ABSTRACT

The aim of this study was to investigate the expression patterns of p16, p53 and VEGF in affected tissue and serum levels of cytokines TNF-α, IL-6, TGF-β and IL-17 in patients with ulcerative colitis (UC) and fistulating Crohn’s disease (CD). Serum levels of cytokines in patients with ulcerative colitis (n=24) and with Crohn’s disease (n=7) were analysed by ELISA. In colonoscopically obtained biopsies, p16, p53 and vascular endothelial growth factor expression were evaluated by immunohistochemistry.

The results of this study clearly show the predominance of pro-inflammatory type 1 and 17 immune response in patients with CD compared to those with UC. We believe that altered p16 and p53 induce enhanced VEGF expression and implicates enhanced production of pro-inflammatory TNF-α and IL-6. TNF-α and IL-6 further facilitate development of type 1/17 immune response.

Key words: ulcerative colitis, Crohn’s disease, p16, p53, VEGF, cytokines

ABBREVIATIONS

ABC - Avidin-Biotin peroxidase Complex
CD - Crohn’s disease
CDK - Cyclin dependent kinase
CARD - Caspase activating recruitment domain
DNA - Deoxyribonucleic acid
ELISA - Enzyme Linked Immunosorbent Assay
IBD - Inflammatory bowel disease (IBD)
IFN-γ - Interferon-γ
IL - Interleukins
MAPK - Mitogen Activated Protein Kinase
NF-kB - Nuclear factor kappa light chain enhancer of activated B cells
NOD2 - Nucleotide binding oligomerization domain containing protein 2
NK cells - Natural Killer Cells
VEGF - Vascular endothelial growth factor
TNF-α - Tumour necrosis factor alpha
TGF-β - Transforming growth factor beta
UC - Ulcerative colitis
INTRODUCTION

The inflammatory bowel diseases (IBDs) are a group of inflammatory conditions of the colon and small intestine. IBDs include Crohn’s disease (CD) and ulcerative colitis (UC) and are characterized by spontaneous remittances and relapses (1). The exact cause of IBDs remain unknown. Available evidence suggests that an abnormal immune response against the microorganisms of the intestinal flora is responsible for the diseases in genetically susceptible individuals (2). Ulcerative colitis is characterized by continuous inflammation of the intestinal lamina propria, starting from the rectum and potentially involving the whole colonic mucosa. At present, its pathogenesis is still unclear, but evidence suggests that the disease occurs in genetically susceptible subjects and is trigged by environmental factors, which lead to an exaggerated and uncontrolled immune response (3). Cytokines have a crucial role in the pathogenesis of UC, where they control multiple aspects of the inflammatory response. In particular, the imbalance between pro-inflammatory and anti-inflammatory cytokines that occurs in UC impedes the resolution of inflammation and instead leads to disease perpetuation and tissue destruction (4). Beside monocyte/macrophages, CD4+ helper T lymphocytes are major producers of cytokines and can be classified according to the type of cytokines they produce: Th1 (IFN-γ and TNF-α), Th2 (IL-4, IL-5 and IL-13), Th17 (IL-6 and IL-17) and Tregs (IL-10 and TGF-β) (5).

Crohn's disease, also known as Crohn syndrome and regional enteritis, is a type of inflammatory bowel disease (IBD) that may affect any part of the gastrointestinal tract from mouth to anus (6). Crohn's disease is caused by a combination of environmental, immune and bacterial factors in genetically susceptible individuals (7-9). It results in a chronic inflammatory disorder, in which the immune system attacks the gastrointestinal tract, with possible direction at microbrial antigens (8-10). While Crohn’s is an immune related disease, it does not appear to be an autoimmune disease (in the sense of Good Clinical and Laboratory Practices). The inflammatory and anti-inflammatory cytokines that occurs in UC leads to disease perpetuation and tissue destruction (4). Beside monocyte/macrophages, CD4+ helper T lymphocytes are major producers of cytokines and can be classified according to the type of cytokines they produce: Th1 (IFN-γ and TNF-α), Th2 (IL-4, IL-5 and IL-13), Th17 (IL-6 and IL-17) and Tregs (IL-10 and TGF-β) (5).

