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Case Control Study

Local inflammatory response to gastroesophageal reflux: Association of gene expression of inflammatory cytokines with esophageal multichannel intraluminal impedance-pH data

Morozov S *et al*. Cytokines' genes expression in GERD

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Abstract

BACKGROUND

Expression of inflammatory cytokines' genes may take part in pathophysiology of different forms of gastroesophageal reflux disease (GERD).

AIM

To explore the expression of inflammatory cytokines' genes in esophageal mucosa in patients with erosive esophagitis (EE) and non-erosive forms of GERD (NERD) and its association with data of esophageal multichannel intraluminal impedance-pH (MII-pH) measurements.

METHODS

This was a single-center prospective study. Esophageal mucosa samples were taken from lower part of the esophagus during endoscopy. Expression of interleukin (*IL*)-*1β*, *IL-10*, *IL-18*, tumor necrosis factor α (*TNFA*), toll-like receptor 4 (*TLR4*), GATA binding protein 3 (*GATA3*), differentiation cluster 68 (*CD68*) and β-2 macroglobulin (*B2M*) genes in esophageal mucosa was assessed with ImmunoQuantex assays. MII-pH measurements were performed to all the participants. Diagnosis of GERD was confirmed by the results of MII-pH data. Based on the endoscopy, patients were allocated to the groups of EE and NERD. The control group consisted of non-symptomatic subjects with normal endoscopy and MII-pH results. We used non-parametric statistics to compare the differences between the groups. Association of expression of the mentioned genes with the results of MII-pH data was assessed with Spearman rank method.

RESULTS

Data of 60 patients with GERD and 10 subjects of the control group were available for the analysis. Higher expression of *IL-18* (5.89 ± 0.4 *vs* 5.28 ± 1.1, $P = 0.04$) and *GATA3* (2.92 ± 0.86 *vs* 2.23 ± 0.96, $P = 0.03$) genes was found in EE group compared to NERD. Expression of *IL-1β*, *IL-18*, *TNFA*, and *TLR4* genes was lower ($P < 0.05$) in the control group compared to EE and NERD. Esophageal acid exposure correlated

with the expression of *IL-1β* (Spearman rank r = 0.29), *IL-18* (r = 0.31), *TNFA* (r = 0.35), *GATA3* (r = 0.34), *TLR4* (r = 0.29), *CD68* (r = 0.37) genes. Mean esophageal pH correlated inversely with the expressions of *IL-18*, *TNFA*, *GATA3*, *TLR4*, *CD68* genes. No association of genes expression with number of gastroesophageal refluxes was found.

CONCLUSION

In patients with EE, local expression of *IL-18* and *GATA3* genes was higher compared to subjects with NERD. Esophageal acid exposure correlated directly with expression of *IL-1β*, *IL-18*, *TNFA*, *TLR4*, *CD68* and *B2M* genes. Inverse correlation was revealed between expression of *IL-18*, *TNFA*, *GATA3*, *TLR4*, *CD68* genes and mean esophageal pH.

Key Words: Gastroesophageal reflux disease; Gene expression; Cytokines; Erosive esophagitis; Non-erosive gastroesophageal reflux disease; Esophageal multichannel intraluminal impedance-pH; Gastroesophageal reflux

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Core Tip: Local expression of cytokines' genes may be involved in pathophysiology of different forms of gastroesophageal reflux disease (GERD). In this study, we found different profile of local expression of cytokines genes in subgroups of patients with erosive esophagitis and non-erosive forms of GERD. For the first time we have revealed correlation between expression of interleukin (*IL*)*-18*, tumor necrosis factor alpha (*TNFA*), GATA binding protein 3 (*GATA3*), toll-like receptor 4 (*TLR4*), differentiation cluster 68 (*CD68*) genes and mean esophageal pH, and association of acid exposure with expression of *IL-1β*, *IL-18*, *TNFA*, *TLR4*, *CD68* and β-2 macroglobulin genes.

