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Original article

Runt-related transcription factor 2 (RUNX2) inhibits apoptosis of intestinal epithelial cells in Crohn's disease

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ABSTRACT

Apoptosis in intestinal epithelial cells (IECs) prevents the development of Crohn's disease (CD), a type of inflammatory bowel disease (IBD). Runt-related transcription factor 2 (Runx2) inhibits apoptosis in osteosarcoma-derived U2OS cells via down-regulating the transcriptional activity of p53. However, the expression and function of Runx2 in CD remain unclear. In this study, Runx2 protein levels were decreased in the intestinal epithelial cells (IECs) of CD patients and in a mouse 2, 4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis model; in contrast, the expression levels of p53 and Bax, a p53-target gene, were increased. In a TNF-α-treated HT29 cell colitis model, the down-regulation of Runx2 was accompanied by the up-regulation of apoptotic markers, including cleaved caspase-3 and Bax. Furthermore, Runx2 overexpression effectively decreased TNF-α-induced Bax and cleaved caspase-3 expression levels. In conclusion, our data indicated that Runx2 might protect IECs from apoptosis in CD, thus revealing a novel molecular target for treating CD.

1. Introduction

Crohn's disease (CD) is a type of inflammatory bowel disease (IBD); IBDs are a heterogeneous family of chronic gastrointestinal inflammatory disorders. The clinical manifestations of CD include stomach ache, diarrhoea, intestinal obstruction accompanied by fever, nutritional disorders and other parenteral symptoms [1]. The disease is prone to recurrent attacks, and especially when aggravated, it can seriously decrease the work productivity and life quality of CD patients [2,3]. The rising incidence and prevalence of IBD has been noted over time worldwide, including the countries of England, Persia, Iran and China [4]. In recent years, a number of studies have focused on the aetiology of CD [5–7]. However, due to the complex interactions among hereditary predisposition [8] and variations in the microflora and microenvironmental factors [9], which induce intestinal immune regulation disorders, the pathogenesis of CD remains unclear. Intestinal epithelial cells (IECs) play critical roles in maintaining host-microbial interactions and tissue homeostasis [10], and IEC apoptosis may destroy the structural integrity of the gut, contributing to the expansion of acquired immune responses to abnormal gut microflora in CD [11]. From recent studies of the relationship between IEC apoptosis and CD development [12], it is apparent that IEC apoptosis plays a significant role in the pathogenesis of CD.

Runt-related transcription factor 2 (Runx2) was originally recognized as a master protein regulating bone development [13]. Later, Runx2 was found to interact with other transcriptional factors, such as p53, which induces apoptosis [14]. Runx2 inhibits the pro-apoptotic activity of p53 to protect cells from DNA damage [14], while p53 is closely linked to IEC apoptosis in colitis. In addition, Runx2 and Myc proto-oncogene protein (MYC) contribute to lymphoma development by inhibiting apoptotic pathways [15]. Runx2 can mediate Bcl-2 expression to cause apoptosis resistance [16]. Moreover, Runx2 activates phosphoinositide-3-kinase (PI3 K)/protein kinase B alpha (Akt) signalling to increase survival in epithelial cell lines via mammalian target of rapamycin complex-2 (mTORC2) [17]. According to previous studies, Runx2 is associated with cell apoptosis. However, the expression and function of Runx2 in CD are unclear.

In the present study, we aimed to determine the underlying molecular mechanisms of CD. First, the expression levels of Runx2, p53, and Bax in samples from CD patients were measured. Then, the function of Runx2 in p53-induced apoptosis pathways in IECs during CD development in a 6-trinitrobenzenesulfonic acid (TNBS)-induced mouse colitis model and a TNF-α-treated HT29 cell colitis model was analysed. Our findings indicate that Runx2 is a potential therapeutic target for CD.

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2. Materials and methods

2.1. Clinical specimens

Colon tissue samples were collected from CD patients (n = 10) and healthy subjects (n = 10) by endoscopy at the Affiliated Hospital of Nantong University (Nantong, Jiangsu, China) from 2011 to 2016. A portion of each specimen was used for western blotting, and the other portion was immediately fixed in formalin and embedded in paraffin to section for immunohistochemistry. Written informed consent was obtained before specimen collection.

2.2. Colitis animal model

The care and handling of animals were in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Research Council in 1996 and the Chinese National Committee for the Use of Experimental Animals for Medical Purposes, Jiangsu Branch. BALB/c mice (females, weighing 18–20 g) were supplied by the Department of Laboratory Animals Center, Nantong University. All mice were maintained under SPF conditions and allowed free access to food and water. The mice were not used for experiments until they had adapted to the environment. To create a Crohn's disease model, enteritis was induced by 2, 4,6-trinitrobenzenesulfonic acid (TNBS) (Sigma Chemical Co., USA) as previously described [18]. In addition, 0.1 ml of 2.5% (w/v) TNBS solution in 50% ethanol was injected into the colon to create a colitis model. For the control group, 0.1 ml of 50% ethanol was injected into the colon.