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MATERIAL AND METHODS

Patients

From May 2011 until March 2013, patients were enrolled at the Center for Gastroenterology, Clinical Centre Kragujevac. All immunological measurements were conducted at the Center for Molecular Medicine and Stem Cell Research, Faculty of Medical Sciences, University of Kragujevac. All ethical approvals were obtained, and research was conducted in accordance with the regulations of Good Clinical and Laboratory Practices.

We investigated a total of 24 patients with ulcerative colitis (15 males and 9 females; mean age: 48.38 ± 17.94 years) and 7 patients with Crohn’s disease (4 males and 3 females; mean age: 20.75 ± 1.71 years). The diagnoses of ulcerative colitis and Crohn's disease were based on endoscopic and histopathological criteria. The study did not include patients with inflammatory bowel disease who were previously treated with antibiotics, aminosalicylates, corticosteroids, immunosuppressive agents, or biological therapy. All subjects had a complete medical history, including physical examination, routine laboratory tests and diagnostic imaging (chest X-ray, abdominal ultrasound, abdominal computed tomography scan and endoscopy).

Assessment of serum level TNF-α, IL-6, IL-17 and TGF-β

Blood samples were obtained before application of therapy. The control group consisted of 37 healthy male and female blood donors at the Clinical Centre of Kragujevac. The control group was matched with the experimental groups on the basis of gender. Serum was separated, and
all serum samples were kept at -20°C before use. Serum levels of cytokines were measured as described before (28), using sensitive enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Minneapolis, MN, USA for IL-6, TNF-α, TGF-β and IL-17), specific for human cytokines, according to the manufacturer’s instructions.

**RESULTS**

**Immunohistochemical study of VEGF, p16 and p53**

Immunohistochemical staining was performed using the streptavidin-biotin technique, as previously described (29). Immunohistochemistry was performed on multiple endoscopically obtained colonic mucosal biopsy specimens collected from patients with ulcerative colitis and patients with Crohn’s disease. Paraffin-embedded tissue samples were sectioned at 4-5 μm. Briefly, tissue samples were deparaffinised. Antigen retrieval was performed by microwave heating for 20 min in 10 mM sodium citrate buffer (pH 6.0). The sections were incubated with 3% hydrogen peroxide to block endogenous peroxidase activity and then incubated with mouse monoclonal antibodies against VEGF (ab16883, Abcam, Cambridge, UK, at a 1:200 dilution), p53 (ab17869-250, Abcam, Cambridge, UK, at a 1:200 dilution) and p16 (sc-81156, Santa Cruz Biotechnology, Santa Cruz, CA, USA, at a 1:50 dilution) for 60 min in a humidity chamber, followed by incubation with biotinylated secondary antibodies. The ABC (Avidin-Biotin peroxidase Complex) method was used for detection. The slides were examined by conventional light microscopy. Negative controls were treated identically, but with the primary antibodies omitted. Positive controls consisted of tissue known to contain the protein of interest. The VEGF, p16 and p53 stained sections were assessed semiquantitatively by two pathologists. The staining score was evaluated as the percentage of stained cells out of the total number of evaluated cells. The extent of each staining pattern of p16 and p53 was recorded as the number of positive nuclei per 250 cells. The same pattern was used for VEGF in cytoplasm. Percentage of positive cells was determined by counting 5 non overlapping microscopic fields at 400x magnification. Staining for p16, p53 and VEGF was defined as positive when >10% of the cells were stained and as negative when ≤ 10% of the cells were stained (30, 31).

**Serum concentrations of pro- and anti-inflammatory mediators and their ratios in UC patients and patients with Crohn’s disease**

Next, we examined the serum levels of pro-inflammatory (IL-4, IL-6, TNF-α, IFN-γ and IL-17) and anti-inflammatory mediators (IL-10) in patients with ulcerative colitis and Crohn’s disease. Twenty-four patients with ulcerative colitis, 15 males and 9 females with a mean age of 48.38 ± 17.94, and seven patients with Crohn’s disease, 4 males and 3 females
with mean age 20.75 ± 1.71 years, were studied. We obtained a significantly higher serum level of TNF-α (p=0.032; Fig 3A, left panel) and IL-6 (p=0.039; Fig 3A, right panel) in patients with Crohn’s disease, in comparison with the UC patients. It had been suggested that the ratio of counterregulatory cytokines could be a marker of disease progression. Therefore, we analysed ratios of pro- and anti-inflammatory cytokines and found a significantly higher value of IL-6/TGF-β ratio (p=0.045; Fig 3B, left panel) and IL-17/TGF-β ratio (p=0.024; Fig 3B, right panel) in patients with Crohn’s disease compared to patients with UC.

