinal microflora could affect DSS-induced intestinal inflammation. *E. coli* O83, *E. coli* Nissle and *Lactobacillus casei* (Lc) ameliorate the clinical signs of intestinal inflammation, as was demonstrated by a lower symptom score, healthier clinical appearance of the animals and partial restoration of normal in colon length. Lc-precolonized mice had also a significantly lower mass loss at

the end of the experiment and lower histological inflammation score in comparison with untreated mice with DSS-induced colitis. A significant difference between probiotic-colonized mice with DSS-induced colitis and healthy probiotic-colonized mice was found in the level of specific IgA against bacterial strains in enteral contents. Moreover, a significant difference in total specific IgG in serum between DSS-colitic and healthy mice was determined in Lc-pretreated mice.

Our data corroborate the evidence that nonpathogenic luminal bacteria participate in the pathogenesis of chronic intestinal inflammation. Administration of probiotic bacteria influence the composition of intestinal microflora may be relevant to the development of a novel strategy in the prevention and management of IBD patients.

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