Intensity of Proliferative Processes and Degree of Oxidative Stress in the Mucosa of the Ileum in Crohn's Disease

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The intensity of proliferative processes (estimated from Ki-67 expression) and degree oxidative stress (chemiluminescence assay) in biopsy specimens from the terminal portion of the ileal mucosa were studied in patients with Crohn's disease. Crohn's disease is characterized by hyper-regenerative processes in the ileal mucosa. The labeling index (Ki-67 expression) in biopsy specimens from the intact ileal mucosa in patients with the irritable bowel syndrome (reference group) was 10.64±0.62%. The corresponding values in patients receiving monotherapy with mesalazine (group 1) and combination therapy with mesalazine and dalargin (group 2) were 24.05±1.17 and 22.90±0.92%, respectively. Analysis of free radical oxidation showed that this state is accompanied oxidative stress. Spontaneous and H_2O_2-induced luminol-dependent chemiluminescence in biopsy specimens from the ileal mucosa was 1.8-2.3-fold higher compared to the reference group. After therapy, the labeling index in groups 1 and 2 decreased to 18.60±1.18 and 14.38±0.81%, respectively. Histologically, normalization of the disease symptoms was more pronounced after combination therapy. The decrease in free radical oxidation in this group of patients was more pronounced than after mesalazine monotherapy. Our results suggest that oxidative stress plays a role in the hyper-regenerative reaction.

Key Words: *proliferation; free radical oxidation; Crohn's disease; mesalazine; dalargin*

Our previous studies showed that lichen ruber planus and atopic dermatitis are accompanied by hyper-regenerative reaction in the site lesion, which accompanies the development of local oxidative stress [6]. After therapy partial (atopic dermatitis) and complete normalization (lichen ruber planus) of proliferative processes was accompanied by a decrease in the degree of local oxidative stress. These data suggest that free radicals play a role in the progression of hyper-regenerative processes. The present study was performed to test this hypothesis under other pathological conditions.

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MATERIALS AND METHODS

We examined 45 patients (24 men and 21 women, average age 40.29±11.20 years) with the first diagnosed Crohn's disease (CD) of mild or moderate severity. These patients were characterized by acute damage to the terminal portion of the ileum. The diagnosis of CD was made by the criteria of the European CD Organization (2004).

The patients were randomized into 2 groups. Group 1 patients (N=30) received the standard therapy with mesalazine in a daily dose of 3 g for 8 weeks. Group 2 patients (N=15) received the combination therapy with mesalazine (daily dose 3 g, 8 weeks) and dalargin (daily dose 2 mg, 4 weeks). The reference group consisted of 30 patients (22 women and 8 men) with functional disorders (irritable bowel syndrome). The diagnosis of irritable bowel syndrome was made

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by the Roman criteria III (2007). Detailed examination did not reveal the organic substrate of inflammation in patients of the reference group. They had only symptoms of functional disorders.

Target biopsy (10-15 cm from the Bauhin's valve) was performed to examine the terminal portion of the ileum. Biopsy specimens were fixed in 10% neutral formalin in phosphate-buffered saline. The sections (5 μ) were embedded into paraffin and mounted on polylysine-coated glasses. A pathohistological study was conducted to examine the mucosa. Proliferative activity was evaluated. Expression of the Ki-67 antigen was determined using a Novolink polymer detection system (Noocastra™). The labeling index (LI) was evaluated from the expression of Ki-67-positive cells in longitudinal sections of the glands (%).

Chemiluminescence (CML) assay was performed for complex evaluation of free radical oxidation in homogenized biopsy specimens from ileal mucosa. CML was recorded on a LS 50B luminescence spectrometer (Perkin Elmer). Standardization of the signal and mathematical processing of the curves were performed with Finlab software. Spontaneous and Fe^{2+}-induced CML was estimated [2]. The sum of light energy was measured over 1 min of spontaneous CML (S_{SP}). This parameter correlates with the intensity of free radical processes. The flash maximum (h) of induced CML reflects the content of lipid hydroperoxides. The sum of light energy over 2 min of the post-flash period ($S_{IND}1$) reflects the rate of peroxide radical generation. Kinetic parameters of H_2O_2-induced luminol-dependent CML were estimated as described previously [1,11]. The maximum luminescence (H) reflects the ability of a biological object to undergo peroxidation. The sum of light energy over 2 min ($S_{IND}2$) depends on activity of the antioxidant and antiradical defense system. The intensity of CML (in mV) was calculated per 1 g wet tissue weight and expressed in relative units.

The results were analyzed by Microsoft Excel software (Office 2003 and Primer of Biostatistics 4.03 for Windows). Pairwise Student's *t* test was used for repeated measurements (before and after therapy). The differences were significant at $p<0.05$. The differences between two variational series were evaluated by Fisher's *F* test [3].

RESULTS

Before therapy, morphological changes in the ileal mucosa of CD patients were typical of a severe inflammatory reaction.

This conclusion was derived from the presence of aphthae, transmural lesion and infiltration of the gastric mucosa with lymphoid and plasma cells, formation of submucosal granulomas, and inflammation of the crypts (crypt abscess). Morphological changes were evaluated from histomorphological activity of the process.