Logistic regression analyses of p53 expression and TNF-α serum level in colorectal inflamed tissue

Binary logistic regression showed that higher expression of p53 strongly correlated with the presence of Crohn’s disease. Analysis showed that p53 can be a valuable marker for differentiating Crohn’s disease from UC (sensitivity 78.6%, specificity 95.2%, cut-off >0; Fig 4, right panel). Analysis also showed that TNF-α (sensitivity 78.7%, specificity 87.5%, Fig 5, left panel) can be a valuable marker for distinguishing UC and Crohn’s disease. The optimal cut-off value estimated for TNF-α that allows discrimination between UC and Crohn’s disease patients was 35 pg/ml. For this cut-off, we determined sensitivity to be 78.7% and specificity 87.5%.

DISCUSSION

In the present study, we found a predomination of pro-inflammatory cytokines in patients with Crohn’s disease compared to patients with UC. Positive staining of p16, p53 and VEGF was detected in the mucosa of both groups, and its expression was significantly higher in Crohn’s disease compared to ulcerative colitis. Furthermore, the expression of p53 and serum values of TNF-α higher than 35 pg/ml present a highly sensitive and specific marker in the differentiation between Crohn’s disease and UC.

The type of immune response is different in CD and UC. CD is characterized by granuloma formation, while the hallmark of UC is infiltration of neutrophils with the destruction of the epithelium (32). Additionally, CD is dominantly followed by a Th1-type immune response (with
the production of IL-2, IL-8, IL-12, IFN-γ and TNF-α) (32), while in UC, a Th2 type immune response dominates (production of IL-4, IL-5, IL-6, IL-10 and IL-13), which stimulates humoral immunity (32). In this study, we estimated significantly higher concentrations of TNF-α and IL-6 in the serum of patients with CD compared to those with UC (Fig 4A). The role of TNF-α in the pathogenesis of CD is well known (33). The high systemic values of TNF-α in patients with CD is in accordance with data from the literature (32-34). According to the aforementioned data, it may be assumed that the increased TNF-α in sera induces IL-6 production, with subsequent elevation of IL-6 in the serum of patients with CD. Increased concentrations of IL-6 in patients with CD, in comparison to UC, can also be explained by the fact that all patients had the fistulising form of CD. The fistulising form of CD can cause changes in colon microenvironment, with subsequent changes in the type of immune response (33).

Furthermore, we analysed ratios of contra-regulatory cytokines IL-6, IL-17 and TGF-β, and documented that patients with CD (compared to patients with UC) had significantly higher IL-6/TGF-β and IL-17/TGF-β ratios (Fig 4B). TGF-β is an inhibitory cytokine and the key regulator of immune homeostasis and inflammation (35). It prevents proliferation of leukocytes and their activation and takes a part in the inhibition and spread of the inflammatory process (35). Decreased activity of the TGF-β is considered to be responsible for the development of a variety of autoimmune diseases, including inflammatory bowel disease (35). TGF-β is a negative regulator of inflammation of the bowel mucosa (36) and also an important factor for the development of regulatory T lymphocytes (37). Regulatory T cells produce a large amount of TGF-β, which plays a significant role in the maintenance of peripheral immune tolerance and immunosuppression (38). Th17 cells are a subclass of lymphocytes, which produce a large amount of IL-17 and IL-23 (39), it is believed that Th17 cells promote tissue destruction during inflammatory processes and are significant in the genesis and development of chronic inflammatory diseases (40). TGF-β, in combination with IL-6, directs the differentiation of naive T cells into Th17 cells (36, 40-43). Participation of TGF-β in the genesis of Th17 lymphocytes is quite surprising because TGF-β alone has an anti-inflammatory effect and is important for the generation of regulatory T lymphocytes (40). It has been assumed that there is a natural antagonism be-

