Biomedicine & Pharmacotherapy 103 (2018) 346–354

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

GZMB gene silencing confers protection against synovial tissue hyperplasia and articular cartilage tissue injury in rheumatoid arthritis through the MAPK signaling pathway

Cui-Xia Bao[a,1], Hai-Xia Chen[b,1], Xue-Jie Mou[c], Xiang-Kui Zhu[d], Qi Zhao[a], Xin-Guang Wang[e,*]

[a] Clinical Laboratory, Yantai Yuhuangding Hospital, Yantai 264000, PR China
[b] Clinical Laboratory, Yeda Hospital, Yantai 264000, PR China
[c] Clinical Laboratory, Yantai Taocun Central Hospital, Yantai 265301, PR China
[d] Department of Radiology, Yantai Oral Hospital, Yantai 264000, PR China
[e] Department of Blood Transfusion, Yeda Hospital, Yantai 264000, PR China

ARTICLE INFO

Keywords:
GZMB
MAPK signaling pathway
Rheumatoid arthritis
Synovial tissue hyperplasia
Articular cartilage tissue injury

ABSTRACT

Introduction: Rheumatoid arthritis (RA) represents the most commonly occurring inflammatory type of arthritis and is a major cause of disability. Reports have placed emphasis on the potential of, granzyme B (GZMB) as a potentially valuable prognostic marker in early RA, the mechanism of which still remains largely unclear. Thus, the aim of the current study was to investigate the effects GZMB gene silencing influences synovial tissue hyperplasia and articular cartilage tissue injury of RA through the regulation of the MAPK signaling pathway.

Methods: Following the successful establishment of the collagen-induced animal model of RA in rats, a five-grade scoring method was applied to evaluate the swelling degree measurement of the rats for model identification. The various rat responses to GZMB shRNA and U-46619 (activator of the MAPK signaling pathway) were subsequently detected. The general status of rats was observed and recorded, with their weight and ankle diameter kept accurate record of. ELISA was employed to detect the levels of inflammatory cytokines, while RT-qPCR and Western blotting techniques were applied to determine the expressions of GZMB and pathway-related genes and proteins.

Results: GZMB gene silencing was observed to aid in the maintenance of rat weight increases, while acting to reduce the degree of ankle swelling, while hypertrophy of the synovial tissue and the injury of the articular cartilage tissue were not obvious. GZMB gene silencing was shown to decrease inflammatory cytokine levels, as well as decreased bcl-2, Cyclin D1, VEGF and bFGF while increasing caspase 3. Notably, GZMB gene silencing suppressed the activation of the MAPK signaling pathway by reducing the phosphorylation extent of ERK and MEK.

Conclusion: Taken together, the key findings of the present study ultimately suggest that GZMB gene silencing acts to inhibit MAPK signaling pathway through regulating the expressions of inflammatory factors, factors correlated with apoptosis (bcl-2 and caspase), as well as factors associated with angiogenesis (VEGF and bFGF), thus relieving synovial tissue hyperplasia and articular cartilage tissue injury brought about by RA. The GZMB gene could well be a new therapeutic target for RA treatment.

1. Introduction

Rheumatoid arthritis (RA) remains the most commonly occurring chronic inflammatory diseases, affecting millions worldwide [1]. RA primarily affects joints by eliciting an immunological response, triggered at various mucosal sites, manifested to be warm, swollen, and painful joints [2]. RA is characterized by the erosion of the underlying bone and destruction of the articular cartilage [3]. The incidence of the condition has been shown to occur at any age, however 30–50 years olds represent the most at risk age group, with the lifetime prevalence of RA reported to have risen to 1% all globally [4]. RA affects patients primarily through its negative effect on patients' daily activities and health-related quality of life, ultimately increasing mortality [5]. At present, treatments available for RA are largely aimed at alleviating

* Corresponding author at: Department of Blood Transfusion, Yeda Hospital, No. 11, Taishan Road, Yantai, 264000, Shandong Province, PR China.
E-mail address: xinguangwangxgw@126.com (X.-G. Wang).
[1] These authors are regarded as co-first authors.

https://doi.org/10.1016/j.biopha.2018.04.023
Received 8 February 2018; Received in revised form 3 April 2018; Accepted 3 April 2018

pain, reducing inflammation, in a bid to improve the overall function of RA patients [6]. Previous reports have indicated that targeted immune treatments and aggressive therapeutic strategies may contribute significantly to the clinical outcomes and pathogenetic understanding of RA. At present, there is no existing cure for RA [7]. Moreover, other existing treatments available for RA are not by any means considered to be curative or definitive in nature [8]. Therefore, elucidating the potential molecular mechanism is an absolute necessity in order to reach the eventual goal of developing new treatment strategies for RA.

Granzyme B (GZMB) represents a serine proteinase, which assembles in the cytoplasmic granules of activated cytotoxic T lymphocytes and natural killer cells [9]. GZMB plays a central role in killing human tumor cell lines. Furthermore, GZMB of human origin has been reported to be a reliable candidate for tumor therapy considering its being recombinantly expressed [10]. Studies have attested to the notion that GZMB can induce cell death and apoptosis [11]. Likewise, a previous study demonstrated that GZMB could act to repress viral replication, which may promote the antiviral immunity [12]. More importantly, GZMB is likely to be a useful prognostic marker in early RA and may provide crucial inklings aiding in the elucidation of the pathogenesis of RA [13]. Mitogen-activated protein kinases (MAPKs) are a class of serine-threonine kinases, which mediate intracellular signaling in regard to an array of cellular activities including that of cell proliferation, inflammation, and survival [14]. Studies have demonstrated as regulators of inflammation and stress responses, p38, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK) as three crucial components of the MAPK pathway [15]. The MAPK pathway has been a deeply investigated, findings of which have identified key signaling mechanism in plants, insects and mammals [16]. Besides, MAPK has been reported to play an essential role in the pathogenesis of RA, with ERK inhibitors expected to be new therapeutic method for RA [17]. A previous study highlighted GZMB contributory involvement in the incidence of inflammation in cases of human disease *via* the p38 MAPK signaling cascade [18]. Furthermore, U-46619, a thromboxane receptor agonist [19,20] has been to play a role in the MAPK signaling pathway [21], and hence, was utilized in the cell transfection process in the present study. Previous evidence has been provided indicating an association between U-46619, improved recovery and tissue repair in people suffering from toxin-induced acute liver injury [22]. Based on the exploration of current literature, we subsequently asserted a hypothesis that silencing of the GZMB exerts influence on RA through the regulation of the MAPK pathway. Therefore the aim of the current study was to confirm the hypothesis so as to propose a novel therapeutic target for RA.

2. Materials and methods

2.1. Ethics statement

The study was conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The applied protocols of the study were approved by the Institutional Animal Care and Use Committee of Yeda Hospital. All efforts were made in order to minimize both the number of animals used as well as their respective suffering (the number of the ethical committee's sanction: 201705003).

2.2. Model establishment of RA

Sixty Wistar adult male rats weighing 150–180 g provided by the laboratory animal center of Yantai Medical College were recruited for the purposes of this study. The rats were confirmed to be in good health, and were housed and fed in a clean environment with access to Co^{60} sterilized commercial feed that conformed to national standards by obtaining a production license, as well as administration of an amicrobic filtered water. The feeding environment was controlled according to the following: air exchange rate 8–15 times per hour, ammonia concentration was below 20 ppm, while relative humidity was between 40 and 80%, filtered air purity was at grade 10,000, noise levels were below 50 dB, with the temperature set at controlled levels between 20 and 23 °C.

According to the Freunds Adjuvant Incomplete (08642852, MP Biomedical Inc, California, USA) formula, the complete Freund's adjuvant (CFA) was compounded to establish the model. Based on the formula of the IMCII-Immunization grade chick type II collagen (20011, Chondrex Inc, Washington, USA), the type II collagen with concentration of 2 g/L was compounded, which was then mixed with 2 g/L of CFA into 1 g/L of stable emulsion. Post anesthesia administration by aether, the coats on the backs of rats were shaved off, with the emulsion subcutaneously injected administered to the rats via their tails to both sides of spine at different sites. Each rat was administered with 1.0 mL, and after 1 week had passed, they were injected once more to boost their immunity. Collagen (0.5 mg) was dissolved into 0.5 mL/1 M of ethylic acid, which was then injected into rats (0.5 mL for each). The collagen-induced animal model of OA in rats was then presumed to be established. Rats in control group were injected with normal saline in the corresponding period.

2.3. Model identification

The initial subcutaneous injection of emulsion was the primary immunization. A five-grade scoring system was applied in order to measure the arthritis index of rats on the 21st day, 28th, 35th and the 42nd day, from the day when small ulcers (would self-heal 7 days later) were observed in the primary immunization sites (Table 1). The evaluation score was marked based on the degree and range of redness and swelling of joints of rats as well as arthrophyma and arthrentasis. 0 point were given for rats without arthritic symptoms; 1 point for rats with mild symptoms and ankle swelling; 2 points for rats with moderate ankle swelling; 3 points for severe swelling on the entire paw including the toes; and 4 points for severe joint swelling with dysfunction. The average was calculated based on the sum of scores in relation to the symptoms on the two paws and toes. After the booster injection, the growth of ankle diameter of rats was measured accordingly.

2.4. Vector construction

The Gen Bank database was utilized in order to search for the mRNA sequence of GZMB in rats [23]. The sequence was designed by RNAi software, with three shRNA sequences subsequently selected for homology analysis purposes by the Basic Local Alignment Search Tool (BLAST). Chemical synthesis methods were employed in order to synthesize the shRNA sequence, while the matched single chains were synthesized into double chains. The shRNA sequence (Table 2) was as follows:

The establishment of RNAi retroviral vector: two segments of shRNA sequence were added respectively with the BamHI and Hind III endonuclease sites, and the BamHI and Hind III double enzyme digestion pLXIN vectors, followed by reclaiming of the target gene segments. The connection reaction system comprised of 10 μL, including 5 μL of connection reaction buffer (2 ×), 1 μL of retroviral vector pLXIN (Biovector Co., Ltd), 1 μL of insertion segment (approximately 0.25 μg), 1 μL of T4

Table 1
Score table of arthritic index.

Score	Arthritic symptoms
0	No arthritis
1	Mild symptoms, redness and swelling appeared on ankle or wrist
2	Moderate redness and swelling on ankle and wrist
3	Severe redness and swelling on whole paws including toes
4	Severe swelling and dysfunction on joints