Before therapy, 45 patients were characterized by moderate histological changes in the ileal mucosa (Table 1). The degree of histological changes in 6 patients (20%) was significantly decreased after mesalazine monotherapy. The severity of histological signs in 11 patients (73.3%) was low after combination therapy. A between-group comparison (Fisher's *F* test) showed that after combination therapy the number of patients with low histological activity was much higher compared to that observed in the mesalazine monotherapy group ($p<0.05$).

Analysis of epithelial proliferation in the ileal mucosa showed that LI in patients of the reference group (no morphological changes) is 10.64±0.62%. Proliferative processes were activated in non-treated patients with CD (as compared to the intact mucosa). LI in patients of the mesalazine monotherapy group and combination therapy group was 24.05±1.17 and 22.91±0.92%, respectively. After therapy, LI was reduced and approached that in the reference group. LI in patients of the mesalazine monotherapy group was 18.60±1.18%. Histologically, normalization of disease symptoms was more pronounced after combination therapy. Moreover, LI in these patients was decreased

TABLE 1. Histological Activity of the Disease in CD Patients after Monotherapy and Combination Therapy

Parameter	Mesalazine monotherapy (*N*=30)		Combination therapy with mesalazine and dalargin (*N*=15)	
	before therapy	after therapy	before therapy	after therapy
Low histological activity	0	6*	0	11*
Moderate histological activity	30	24	15	6

Note. *$p<0.05$ compared to pre-treatment parameter.

TABLE 2. Free Radical Status of the Ileal Mucosa in CD Patients

CML parameter, rel. units	Reference group (*N*=30)	Mesalazine monotherapy (*N*=30)		Combination therapy with mesalazine and dalargin (*n*=15)	
		before therapy	after therapy	before therapy	after therapy
S_{SP}	0.11±0.02	0.22±0.02*	0.14±0.01^{+}	0.22±0.02*	0.15±0.01^{+}
h	0.12±0.01	0.28±0.03*	0.21±0.01*	0.22±0.02*	0.11±0.01^{+o}
$S_{IND}1$	0.28±0.01	0.51±0.02*	0.36±0.03^{+}	0.50±0.03^{+}	0.29±0.03^{+o}
H	0.17±0.01	0.36±0.02*	0.31±0.02*	0.39±0.03*	0.19±0.02^{+o}
$S_{IND}2$	0.24±0.02	0.52±0.02*	0.28±0.02^{+}	0.43±0.03*	0.24±0.02^{+}

Note. $p<0.05$: *compared to reference group; $^{+}$compared to pre-treatment parameter; ^{o}compared to monotherapy group.

more significantly (14.38±0.81%) than in the mesalazine monotherapy group.

Analysis of free radical oxidation in biopsy specimens from the ileal mucosa of non-treated patients with CD showed that this state is accompanied by the development of oxidative stress. This conclusion was derived from a significant increase in CML parameters in CD patients (as compared to the reference group). We revealed the increase in S_{SP} (by 2 times), $S_{IND}1$ (by 1.8 times), h (by 1.8-2.3 times), $S_{IND}2$ (by 1.8-2.1 times), and H (by 2.1-2.3 times; $p<0.05$). The standard therapy with mesalazine was followed by a significant decrease in CML parameters. S_{SP}, $S_{IND}1$, and $S_{IND}2$ decreased by 1.5, 1.4, and 1.8 times, respectively ($p<0.05$). CML assay of oxidative state in patients of the combined therapy group revealed a strong improvement of study parameters in biopsy specimens from the ileal mucosa (as compared to the pre-treatment parameters). S_{SP}, $S_{IND}1$, h, $S_{IND}2$, and H were reduced by 1.5, 1.7, 2, 1.8, and 1.8 times, respectively ($p<0.05$; Table 2). Our results support the data that oxidative stress has a role in the pathogenesis of CD [1,5,10].

These data indicate that the hyper-regenerative process in CD (similar to that in lichen ruber planus and atopic dermatitis [6]) is accompanied by oxidative stress. This conclusion is derived from activation of proliferative processes in CD patients (as compared to the reference group). The decrease in the degree of oxidative stress was accompanied by a partial recovery of proliferative processes. The introduction of dalargin into combination therapy for these patients (to increase the antioxidant and antiradical defense and to reduce the severity of oxidative stress [4]) was following by a greater improvement of proliferative processes as compared to the mesalazine monotherapy group.

Published data show that oxidative stress is accompanied by not only DNA damage, but also impairment of ^{3}H-thymidine incorporation (reflecting reduced DNA synthesis rate) [9]. Free radicals play an important role in the stimulation of cell passage through the cell cycle. The hyper-regenerative reaction in oxidative stress is probably characterized by a specific dynamics and depends on the degree of oxidative stress. Reactive oxygen metabolites, which determine the development of oxidative stress, initially served as the major factor for the defense from bacterial aggression. In the follow-up period, these agents became involved in the mechanisms for intercellular and intracellular regulation [8]. It concerns the maintenance of tissue homeostasis. Reactive oxygen metabolites have a role in the regulation of kinase phosphorylation and transcription factor activity. The hyper-regenerative reaction in oxidative stress serves as an adaptive process to maintain the integrity of the epithelial barrier. This state is accompanied by the impairment of cell differentiation. Chronic inflammation is characterized by high risk for malignant transformation. It should be emphasized that the epithelial barrier integrity can be impaired when the rate of cell division does not increase [7]. Therefore, the problem of toxicity or necessity of reactive oxygen species should be solved in each specific case.

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