Figure 3. Serum values of mediators of inflammation in patients with UC and CD.
A. Higher serum concentration of TNF-α and IL-6 in patients with Crohn’s disease.
B. Higher values of IL-6/TGF-β and IL-17/TGF-β ratios in patients with Crohn’s disease. IL-6/TGF-β and IL-17/TGF-β ratio was evaluated for each patient, separately. Serum levels of all mentioned cytokines were determined by ELISA. Statistical significance was tested by a Mann–Whitney Rank Sum test or independent samples t-test, where appropriate.
between Th17 and regulatory T cells, and in the absence of an inflammatory environment, the TGF-β produced will induce suppression of the effector T cells and the formation of regulatory T cells, which is important for maintenance of immuno-tolerance (37). In our study, elevated IL-6 in the sera of patients with CD may suppress formation of regulatory T cells and induce the development of a Th17 immune response (37). Additionally, increased production of pro-inflammatory cytokines, such as TNF-α, reduce the secretion of TGF-β (35). IL-17 mediates the pathogenesis of CD, and it is a potent inducer of local infiltrations with neutrophils (44). Additionally, IL-17 induces expression of other pro-inflammatory cytokines (TNF-α, IL-1, IL-6) (45), nuclear growth factor (NF-kB) and the MAPK kinases (46). IL-17 has an effect on fibroblasts and induces the secretion of metalloproteinases, which further contribute to tissue damage (47).

We also analysed expression of anti-oncogenes p16 and p53 and VEGF in affected tissue.

The percentage of patients with CD and a positive expression of p16, p53 and VEGF was higher than those with UC (Fig 1). Vascular proliferation in the submucosa is typical for CD (33). In this study, all subjects with CD had the highest histological grade of inflamed colonic mucosa (histological grade III), which was not the case with subjects with UC. In inflammatory bowel disease, higher expression of p16 and p53 genes correlates with a higher histological grade of inflamed mucosa (48, 49). We assume that a higher degree of histological damage of inflamed mucosa correlates with a higher alteration of p16 and p53 genes. Various studies have revealed that wild type p16 and p53 reduce angiogenesis via down regulation of VEGF (50-52). We proposed that altered p16 and p53 may induce VEGF overexpression and stimulate angiogenesis (29). Higher expression of anti-oncogenes p16 and p53 indicates a loss of function and facilitates neovascularization via VEGF. Our results suggest that enhanced systemic pro-inflammatory immune response correlates with local tissue angiogenesis, in patients with CD.

VEGF also has a role in inflammation and immunity, as a key mediator in recruitment of inflammatory cells and enhanced expression of co-stimulatory molecules on recruited and resident mononuclear cells, leading to up-regulation of the pro-inflammatory cytokines Th1 and Th17 (53, 54).

Finally, we assessed the expression of studied tissue markers and cytokines as useful markers in the assessment of CD. P53 expression showed a high sensitivity and specificity for CD in differentiating from UC (Fig 4). We also envisioned the possible role of TNF-α as a biomarker in distinguishing CD from UC and showed that increased levels of TNF-α enhanced the likelihood of UC, compared to CRC (Fig 4). The results of this study clearly show the predominance of pro-inflammatory types 1 and 17 of immune response in patients with CD compared to those with UC. We believe that altered p16 and p53 induce enhanced VEGF expression that implicates enhanced production of pro-inflammatory TNF-α and IL-6. TNF-α and IL-6 will further facilitate development of Type 1/17 immune response.

Acknowledgements

We thank Aleksandar Ilic, Katerina Martinova, Branislav Stevanovic and Milan Milojcic for their excellent technical assistance. This work was supported by the Serbian Ministry of Science and Technological Development (Grants OP 175071, OP 175103 and OP 175069) and by the Faculty of Medical Sciences, University of Kragujevac, Serbia (Grants JP 09/10).

Competing Interests

The authors have declared that no competing interests exist.
REFERENCES