INTRODUCTION

Gastroesophageal reflux disease (GERD) is a common disorder that significantly impairs patients' quality of life due to persistent symptoms, caused by the reflux of gastric content into the esophagus. Beside typical symptoms, like heartburn and regurgitation, it has a vast spectrum of manifestations[1]. Pathogenesis of GERD is complex, with a paramount role of impaired esophageal-gastric junction motility, delayed gastric emptying and damage of esophageal mucosa with aggressive content of the refluxate[2]. Traditionally, the grade of esophageal mucosa damage and intensity of symptoms were thought to be directly related to the acidity of the reflux content[3]. However, recent data suggest that this relationship is not linear, and the spectrum of manifestations may be genetically determined or be a result of balance in factors responsible for local inflammatory response in esophageal mucosa, perception of refluxate and processing of signals by the peripheral and the central nervous systems[4,5]. For example, it was shown that demographic profiles of patients with GERD differ: Severe grades of esophagitis are commonly found in elder men of white race, and are associated with abdominal-type obesity, while persistent symptoms of the disease in the absence of esophageal mucosa lesions are more typical for young women[6]. When not treated, esophageal mucosa breaks remain stable with time in most of cases[7]. This allows to distinguish different forms of GERD: Erosive esophagitis (EE), Barrett's esophagus and non-erosive form of GERD (NERD), which despite the common etiology, may differ by pathophysiology.

Experimental studies suggest that an exposure of refluxate lead not to a chemical burn, but induce inflammatory cytokines synthesis [like interleukin (IL)-1β and IL-8], followed by migration of lymphocytes and neutrophils into the mucosa and its further damage[8]. These data formed the basis of the theory of "cytokine sizzle", as the basis of the pathogenesis of GERD[9]. The endoscopic phenotype seen in a patient is a result of balance between pro-inflammatory and anti-inflammatory factors that counteract. This balance is genetically determined, as cytokines' production depends on the expression of the related genes. Regulation of cytokines genes expression is complex, and the data on its' dependence on the acidity of the refluxate and types of gastroesophageal refluxes (GER) are insufficiently studied yet.

Moreover, cytokines' profiles studied in humans are not that large, and reports on the involvement of IL-18, transcription factor GATA binding protein 3 (GATA3), toll-like receptor 4 (TLR4), and differentiation cluster 68 (CD68) in local inflammatory response in subjects with different forms of GERD are still lacking.

The aims of the present work were to study: (1) Expression of cytokines' genes in esophageal mucosa of patients with EE and NERD; and (2) Association of the expression with the data of esophageal multichannel intraluminal impedance-pH (MII-pH) measurements.

MATERIALS AND METHODS

Inclusion criteria

The study was approved by the Ethics Committee of the Federal Research Center for Nutrition and Biotechnology. The data of complex examinations of patients served as the source for the study.

Inclusion criteria in the present study including: (1) Males and females, ≥ 18 years and ≤ 80 years; and (2) Written informed consent to participate in this study.

Exclusion criteria

(1) The use of medications that could influence manifestations of GERD, damage esophageal mucosa, affect esophageal motility or influence cytokine response. This included, but was not limited to the use of the following drugs: Glucocorticosteroids, non-steroid anti-inflammatory drugs (except topical agents more than 2 wk before the enrollment), calcium channel antagonist, nitrates and agents that affect adrenergic or acetylcholine receptors, or have local irritating effect on the gastrointestinal mucosa. The use of antisecretory agents (proton pump inhibitors or H2-histamine receptors blockers) was not allowed for at least 4 wk before the enrollment; (2) History of chest and/or abdominal surgery in the anamnesis (excluding appendectomy and cholecystectomy performed at least 6 mo prior to the enrollment); (3) History of esophageal varicose bleeding or presence of esophageal varices on endoscopy; (4) Impossibility to perform at least one type of the examinations required by the study protocol; and (5) Active systemic connective

tissue or autoimmune disorders, decompensated condition of any organ or system, and general condition of a patient that made him inapplicable for the study on the discretion of investigator.

The data of subjects were not included into the final analysis in the case when the data of examinations required by the study protocol were missing or incomplete.

Verification of GERD

(1) Clinical data (presence of heartburn and/or acid regurgitation at least once per week; presence of symptoms more than 6 mo and they should have stayed relevant for at least 3 last mo; history of treatment with proton pump inhibitors with positive effect in the past); (2) GERD-Q score ≥ 8 (validated language-specific version of the international questionnaire was used)[10,11]; and (3) Results of 24 h esophageal MII-pH accordaning to the Lyon consensus[12].