2.3. Evaluation of experimental colitis

Mouse body weights were recorded at the beginning of the study and before they were sacrificed, and the change rate was calculated as a percentage compared with the initial weight. Assessment of the colitis severity was based on a previously published standard [19] as follows: 0 = no macroscopic changes; 1 = mucosal erythema changes only; 2 = mild mucosal edema, slight bleeding or small erosions; 3 = moderate edema, bleeding ulcers or erosions; 4 = severe ulceration, erosions, edema, and tissue necrosis.

2.4. Cell culture and treatment

HT29 is a human colon epithelial cell line (Oulu Biotechnology, Shanghai, China). Cells were cultured in RPMI 1640 medium (Gibco, UK) containing 10% foetal bovine serum (FBS) with 100 μg/ml penicillin and 100 μg/ml streptomycin at 37 °C with 95% air/5% CO_2. The medium was replaced every other day. For subsequent experiments, the cells were stimulated with TNF-α (Sigma Chemical Co., USA) at different concentrations and times.

2.5. Western blotting

Colon samples from near the anus were lysed in lysis buffer (1% NP-40, 50 mmol/l; Tris, pH 7.5; 5 mmol/l EDTA; 1% sodium dodecyl sulfate (SDS); 1% sodium deoxycholate; 1% Triton X-100; 1 mmol/l PMSF; 10 g/ml aprotinin; and 1 g/ml leupeptin) and then centrifuged at 13,000*g* at 4 °C for 20 min to collect the supernatant. The protein extracts were harvested with lysis buffer. Protein concentrations were determined by Bradford protein assays (Pierce, USA). Equal amounts of protein were separated by 10% SDS-PAGE and transferred onto PVDF membranes (Millipore, USA) using the semidry transfer method; the membranes were blocked with skim milk in Tris-HCl-buffered saline for 2 h. Then, the membranes were incubated overnight at 4 °C with the primary antibodies, including anti-Runx2 (mouse, 1:500; Cell Signaling Technology, USA), anti-cleaved caspase-3, anti-Bax, anti-GAPDH, anti-β-actin and anti-p53 (rabbit, 1:500; Santa Cruz, USA). Then, the membranes were incubated with HRP-conjugated secondary antibodies (Jackson ImmunoResearch, USA) for 2 h at room temperature. The membranes were visualized using an enhanced chemiluminescence system (ECL, Pierce Company, USA).

2.6. Cell and tissue immunofluorescence

Cells were plated on small wafers placed in 24 well-plates, washed with PBS, fixed in 4% paraformaldehyde for 30 min, permeabilized with TritonX-100 diluted in PBS for 10 min, sealed for 2 h in a special blocking fluid of 5% BSA diluted in PBS and incubated with primary antibodies (anti-Runx2 and anti-p53; 1:50) in PBS at 4 °C overnight. After incubation, the cells were washed for 15 min (5 min × 3 times) with PBS. Then, the cells were incubated for 2 h with special fluorescence antibodies (1:2000; Eugene, Oregon, USA) and 4′, 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, USA) for 2 h in the dark, rinsed for 45 min (15 min × 3 times), and sealed with mounting medium (50% glycerol and 50% PBS). Images were collected using a fluorescence microscope (Olympus BX41, Olympus Corporation, Japan). Tissue fluorescence steps were similar to those for cell fluorescence.

2.7. Frozen sections and immunohistochemistry (IHC)

The mouse colon samples from near the anus were fast cut and fixed in 4% paraformaldehyde for 14 h. The samples were subsequently subjected to 20% and 30% sucrose gradient dehydration for one day until the colon sample sunk completely to the bottom. Then, the specimens were embedded in OCT and cut into 6 μm slices. Finally, the sections were stored at −20 °C. The paraffin sections (human colon tissue) were baked in an oven at 60 °C for 6–8 h, and the frozen sections (mice colon tissue) were baked for 1–2 h. Then, the antigens in the specimens were detected with an IHC kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions.

2.8. Apoptosis detection

The PE Annexin V Apoptosis Detection Kit I (BD Pharmingen, USA) was used to detect HT29 cell apoptosis. Cold PBS was used to wash the cells twice; then, the cells were spun down and resuspended in binding buffer at a density of 1×10^6 cells/ml. Next, 1×10^5 cells were added to a 10 ml reagent tube with 100 μl of solution. Then, 5 μl of PE Annexin V and 7-AAD were added. Cells were vortexed and incubated for 15 min at RT (25 °C) in the dark. Lastly, 400 μl of binding buffer was added to each tube, and flow cytometry (BD FACSAria II, USA) was used to measure apoptosis for 1 h.

2.9. Co-immunoprecipitation (Co-IP)

Transfected cells were incubated with the appropriate antibodies and protein A/G beads (Life Technologies, Oslo, Norway) overnight. The immunoprecipitates were washed 3 times and then subjected to immunoblot analyses.

2.10. Plasmids and transfection

The cDNA templates for human Runx2 (Origene, Beijing, China) and Runx2-HA were cloned into the pcDNA3.1 (+) plasmid. HT29 cells were digested with trypsin for 2 min and then plated at a density of 90% before transfection. Serum-free medium was used to culture cells for 24 h. The same amount of medium was used to dilute the plasmids and the transfection reagent Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany). After that, the plasmids and transfection reagents were mixed for 20 min, and the mixture was added to plates. Serum-containing medium was replaced with serum-free medium after 4–6 h.