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NADPH oxidase p22phox gene expression in ulcerative colitis

BOWEL

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Original Article

ABSTRACT

Background/Aims: Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which catalyzes the formation of reactive oxygen species (ROS) in phagocytic cells, has five subunits: p67phox ("phox"refers to "phagocyte oxidase"), p47phox, p40phox, p22phox, and gp91phox (catalytic subunit). Oxidative stress resulting from the accumulation of ROS and/or defective removal of ROS by antioxidants has detrimental effects on cellular functions and may contribute to chronic inflammation. Disruption of the colonic mucosa due to the dysregulation of antioxidants or transformation enzymes may play a role in the pathogenesis of ulcerative colitis (UC) and influence the clinical features of this disease. In this study, we examined the expression of the gene encoding NADPH oxidase subunit p22phox cytochrome b-245, alphapolypeptidein the colonic mucosa to test its possible contribution in the pathogenesis of UC.

Materials and Methods: Expression levels of mRNA in the inflamed and non-inflamed colonic mucosa (determined using colonoscopy)of 22 patients with UC and in the normal mucosa of 22 healthy controls were analyzed using real-time polymerase chain reaction.

Results: Expression levels of mRNA were not significantly different between patients with inflamed and non-inflamed colonic mucosa (p>0.05) and betweenpatients with inflamed colonicmucosa and healthy controls (p>0.05).

Conclusion: Although our data suggest that expression of the gene encoding p22phox is not associated with chronic inflammation in patients with UC, other mechanisms can affect oxidative stress in these patients.

Keywords: Ulcerative colitis, oxidative stress, inflammation, NADPH oxidase, p22phox

INTRODUCTION

Ulcerative colitis (UC) is a chronic, non-specific inflammation of the colon with an unidentifiedpathogenesis. Like mostcomplex diseases, UC results from a combination of various factors such as environmental triggersand immune responsesthat contribute to chronic intestinal inflammation in genetically susceptible individuals (1,2).

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase was first described in phagocytic cells as an enzyme involved in the generation of reactive oxygen species (ROS) (3). NADH and NADPH oxidation by the granules of resting and phagocytizing cells). This enzyme comprises two membrane-bound proteins (p22phox and gp91phox), three cytosolic proteins (p67phox, p47phox, and p40phox), and a small G-protein Rac. Gp91phox and p22phox form a heterodimer that is bound to the plasma membrane. This complex is called flavocytochrome b558 because it shows maximum absorption at 558nm and is catalytically inactive in resting cells. It moves toward cytosolic subunits of the cell membrane and forms an active complex with NADPH oxidase (4). ROS levelsin cells should be low because cells participate in several processes, including regulatory mechanisms, intracellular signaling, and

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Table 1. Characteristics and clinical features of patients with UC

Patient number	Age/ gender	UC extension	Endoscopic activity index	UC disease activity index	Treatment	CRP	P22phox inflamed mucosa ($2^{\Delta Ct}$ values)	P22phox Non-inflamed mucosa ($2^{\Delta Ct}$ values)
1	24, M	Distal	12	8	Sulfasalazineand mesalazine	3	290.0	310.8
2	59, M	Extensive	12	12	Mesalazineand budesonide	21	1499.2	1217.7
3	53, M	Extensive	10	10	Mesalazine	40	2778.3	8599.2
4	20, F	Extensive	9	8	-	23	652.5	3191.4
5	47, M	Extensive	12	6	Mesalazineand azathioprine	21	2702.3	1082.3
6	40, F	Extensive	6	5	Mesalazine	6	43.1	448.8
7	23, F	Extensive	12	12	-	20	1024.0	333.1
8	33, F	Distal	7	4	-	5	8.0	138545
9	33, M	Distal	8	10	Mesalazine	6	1807.7	3983.9
10	54, F	Distal	5	7	Mesalazine	5	11190.6	17559.9
11	43, F	Extensive	3	4	Mesalazine	13	6888.6	1136.2
12	33, M	Extensive	7	10	-	3	1370.0	6936.5
13	29, M	Extensive	10	8	Mesalazineand methylprednisolone	6	15825.9	278.2
14	79, M	Extensive	3	7	Mesalazineand methylprednisolone	11	3666.0	22693.6
15	52, F	Distal	9	10	Mesalazine	3	1910.8	4672.5
16	42, M	Distal	8	8	Mesalazine	3	494.5	77935.9
17	34, M	Distal	10	3	Sulfasalazine	58	220436.0	1541.3
18	21, M	Proctitis	10	6	-	3	378517.4	3565.7
19	57, M	Distal	10	4	Mesalazine	3	18179.1	9741.9
20	36, M	Extensive	8	6	Mesalazine	28	7332.0	5330.3
21	27, F	Extensive	2	5	Mesalazineand fluocortolone	3	1105.1	85.6
22	61, F	Proctitis	10	4	-	5	31000.4	903.8