Study procedures

Twenty four hours MII-pH recordings: Twenty four hours MII-pH recordings were performed with the use of pH-impedance recorder Ohmega (MMS, the Netherlands) and manufacturer's software (Solar Gastro, MMS, the Netherlands), and standard pH-impedance catheters with 2 pH- and 6 impedance channels (Unisensor, AG, United States). The distal pH-sensor was placed 5 cm above esophago-gastric junction, based on the results of high-resolution esophageal manometry. Values of acid exposure time (AET) in the lower esophagus more than 6% during the 24 h and/or number of GER > 80/d were considered abnormal. When AET and number of refluxes were lower than the above mentioned limits, association of symptom with GER was taken into account (sensitivity index > 80%, symptom index > 50%)[12].

Endoscopic evaluation: Endoscopic evaluation of esophageal, stomach and duodenal mucosa was necessary to exclude concomitant pathology and to establish presence of esophagitis. Olympus Exera II CV-180 panendoscope (Olympus, Japan)

was used to perform endoscopy. EE was described according to the Los Angeles classification, 1999[13].

Based on the results, patients with GERD were allocated either to the group of EE, or to the group of NERD. The control group consisted of the subjects eligible for the study according to the inclusion/exclusion criteria, who did not experience symptoms of GERD, had normal esophageal endoscopy and data of MII-pH measurements.

Gene expression: Samples of esophageal mucosa were taken from the distal part of the esophagus (5 cm above the esophago-gastric junction) with sterile forceps during endoscopy. In the case of EE, the samples were obtained from the margin of erosions. The tissues were immediately placed to 1.5 mL plastic tubes containing 500 μL RNA stabilizer (IntactRNA, Evrogen, Russia) and stored up to 2 wk at -20°C. RNA purification was performed as previously described with the use of PREP-NA extraction kit (DNA-Technology, Russia) followed by ImmunoQuantex assay (DNA-Technology, Russia)[14,15]. This test system provides a reverse transcription reaction to obtain an mRNA template complementary to DNA from the previously isolated pool of DNA and RNA. Further, this mRNA template is amplified by real-time polymerase chain reaction (PCR). The reverse transcription reaction (synthesis of cDNA on the obtained RNA) was carried out in a volume of 40 μL. Specific oligonucleotides and M-MuLV reverse transcriptase (Evrogen, Russia) were used as primers for reverse transcription. We traced reproducibility and linearity of real time quantitative PCR assays to ensure quality control. The assessment of genes' expression was based on the comparison of threshold cycles method (2 delta Cq method with normalization to reference genes [β-2 macroglobulin (*B2M*)]. The relative value of gene X expression was calculated by the formula: Expression (X) = $2^{-(Cp\,(x)\,-\,NF)}$. After the amplification stage, the mRNA expression level was calculated (based on the indicator of the cycle) for the following genes: *IL-1β*, *IL-10*, *IL-18*, tumor necrosis factor α (*TNFA*), *TLR4*, *GATA3*, *CD68*, *B2M*. Study design, subjects flow and allocation chart are shown in Figure 1.

Statistical processing: Statistical processing of the results was carried out with commercially available software: MS Excel 2016 (Microsoft, United States) and Statistica 10 (StatSoft, United States). The data are presented as means and standard deviation (mean ± SD). Nonparametric statistics (Mann-Whitney's *U*-criteria, Spearman rank R) were used to compare the results between the groups. Values of $P < 0.05$ were considered significant.

RESULTS

Overall, 111 patients were enrolled, 23 subjects were not eligible according to inclusion/exclusion criteria; incomplete results were obtained in 18 patients. The data of 60 patients with GERD [26 (43.3%) men and 34 (56.7%) women]; mean age (mean ± SD): 54.6 ± 15.6 years (18.3% Asians, 81.7% Caucasian) and 10 of the control group (60% men; 46.2 ± 13.0 years old; 20% Asians) were available for the final analysis (Figure 1). Those with EE were younger: 47.5 ± 13 years *vs* 58.2 ± 15.8 years, $P = 0.007$. No significant difference in distribution by sex was found between the groups: The relative number of women in EE group was 65%, and in NERD group, it was 40%, $P = 0.06$.

The results of MII-pH and gene expression analysis are shown in Tables 1 and 2. The total number of GER, number of acid GERs, acid exposure time were higher in EE group compared to NERD. However, the number of weak-acid, non-acid refluxes and mean pH values in the distal part of the esophagus did not differ in the GERD groups.

We found direct correlation between genes expression of *IL-1β*, *IL-18*, *TNFA*, *TLR4*, *CD68*, *B2M* and acid exposure time in the distal esophagus (Table 3). There was inverse correlation between the expression of *IL-18*, *TNFA*, *GATA3*, *TLR4*, CD68 genes and mean pH values.

DISCUSSION

In this study, we showed association of local inflammatory response of esophageal mucosa with data of MII-pH recordings in patients with different forms of GERD, confirmed according to the current standards. Expression of inflammatory cytokines

was assessed in a number of previously published works[16-18]. In most of them, symptoms and endoscopic data were used to confirm GERD. Nowadays, this approach is not fully reliable because esophageal erosions may be caused by different factors and subjects with other diseases (eosinophilic esophagitis, depression) often experience similar symptoms[12]. In our study, diagnosis of GERD was confirmed by the data of MII-pH measurements. The use of this technique allowed us not only to confirm the presence of pathological reflux, but also to analyze association of the expression of inflammatory cytokines' genes with different types of the GER. According to the results, acid exposure time (proportion of time with pH < 4.0 per day at 5 cm above upper border of gastroesophageal sphincter) and, to a lesser extent, mean esophageal pH were the factors associated with the expression of inflammatory cytokines' genes. Zavala-Solares *et al*[19] succeeded to show that the expressions of *IL-1β* and *TNFA* were higher when an abnormal acid exposure in the esophagus (pH < 4 more than 4.2% of time per day) was present. Unfortunately, in the mentioned study diagnosis of GERD was based predominantly on symptoms and endoscopy and did not include esophageal impedance monitoring. Similar to our results, they failed to reveal association of *IL-10* expression with acid exposure in the esophagus[19]. This cytokine plays anti-inflammatory and modulatory effect on the inflammation; reduces production of *TNFA*, *IL-1β*, *IL-12*, and secretion of interferon gamma[20]. The lack of inhibitory effect may cause imbalance in inflammatory response and predominance of pro-inflammatory factors, that leads to the tissue damage[21].

To our knowledge, negative correlation of mean pH values at the lower esophagus with the expression of *IL-18*, *TNFA*, *GATA3*, *TLR4*, *CD68* genes has not been reported yet. The obtained results may be important from both, scientific and practical viewpoints. Indeed, it is difficult to explain the presence of endoscopic or histological changes within the mucosa only by aggressive nature of the reflux content. Significant overlap in the acid exposure in the esophagus was revealed in subjects with different forms of GERD[22,23]. Similarly, in the present study the mean pH values in the esophagus did not differ in subjects with EE and NERD. This suggests that different response may be caused by genetically determined local

inflammatory response[24]. Increased expression of inflammatory cytokine genes causes higher local concentrations of the cytokines and triggers a cascade of corresponding reactions that impair integrity of the mucosa[17,24]. The correlation analysis does not provide information on the direction of influence. However, previous studies with the use of proton pump inhibitors (lansoprazole 30 mg/d for 8 wk) showed significant decrease in expression of another inflammatory cytokine's (*IL-8*) gene after the treatment[16,25].

It was reported that *GATA3*, *TNFA* and *IL-10* may increase secretion of immunoglobulin E and stimulate migration of eosinophils[26,27]. It may have diagnostic value and help understand why some of the patients with eosinophilic esophagitis respond to the therapy with proton pump inhibitors[28,29]. Eosinophilic inflammation may develop in response to the overexpression of these cytokines caused by GER.

The obtained results may be of use for the search of new treatment options in cases when refractory symptoms of GERD are present[30]. In such cases, the use of immune modulators affecting local immune response may help to achieve better results[26].

The limitation of the present study is a relatively small sample size. However, strict eligibility criteria could minimize the risk of inclusion of subjects with non-confirmed diagnosis in the GERD group. We did not divide subgroups depending on the severity of esophagitis. These subgroups could help us to obtain more details, but at the same time, this approach increases the risk of type II error.

The presence of bile acids in the reflux content may lead to greater impairment of esophageal mucosal integrity and less effective chemical clearance[31,32]. We did not detect bile in the refluxate in the present study; however, it seems very promising to analyze association of the cytokines' genes expression with reflux of bile acids.

Our study provides additional information on pathogenesis of GERD. However, larger studies are necessary to confirm the results.

CONCLUSION

In patients with EE, local expression of *IL-18* and *GATA3* genes was higher compared to subjects with NERD. Direct correlation was found between the local expression of *IL-1β*, *IL-18*, *TNFA*, *TLR4*, *CD68* and *B2M* genes and acid exposure time in the distal esophagus. Expression of *IL-18*, *TNFA*, *GATA3*, *TLR4*, *CD68* genes correlated inversely with mean pH values in the distal part of the esophagus. No correlation was found between the expression of cytokines' genes in esophageal mucosa and the number of GER.

ARTICLE HIGHLIGHTS

Research background

Gastroesophageal reflux disease (GERD) has a number of manifestations, including erosive esophagitis (EE) and non-erosive form of GERD (NERD). Similar levels of intraesophageal acidity are often found in subjects with EE and NERD and little is known about the reasons.

Research motivation

Several reports suggest involvement of inflammatory cytokines into pathogenesis of different forms of GERD. Local inflammatory response of esophageal mucosa to gastroesophageal reflux (GER) may depend on cytokines genes' expression. However, data on the dependence of the expression on esophageal acidity are still lacking.

Research objectives

The objective of the study was to explore the expression of inflammatory cytokines' genes in esophageal mucosa in patients with EE and NERD and its association with data of esophageal multichannel intraluminal impedance-pH (MII-pH) measurements.

Research methods

The data were obtained in a single-center prospective study. We analyzed the expression of interleukin (*IL*)-*1β*, *IL-10*, *IL-18*, tumor necrosis factor α (*TNFA*), toll-

like receptor 4 (*TLR4*), GATA binding protein 3 (*GATA3*), differentiation cluster 68 (*CD68*) and β-2 macroglobulin (*B2M*) genes in esophageal mucosa samples obtained during endoscopy. All the subjects underwent MII-pH measurements. Based on the presence of abnormal results they were allocated either to the GERD groups or to the control groups. The GERD group was further divided into the EE group and NERD group. Spearman ranks' correlation analysis was used to analyze association of cytokines' genes expression with esophageal acidity, number and types of GER.

Research results

We found higher expression of *IL-18* and *GATA3* genes in EE group compared to NERD. Expression of *IL-1β*, *IL-18*, *TNFA*, and *TLR4* genes was lower ($P < 0.05$) in the control group compared to EE and NERD. Esophageal acid exposure correlated with the expression of *IL-1β* (Spearman rank r = 0.29), *IL-18* (r = 0.31), *TNFA* (r = 0.35), *GATA3* (r = 0.34), *TLR4* (r = 0.29), *CD68* (r = 0.37) genes. Mean esophageal pH correlated inversely with the expression of *IL-18*, *TNFA*, *GATA3*, *TLR4*, *CD68* genes. No association of the genes' expression with number of GER was found.

Research conclusions

In patients with EE, local expression of *IL-18* and *GATA3* genes was higher compared to subjects with NERD. Esophageal acid exposure correlated directly with expression of *IL-1β*, *IL-18*, *TNFA*, *TLR4*, *CD68* and *B2M* genes. Inverse correlation is revealed between expression of *IL-18*, *TNFA*, *GATA3*, *TLR4*, *CD68* genes and mean esophageal acidity. Expression levels of *IL-10* gene did not differ significantly in the studied groups and did not correlate with esophageal acid exposure and number of GER.

Research perspectives

Larger studies are necessary to confirm the obtained results. Assessment of the association of local inflammatory response with bile acids concentration within the refluxate seems promising.

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