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Original article

Digital image analysis of inflammation markers in colorectal mucosa by using a spatial visualization method

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ABSTRACT

The aim of this study was to apply the spatial visualization method of digital images to quantitative analysis of pro-inflammatory cytokines IL-1, IL-6 and TNF-α in various segments of large bowel excised because of colitis ulcerosa in relation with selected clinical symptoms.

Our preliminary study included 17 patients having undergone restorative proctocolectomy. Immunohistochemistry was performed for IL-1, IL-6 and TNF-α. The area fraction and intensity fraction of the cytokines studied were determined by digital image analysis. The results were then categorized using Alfred Immunohistochemistry Score.

The expression of IL-1, IL-6 and TNF-α was significantly higher in the rectum than in colonic segments ($p < 0.01$), and was associated with the patients' clinical condition.

The method of quantitative immunohistochemistry presented here allows for searching associations between the expression of biomarkers and clinical symptoms. Evaluation of inflammatory cytokines could be recommended in the active stage of the disease with present symptoms of bloody and mucus stools. A higher expression of IL-1, IL-6 and TNF in samples beyond large intestine correlates with an intensified clinical course of the disease.

In patients without bleeding and mucus symptoms present in stools, no significant correlations were found. Therefore, the assessment of cytokines during remission or clinically silent stage might not be useful.

Introduction

Research interest in a possible connection between inflammation and cancer has been growing since 1863, when Rudolf Virchow noted the leucocytes in neoplastic tissues and indicated that chronic inflammation supports cancerogenesis [3].

Mediators of the inflammatory response, e.g., cytokines and chemokines, induce the accumulation of immune cells and their activation within the inflamed tissue. This process may lead to the release of reactive oxygen and nitrogen species that can induce genetic and epigenetic changes including point mutations in tumor suppressor genes, DNA methylation and post-translational modifications. This sequence of changes in critical pathways responsible for maintaining normal cellular homeostasis can lead to the development and progression of cancer. Several chronic inflammatory diseases contribute to an increased risk of cancer. Observations that many malignancies are associated with chronic infection and inflammation support this hypothesis, for instance, inflammatory bowel disease is associated with colon cancer. Inflammatory bowel diseases, i.e. Crohn's disease and colitis ulcerosa, are associated with increased rates of colon adenocarcinoma [30]. Tumor necrosis factor can initiate the inflammatory reactions of the immune system, and induces the production of other cytokines (e.g. IL-1, IL-6 and IL-8) and cytotoxic factors (e.g. nitric oxide, reactive oxygen species) by macrophages, which can mediate tumor suppression. Members of the IL-1 family played significant roles in many aspects of the cancerous process as key regulators of the balance between inflammation and immunity in the tumor microenvironment [11,32].

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The importance of IL-1, IL-6 and TNF in the severity of inflammation in ulcerative colitis has been confirmed in clinical and experimental studies [1,12,13].

Increased levels of those proinflammatory cytokines are detected in active inflammatory bowel disease and correlate with the severity of inflammation, indicating that these cytokines may play a key role in the development of inflammatory bowel disease [24].

Analysis of the inflammed mucosa from patients with Crohn's disease and ulcerative colitis have shown increased expression of certain proinflammatory cytokines such as IL-1, interleukin IL-6 and TNF-α [19]. Early production of tumor necrosis factor is prominent in the initiation of a biologically highly complex system involving chemokines, cytokines and endothelial adhesions that recruits and activates neutrophils, macrophages and lymphocytes at the sites of infections. IL-1 and TNF-α increase the expression of high-affinity adhesion molecules on endothelial cells, stromal cells and leukocytes and, by this, promote infiltration of inflammatory cells from the blood into tissues. Dysregulation of tumor necrosis factor has also been implicated in a wide variety of autoimmune diseases, including Crohn's disease; however, how tumor necrosis factor mediates disease-causing effects is not completely explained. The induction of proinflammatory genes by tumor necrosis factor has been linked to most diseases [5]. TNF-α, together with IL-1 and IL-6, is responsible for the development of clinical symptoms and stimulation of acute-phase proteins in ulcerative colitis [8].

Evaluation of severity of inflammatory lesions is an essential component in clinical staging of colitis ulcerosa. Immunohistochemistry (IHC) is an important technique for biomarker validation. It allows direct visualization of biomarker expression in histologically relevant regions of the examined tissue. Our search continues for objective markers enabling unambiguous possible assessment of severity of inflammatory lesions in the course of ulcerative colitis, potential risk of dysplasia and in consequence – of malignant transformation.

