Expression of Transcription Factor FOXO3a is Decreased in Patients with Ulcerative Colitis

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Abstract

Background: Ulcerative colitis (UC) is associated with differential expression of genes involved in inflammation and tissue remodeling, including FOXO3a, which encodes a transcription factor known to promote inflammation in several tissues. However, FOXO3a expression in tissues affected by UC has not been examined. This study investigated the effects of FOXO3a on UC pathogenesis.

Methods: FOXO3a expression, in 23 patients with UC and in HT29 cells treated with tumor necrosis factor-α (TNF-α) for various durations, was detected by quantitative real-time polymerase chain reaction and Western blotting analysis. Enzyme-linked immunosorbent assay was used to quantify interleukin (IL)-8 expression in FOXO3a-silenced HT29 cells treated with TNF-α for various durations.

Results: The messenger RNA and protein expression of FOXO3a were significantly lower in UC tissues than those in normal subjects (P < 0.01). TNF-α treatment for 0, 0.5, 1, 6, and 24 h induced FOXO3 degradation in HT29 cells. FOXO3a silencing increased IL-8 levels in HT29 cells treated with TNF-α for 6 h (P < 0.05).

Conclusion: FOXO3a may play an important role in the intestinal inflammation of patients with UC.

Key words: FOXO3a; Interleukin-8; Ulcerative Colitis

INTRODUCTION

Ulcerative colitis (UC) is a type of nonspecific colon inflammation, with lesions mainly at colonic mucosa and submucosa. Its etiology and pathogenesis are yet to be elucidated. Currently, it is believed that immune dysfunction of intestinal mucosa against bacteria in the lumen, loss of intestinal homeostasis, and excessive inflammatory responses against pathogens leads to increased secretion of proinflammatory cytokines, resulting in intestinal inflammation.1,2 Several studies have found that patients with UC have higher secretion of many cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, and IL-8. This finding indicates that cytokine imbalance plays an important role in the occurrence and the development of UC.

The transcription factor FOXO3a is an important member of the forkhead family. It is widely expressed in many tissues and organs in adults and regulates many cellular functions such as growth, metabolism, differentiation, apoptosis, and immunity through transcriptional regulation.3-5 Some studies suggest that FOXO3a plays an important role in the occurrence and the development of inflammation through regulation of nuclear factor kappa B (NF-κB).6 However, FOXO3a expression has not been reported in UC. In this study, we explored the role of FOXO3a in the pathogenesis of UC by examining FOXO3a levels in the intestinal mucosa of patients with UC and in the intestinal HT29 cells stimulated by TNF-α, as well as by observing changes in IL-8 expression in these cells after silencing FOXO3a using small interfering RNA (siRNA).

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Methods
Tissue collection
Colonic mucosa pinch biopsies were obtained from the sigmoid colon of patients with active UC (n = 23) and healthy subjects (n = 20) undergoing screening colonoscopies at the Institute of Digestive Diseases, Chinese People’s Liberation Army General Hospital from May to November 2011. UC diagnosis was confirmed by colonoscopy and pathological findings. Disease activity was defined using the Mayo criteria.\(^7\) The clinical characteristics of the patient groups are listed in Table 1. The biopsies were either embedded in paraffin blocks or snap frozen in liquid nitrogen and subsequently stored at −80°C. This study was approved by the Chinese People’s Liberation Army General Hospital Institutional Review Board.

Cell culture and tumor necrosis factor-\(\alpha\) treatment
The human intestinal epithelial HT29 cells (ATCC, Rockville, USA) used in this study were routinely cultured in Dulbecco’s modified Eagle medium (DMEM) (Invitrogen, Carlsbad, USA) supplemented with 10% fetal calf serum, penicillin, and streptomycin at 37°C in a 5% CO\(_2\) incubator. For protein analysis, cells were plated in 6-well plates and used at 60–70% confluence. Semiconfluent HT29 monolayers were treated with TNF-\(\alpha\) at a final concentration of 10 ng/ml for 0.5, 1, 6, and 24 h.

Quantitative real-time polymerase chain reaction
FOXO3a expression was validated by an SYBR green-based quantitative real-time polymerase chain reaction (qRT-PCR) assay (Qiagen, Valencia, USA). Primers for FOXO3a and the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtained from Invitrogen and are listed in Table 2. The qRT-PCR was performed in an ABI7900 cycler (Applied Biosystems, Foster City, USA), and the cycle threshold (Ct) value was recorded.

Western blotting analysis
Protein extracts were prepared from the human tissues and cells using a lysis buffer supplemented with ethylenediamine tetraacetic acid (EDTA)-free complete protease inhibitors (Roche, Mannheim, Germany). Proteins were extracted and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel. The bands were transferred to a polyvinylidene difluoride membrane. After blocking with 5% nonfat dry milk in Tris-buffered saline supplemented with 0.1% Tween 20 for 4 h at room temperature, the membranes were incubated overnight with either anti-FOXO3a or anti-\(\beta\)-actin primary antibodies (all used at 1:1000 dilution; Bioworld, Shanghai, China) at 4°C. The membranes were then incubated with a secondary antibody at 37°C for 1 h. Normalization was performed by blotting the same membranes with anti-\(\beta\)-actin antibody.

RNA interference
The siRNAs targeting FOXO3a were designed and synthesized by GenePharma (Shanghai, China) and are listed in Table 2. All siRNA transfections were performed with Lipofectamine 2000 (Invitrogen). HT29 cells were transfected with 100 nmol/L siRNA for 48 h.

Enzyme-linked immunosorbent assay
To quantify IL-8 expression, the culture medium from the monolayers was analyzed using an enzyme-linked immunosorbent assay kit (R and D Systems, Minneapolis, USA) according to the manufacturer’s protocol.

Statistical analysis
All experiments were repeated at least 3 times. The results were expressed as mean ± standard deviation values. The differences between groups were determined by two-tailed Student’s t-tests and one-way analysis of variance (ANOVA) performed using the software SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). \(P < 0.05\) was considered as statistically significant.

Results
FOXO3a expression decreased in patients with active ulcerative colitis
As shown in Figure 1, the messenger RNA (mRNA)
expression of FOXO3a was significantly lower in the colon tissues of active UC patients than in those of the controls ($P < 0.01$). FOXO3a protein level was significantly lower in active UC tissues than in the tissues of healthy subjects ($P < 0.01$) [Figure 2].

**Tumor necrosis factor-α regulates FOXO3a in HT29 cells**

Treatment of HT29 cells with TNF-α for 0, 0.5, 1, 6, and 24 h resulted in decreased FOXO3a mRNA expression [Figure 3]. TNF-α treatment also induced FOXO3 protein degradation [Figure 4].

**Silencing FOXO3a increases interleukin-8 level in tumor necrosis factor-α–treated HT29 cells**

FOXO3a silencing by siRNA was confirmed by Western blotting analysis [Figure 5]. We chose siRNA-1886 for the use in further investigation as it was found to be the most effective. Transfection of HT29 cells with siRNA against FOXO3a inhibited FOXO3a mRNA expression by 90% (data not shown). As shown in Figure 6, FOXO3a silencing significantly increased the level of IL-8 in HT29 cells after 1-h treatment with TNF-α.

**DISCUSSION**

The forkhead family of transcription factors includes at least 17 sub-families from FOXa to FOXq. They play very important roles in regulating the expression of genes related to cellular functions such as apoptosis, cell cycle, oxidative stress, and differentiation.[8-10] In mammals, the FOXO protein sub-family includes FOXO1, O3, O4, and O6, which are important transcription factors that function downstream of the PI3K/Akt signaling pathway. Various external stimuli and cytokines can stimulate PI3K and activate Akt, leading to phosphorylation of the transcription factor FOXO3a and its transport from the nucleus into the cytoplasm. This translocation of FOXO3a decreases the transcriptional activation of the target gene, which finally affects the corresponding cellular signaling pathway.[11,12] Some studies have shown that FOXO3a knockout mice develop spontaneous, multi-organ systemic immune responses, which increase with increasing cytokines production and NF-κB activity.[6]

The pathological basis of UC is nonspecific intestinal inflammation, characterized by infiltration of lymphocytes, plasma cells, and neutrophils into the colon. These activated inflammatory cells can release inflammatory mediators and cytokines, which contribute to the occurrence and the
FOXO3a silencing in HT29 cells treated with tumor necrosis factor-α for 1 h. The medium was collected for interleukin-8 quantification. *P < 0.05.

Figure 5: Effect of three small interfering RNAs on FOXO3a expression as determined by Western blotting analysis. All small interfering RNAs decreased FOXO3a level, but small interfering RNA-1886 was the most effective.

Figure 6: FOXO3a silencing in HT29 cells treated with tumor necrosis factor-α for 1 h. The medium was collected for interleukin-8 quantification. *P < 0.05.

In order to further clarify the regulatory mechanism of FOXO3a in intestinal inflammation, we observed the changes in FOXO3a expression in TNF-α-stimulated HT29 cells, as well as determined the changes in the expression of the inflammatory cytokine IL-8 in FOXO3a-silenced HT29 cells. Our results show that FOXO3a expression decreased gradually while IL-8 level increased significantly with increasing TNF-α stimulation time. IL-8 is linked to many inflammatory diseases and is known to promote inflammation, as well as induces immune responses and tissue damage. IL-8 level in colon mucosa and peripheral blood in patients with UC significantly increases with the severity of inflammation, and it plays an important role in the persistence and amplification of UC inflammation. Some current studies suggest that IL-8 is an essential inflammatory mediator in the development of UC and inflammatory reactions induced by TNF-α, IL-1, and IL-6 are to a large extent mediated by the IL-8-induced production of other chemokines.[14]

NF-κB regulates genes involved in many physiological and pathological processes, including the proliferation of inflammatory immune cells and apoptosis.[15] It also plays an important role in the pathogenesis of a range of diseases that involve cytokine inflammatory mediators and is closely linked to UC pathogenesis.[16,17] Some studies have shown that FOXO3a can inhibit NF-κB activation, reducing downstream production of inflammatory cytokines.[8] This type of regulation can allow for the control of the inflammatory reaction and help maintain the homeostasis of the intestinal mucosa. Therefore, we believe that the reduction in FOXO3a expression in the intestinal mucosa of patients with UC likely leads to increased production of inflammatory cytokines (such as IL-8) through inducing NF-κB activation. This would disrupt the cytokine balance in the intestinal mucosa, thereby inducing inflammation and ultimately damaging the mucosa.

The results of this study indicate that FOXO3a could involve in the inflammation of intestinal mucosa and might play an important role in UC pathogenesis. It could also be a protective factor of the intestine, maintaining the immune hemostasis of the mucosa against lumen bacteria and the balance among inflammatory cytokines. Therefore, FOXO3a could be a potential therapeutic target for UC.

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Conflicts of interest
There are no conflicts of interest.

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