ΔCt: gene expression levels in each sample normalized to those of GAPDH in a given sample; CRP: c-reactive protein; UC: ulcerative colitis

host defense against pathogens. ROS are highly reactive molecules because of the presence of unpaired electrons; hence, increased ROS levels are toxic and damage cell membranes through lipid peroxidation and proteins by exerting oxidative damage (5). ROS can damage the colonic epithelium and increase mucosal permeability. Once the intestinal epithelial barrier is damaged, bacterial antigens can pass through the sterile submucosal layers and initiate a destructive cascade of immune response (6,7). Oxidative stress occurs when there is an imbalance between ROS production and their removal by antioxidants. Oxidative stress is a potential etiological factor in UC because ROS are known to play a role in inflammation (8).

Changes in the expression of NADPH oxidase subunit p22phoxmay be one of the reasons for mucosal inflammation in UC. In this study, we examined the expression of the gene encoding NADPH oxidase subunit p22phox cytochrome b-245, alphapolypeptide (*CYBA*) in the colonic mucosa to test its possible contribution in the pathogenesis of UC. To the best of our knowledge, expression of the gene encoding p22phox has not been studied previously in patients with UC.

MATERIALS AND METHODS

This study included 22 patients with UC and 22 healthy controls who were enrolled at the Department of Gastroenterology, Faculty of Medicine, University of Gaziantep, between May and December 2010. UC was diagnosed based on clinical, endoscopic, and histopathological criteria. The mean age of nine women and 13 men with UC was 39.22±14.54 and 42.08±16.39 years, respectively, while that of nine women and 13 men in the control group was 53.66±15.34 and 48.15±14.76 years, respectively. Before sampling, six patients with UC received no medication, 11 received 5-aminosalicylic acid (5-ASA) compounds alone, four received steroids, and one received azathioprine in combination with 5-ASA compounds. Of the 22 patients with UC, 12 had extensive colitis, eighthad distal colitis, and two had proctitis. Written informed consent was obtained from all the patients and healthy controls. This study was approved by the local ethics committee and was conducted in accordance with

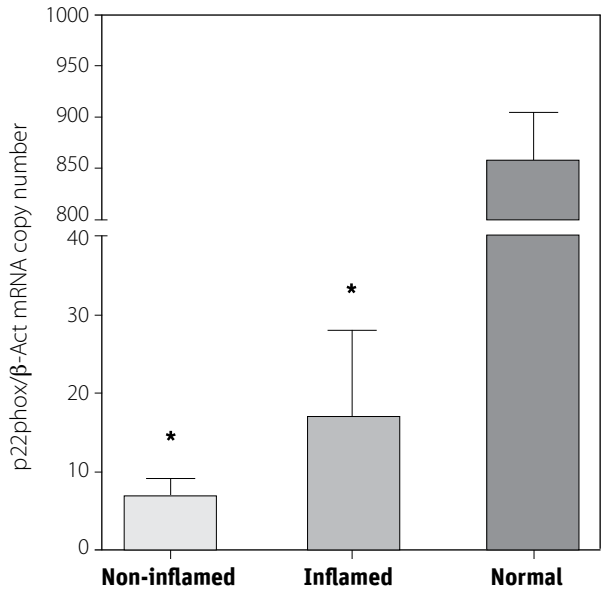

Figure 1. Levels of p22phox mRNA in patients with inflamed and non-inflamed colonic mucosa and in healthy controls. Statistical significance denoted by*.

the Declaration of Helsinki. Severity of UC was graded according to Rachmilewitz endoscopic activity index. The characteristics and clinical features of patients with UC are presented in Table 1.

Total RNA was isolated from samples obtained from colonic biopsy by using High Pure Isolation kit (Cat no.12033674001; Roche, Mannheim, Germany) according to manufacturer's instructions.

The isolated mRNA was transcribed into cDNA by using random hexamers and AMV reverse transcriptase (CatNo.11483188001; Roche, Mannheim, Germany). Primer set for p22phox was 5′-TGGCGGGCGTGTTTGTGT-3′ (sense) and 5′-CCACGGCGGTCATGTACTTC-3′ (antisense). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the housekeeping gene for normalization and was amplified using the following primers: 5′-CCCATGTTCGTCATGGGTGT-3′ (sense) and 5′-TGGTCATGAGTCCTTCCACGATA-3′ (antisense). Real-time polymerase chain reaction (PCR) was performed using TaqMan gene Expression Assay. Reaction conditions for real-time PCR were as follows: initial denaturation at 95°C for 10 min;45cycles at denaturation at 95°C for 10 s, annealing at 60°C for 30s, and elongation at 72°C for 1s;and final elongation at 40°C for 30 s. Gene expression was quantified using comparative Ct method. Briefly, expression levels of gene encoding p22phoxin each sample were normalized to those of *GAPDH* within a given sample (ΔCt). Results were evaluated by using $2^{\Delta Ct}$ method as relative gene expression values.

Data were statistically analyzed using Statistical Package for Social Science (SPSS) program (SPSS for windows, version 14.0).

Table 2. Expression levels of the gene encoding p22phox in healthy controls

Control group	Age/gender	P22phox expression ($2^{\Delta Ct}$ Values)
1	53, M	1379.6
2	62, M	8306.4
3	23, M	3373.4
4	45, M	2592.3
5	60, F	1287.2
6	60, M	262144
7	66, F	294927.33
8	44, F	1727195.55
9	55, M	604.67
10	64, M	1951.0
11	31, F	1176.3
12	31, M	600.49
13	29, M	1562.9
14	54, M	2610.3
15	43, M	5556.7
16	76, F	1871.5
17	34, M	232.32
18	53, F	484.38
19	73, M	7968.0
20	34, F	82953
21	46, F	108701
22	73, F	4705.1

ΔCt: gene expression levels in each sample normalized to those of GAPDH in a given sample; Phox: phagocyte oxidase

Chi-square test was used to compare the groups. Qualitative data were expressed as frequency and percentage, and quantitative data were expressed as mean and standard deviation. Student's *t*-test and Mann-Whitney *U* test were used to compare two groups. P values less than 0.05 were considered statistically significant.

RESULTS

Expression levels of the gene encoding p22phoxwere not significantly different between patients with inflamed (32214±19230 $2^{\Delta Ct}$) and non-inflamed colonic mucosa (14095±69062$^{\Delta Ct}$; p=0.80), between patients with inflamed colonic mucosa (114644±78798 $2^{\Delta Ct}$) and healthy controls (p=0.60),and between patients with non-inflamed mucosa and healthy controls (p=0.40). These expression levels are presented at Table 2 and Figure 1.

No statistically significant differenceswere observed between patients with inflamed and non-inflamed colonic mucosa with

respect to endoscopic activity index (p=0.51 and p=0.14, respectively), UC activity index (p=0.12 and p=0.96, respectively), erythrocyte sedimentation rate (p=0.60 and p=0.889, respectively), and C-reactive protein (CRP) levels (p=0.53 and p=0.69, respectively).

Expression of the gene encoding p22phox was not significantly different between six patients with inflamed (68762±62147 $2^{\Delta Ct}$) and non-inflamed mucosa (25579±22613 $2^{\Delta Ct}$) who did not receive any treatment before sampling (p=0.25). No correlation was observed between expression of the gene encoding p22phox and UC among 16 patients with inflamed (18509±13533 $2^{\Delta Ct}$) and non-inflamed mucosa (9788±4833 $2^{\Delta Ct}$) who received treatment before sampling. Moreover, no difference was observed between patients with inflamed (619 9±2588/31789±27048/190008±188509 $2^{\Delta Ct}$) and non-inflamed mucosa (4251±1871/31786±1774/2391±1174 $2^{\Delta Ct}$) with respect to the three colitis types (extensive, distal, and proctitis).

DISCUSSION

Ulcerative colitis is characterized by the confluent inflammation of the colonic mucosa that extends from the rectum to the proximal colon. Although the cause of colonic inflammation in UC is not completely established, substantial evidence suggests that it is associated with elevated production of ROS. Experiments in both animals and humans have shown that overproduction of ROS and consequent oxidative stress play a critical role in the pathophysiology of UC (9-11).

Nicotinamide adenine dinucleotide phosphate oxidases are a major source of intracellular ROS (4). P22phox is a core component of this enzyme and plays a key role in the production of ROS. P22phox is the α-subunit of cytochrome b558, which is involved in the final electron transport from NADPH to molecular oxygen (12). This study assessed the association between oxidative stress and expression of the gene encoding p22phox as the cause of inflammation in UC. Expression of the gene encoding p22phoxhas not been analyzed in UC thus far.

To the best of our knowledge, the only study investigating the association between UC andthe gene encoding p22phoxis based on polymorphism C242T in this gene. The C242T polymorphism substitutes amino acid histidine with tyrosine at position 72 in p22phox. Presence of the C242T polymorphism in the gene encoding p22phox significantly reduces superoxide production in human neutrophils (13). However, no association has been observed between this polymorphism and different clinicopathological features of UC, such as gender, age, age of onset, clinical type, extension of colitis, and response to treatment (14). Associations between the C242T polymorphism and other diseases, including coronary arterydisease, cerebrovascular disease, and nondiabetic nephropathy have been observed (15-17).

A low level of ROS is necessary for several processes within the cell; it especially plays important physiological roles in innate immunity against pathogenic microorganisms encountered in the gut. However, increased levels of ROS are toxic and can damage cell membranes through lipid peroxidation and proteins through oxidative damage. Thus, there should be a balance between ROS production and oxidative defense within cells. When this balance is disrupted, destructive effects of ROS may appear (5). In response to oxidative stress, tissues produce more antioxidants. Moreover, severe oxidative stress depletes body's antioxidant resources andreduces its ability to produce more antioxidants, thus lowering antioxidant levels (18). Increased levels of ROS and biomarkers of oxidative stress such as lipid peroxidation products (reactive aldehydes, f2-isoprostanes, etc.) and protein modifications (protein carbonyls, etc.),are present in the colonic mucosa of patients with UC (19-21).

Correspondingly, levels of antioxidants such as glutathione, coenzyme Q_{10}, glutathione *S*-transferase, superoxide dismutase, catalase, paraoxonase-1, and metallothionein decrease in patients with UC compared with those in normal individuals (22,23). Antioxidant levels also decrease in the peripheral red blood cells of patients with active UC (24) (Krzystek-Korpacka, 2010, Impaired erythrocyte antioxidant defense in active inflammatory bowel disease: impact of anemia and treatment). Alagozlu et al. quantified advanced oxidation protein products (AOPPs) and total thiol levels as markers of oxidative protein damage, malondialdehyde level as a marker of lipid peroxidation, and myeloperoxidase activity as a marker of neutrophil activation in patients with UC. They found that increased levels of plasma AOPP ssupport the presence of oxidative stress and protein oxidation in patients with UC and that this marker may be used as a simple serum marker to assess disease activity and to predict the disease severity and probably response to therapy (25) (Alagozlu, 2013, Increased plasma levels of advanced oxidation protein products (AOPP) as a marker for oxidative stress in patients with active ulcerative colitis) (Alagozlu, 2013, Increased plasma levels of advanced oxidation protein products (AOPP) as a marker for oxidative stress in patients with active ulcerative colitis) (Alagozlu, 2013, Increased plasma levels of advanced oxidation protein products (AOPP) as a marker for oxidative stress in patients with active ulcerative colitis) (Alagozlu, 2013, Increased plasma levels of advanced oxidation protein products (AOPP) as a marker for oxidative stress in patients with active ulcerative colitis}{Alagozlu, 2013, Increased plasma levels of advanced oxidation protein products (AOPP) as a marker for oxidative stress in patients with active ulcerative colitis}. However, some conflicting results have been obtained regarding severity of the disease and levels of antioxidants or oxidative stress biomarkers. Although majority of studies have failed to show any significant correlation between the severity of the disease and levels of antioxidants and oxidative stress biomarkers, few studies have demonstrated this correlation, even with the extension of intestinal inflammation.

Our results did not show differential expression of the gene encoding p22phoxin patients within flamed or non-inflamed co-

lonic mucosa comparedwith that in healthy controls. In addition, no increase in the expression of this gene in the inflamed colonic mucosa suggested that other mechanisms mightaffect the expression of this gene. There are no data in literature on this interaction. However, because majority of our patients used 5-ASA, an antioxidant, before inclusion in the study, we feel that this treatment may have influenced the expression of p22phox. However, it is conflicting to our idea that there was no significant difference concerning the p22phox gene expression between the treated and untreated groups. Finally, we observed no significant correlation between the indices of severity and expression of the gene encoding p22phoxin patients with UC contradicted our hypothesis. Although our results suggested that expression of this gene was not associated with chronic inflammation in patients with UC, further studies with larger sample size are needed to understand the factors involved in UC development and expression of p22phox.

Ethics Committee Approval: Ethics committee approval was received for this study from Gaziantep University Ethical Commitee.

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

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