Traditionally, pathologists examine visually and assign scores for IHC data in a semi-quantitative fashion incorporating the intensity and the distribution of specific staining. Due to staining heterogeneity of the tumor cells, the pathologist's visual scoring is fraught with problems due to subjectivity in interpretation. Automated IHC measurements promise to overcome these limitations. Therefore, we decided to apply a spatial visualization method for quantitative evaluation of IHC data.

Thus, the aim of this study is to present an application of the spatial visualization method of digital images for quantitative analysis of pro-inflammatory cytokines IL-1, IL-6 and TNF-α in various segments of large bowel excised because of colitis ulcerosa in relation with selected clinical symptoms.

Materials and methods

Our preliminary study included 17 patients having undergone restorative proctocolectomy with J-pouch and temporary loop ileostomy or Hartmann's colectomy with end ileostomy and preservation of a short rectal stump. The patients were operated on at the Department of General, Gastroenterological and Endocrinological Surgery and at the Department of General and Colorectal Surgery of the University of Medical Sciences in Poznan. Indications for surgical treatment included: exacerbating clinical symptoms (diarrhea, lower digestive tract bleeding, progressive loss of body weight, malnutrition, megacolon, fulminating colitis).

Patients' age in the study was on average 43 ± 14 years (range: 21–69 years), and the males to females ratio was 9:8. The mean duration of symptoms was 58 ± 31 months (range: 12–120 months). All patients were treated without the use of steroids agents and biological therapy, as well as immunosuppression in the last 2 months before surgery. The study excluded patients with other systemic diseases such as diabetes or rheumatoidal diseases. Inclusion criteria reduced the number of patients, but resulted in greater homogeneity of the group and reduced the potential impact of other factors on the level of investigated cytokines.

Rectal mucosa was much more altered on macroscopic inspection, showing visible thinning of bowel wall, numerous ulcers and indicators of active bleeding.

Mucosal samples of rectum and colon were collected from surgical specimens directly after resection, up to a maximum of 20 min after artery ligations. Bowel was cut in longitudinal line. The size of specimens was standardized (5 mm × 5 mm), specimens were taken from the transversal colon and from the middle part of the rectum, 5–6 cm below the dental line. Then, specimens were fixed in buffered 10% formalin (pH 7–7.8) for 48 h at stable temperature (22 °C) (air-conditioned room) and processed in a standard fashion. Paraffin sections were stained with hematoxylin and eosin (H + E), and only blocks with the most pronounced lesions were selected for further immunohistochemical studies. The specimens were then histologically evaluated for severity of inflammation and dysplasia.

Inflammation was scored on a scale of 0–2 [14], whereby:

- 0 – lack of active inflammation (no indices of *cryptitis*);
- 1 – mild signs of acute inflammation (*cryptitis* present in less than 50% of crypts);
- 2 – moderate signs of acute inflammation (*cryptitis* present in more than 50% of crypts);
- 3 – severe inflammation (ulcers and defects of mucosal epithelium).

Microscopic study of tissue samples revealed moderate severity of inflammatory process: mean score 2.1 (range: 0–3).

Immunohistochemical studies

Tissue samples were processed using the streptavidin-biotin-peroxidase technique (LSAB kit, DAKO, K0675). Antigens were exposed in a water bath at a temperature of 96 °C in a citrate buffer (pH 6.0) during 90 min. Activity of endogenous peroxidase was inhibited using 3% H_2O_2. Subsequently, preparations were incubated overnight at room temperature with antibodies anti-IL-1β (MAB263), anti-IL-6 (MAB261) and anti-TNF-α (MAB226). During subsequent 30 min incubation, biotinylated antibody was used, which was then incubated with peroxidase–streptavidin complex. Between consecutive incubations, preparations were flushed with TBS buffer (pH 7.6) using DAB-3.3 chromogen (SIGMA-ALDRICH, D5637), and the antigen was located. Preparations were stained with hematoxylin and after dehydration cover glass was applied. Reaction devoid of primary antibody was used as negative control for immunocytochemical studies.

Histological slides were examined with Olympus DP-12 microscope coupled to a digital camera. Color microscopic images of size 2048 × 1536 pixels were acquired and archived using 40× objective (at least 10 images in every slide with an immunopositive reaction). Using the software DP12-BSW, images were downloaded directly from the camera control unit to the PC.

To assess the expression of markers studied within inflammatory infiltrates, a quantitative analysis was used. The area fraction and markers' intensity fraction were determined by using a spatial visualization technique [15]. The received results were then categorized by using Alfred Immunohistochemistry Score [2] in the following way: