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Original Article

High mobility group box protein 1 downregulates acid β-glucosidase 1 in synovial fibroblasts from patients with rheumatoid arthritis

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Abstract: High mobility group box protein 1 (HMGB1) plays an important role in the pathogenesis of rheumatoid arthritis (RA), but the pathogenic mechanisms of HMGB1 in RA and the involvement of the lysosomal enzyme acid β-glucosidase 1 (GBA1) are not fully elucidated. The aim of the present study was to use HMGB1 to treat RA synovial fibroblasts (RASFs) and to examine the changes of transcriptional factors. RASFs were isolated from synovial tissues obtained from five RA patients undergoing synovectomy or joint replacement. RASFs were incubated with 100 ng/mL of HMGB1 for different periods. The changes in transcriptional factors were screened by RNA sequencing (RNA-seq) and results were confirmed by quantitative real-time PCR and western blot. The results showed that the mRNA of >60 genes in RASFs were differentially expressed after HMGB1 treatment. Among them, GBA1 was the most markedly decreased (-3.99 folds, P<0.001). These results were confirmed by qRT-PCR and western blot. The late-stage inflammatory mediator HMGB1 probably exerts its pathogenic role in RA by downregulating GBA1.

Keywords: Rheumatoid arthritis, synovial fibroblasts, high mobility group box protein 1, lysosomal enzyme acid β-glucosidase 1

Introduction

The highly conserved DNA-binding protein high mobility group box protein 1 (HMGB1) exerts vital intracellular and extracellular functions in eukaryotic cells [1]. It is known that proinflammatory cytokines such as tumor necrosis factor (TNF)-α or interleukin (IL)-1β can stimulate HMGB1 translocation into the cytoplasm and its release in different cell types [2]. Extracellular HMGB1 acts as an alarm in binding to multiple cell-surface receptors, cytokines, and chemokines to stimulate the innate immune system and trigger inflammatory responses [3, 4].

The role of HMGB1 in chronic inflammatory diseases such as rheumatoid arthritis (RA) has been extensively studied [5-9]. In patients with RA, extranuclear expression of HMGB1 can be detected locally in their synovial fluid and serum [6]. Taniguchi et al. [5] demonstrated that HMGB1 plays a key role in the pathogenesis of RA; they showed that HMGB1 levels were higher in synovial fluid of RA patients than in those of osteoarthritis (OA) patients, and expression of HMGB1 was prominent in macrophages and endothelial cells. Moreover, HMGB1 also stimulates macrophages derived from synovial fluid to release proinflammatory cytokines (such as TNF, IL-1β and IL-6), activated complement, and hypoxia [2, 5]. In addition, HMGB1-nucleosome complexes lead to the formation of autoantibodies against DNA and nucleosome [2]. HMGB1 also promotes proteolytic enzymes and the osteoclast maturation requires HMGB1 [2]. Nevertheless, the mechanisms underlying the pathogenic effects of HMGB1 in RA are still not fully elucidated.

The lysosomal enzyme acid β-glucosidase 1 (GBA1) cleaves the β-glycosidic linkage of glucosylceramide to release glucose and generate ceramides [10]. Mutations in the GBA1 gene result in the abnormal accumulation of lipid substrates and have been involved in Gaucher's disease and Parkinson's disease [11,

Table 1. Characteristics of the patients who provided synovial samples

	RA patients
N	5
Age (years)	59±8
Sex (female/male)	4/1
Disease duration (years)	20.2±8.0

Table 2. Sequences of PCR primers

Genes	Primers (5'-3')
GBA1	CTGCTGCTCTCAACATCCTT
	GAAGGGGTATCCACTCAACA
GAPDH	GGAGCGAGATCCCTCCAAAAT
	GGCTGTTGTCATACTTCTCATGG

PCR: polymerase chain reaction; GBA1: acid β-glucosidase 1.

12]. GBA1 is involved in sphingolipids metabolism and the salvage pathway of ceramide formation [13]. Ceramides have emerged as a bioactive lipid that mediates a variety of cellular responses, including regulation of cell growth, differentiation, and stress responses [14]. Kitatani et al. [15, 16] had proved that GBA1 played a significant role in the salvage pathway of ceramide formation in PKC-mediated cellular responses, and the GBA1-ceramide pathway was vital in terminating p38δ activation responsible for IL-6 biosynthesis. It is well-known that IL-6 plays a pivotal role in the pathogenesis of RA [17]. Moreover, interleukin-6 receptor inhibition, primarily using tocilizumab, has been used effectively for patients with RA [18]. Taken together, we speculated that the abnormal expression of GBA1 may take part in the progression of RA by disturbing IL-6 biosynthesis. Nevertheless, the link between GBA1 and HMGB1 is very poorly understood. Some previous studies showed that cancer cells treated with apoptosis-inducing doses of ceramides released large amounts of HMGB1 in the medium [19-21]. In addition, a recent study suggested that ceramides and HMGB1 could mediate the toxic metabolic effects of cigarette smoke [22].

Therefore, the roles of HMGB1 and GBA1 in RA remain to be explored. The aim of the present study was to use HMGB1 to treat RA synovial fibroblasts (RASFs) and to examine the changes of transcriptional factors using RNA sequencing (RNA-seq). RNA-seq is a technique that profoundly sequences the transcriptional levels and covers almost all transcripts of a specific tissue at a specific time [23]. The results demonstrated that the mRNA of >60 genes in RASFs were changed significantly after HMGB1 treatment, with GBA1 being decreased. Further investigation confirmed that HMGB1 downregulated GBA1 at the mRNA and protein levels in RASFs.

Materials and methods

Cell cultures

Synovial tissues were obtained from five RA patients undergoing synovectomy or joint replacement (**Table 1**). All RA patients met the American College of Rheumatology 1987 revised criteria for the classification of RA [24]. All samples were obtained with the informed consent from the patients, and the study was approved by the Ethics Committee of First Affiliated Hospital of Jiaxing University (No. 2015-025).

Synovial tissues were minced and digested in DMEM containing 1.0 mg/mL of bacterial collagenase for 2 h at 37°C. The cell suspensions were then filtered through a 100 μm nylon filter and collected by centrifugation. The cells were washed and resuspended with complete medium made of DMEM containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, and cultured in a tissue culture incubator at 37°C with 5% CO_2. The non-adherent cells were washed out. Adherent cells were cultured in complete medium and the culture medium was replaced every week [9]. At 80% confluence, the cells were dispersed by trypsinization and then transferred to new plastic dishes. For subsequent experiments, cells were used at the third to sixth passages, at which time they were a homogeneous population of fibroblasts. Before experiments, cultured RASFs were starved for 2 hours in DMEM supplemented with 50% and 25% fetal bovine serum, respectively. RASFs were incubated with 100 ng/mL of HMGB1 (R&D Systems, Minneapolis, MN, USA; a gene recombinant product with a purity >95% as determined by SDS-PAGE, without DNA and the LPS was <0.10 EU per μg of protein as determined by the LAL method), as previously described [8, 9, 25] for different periods.

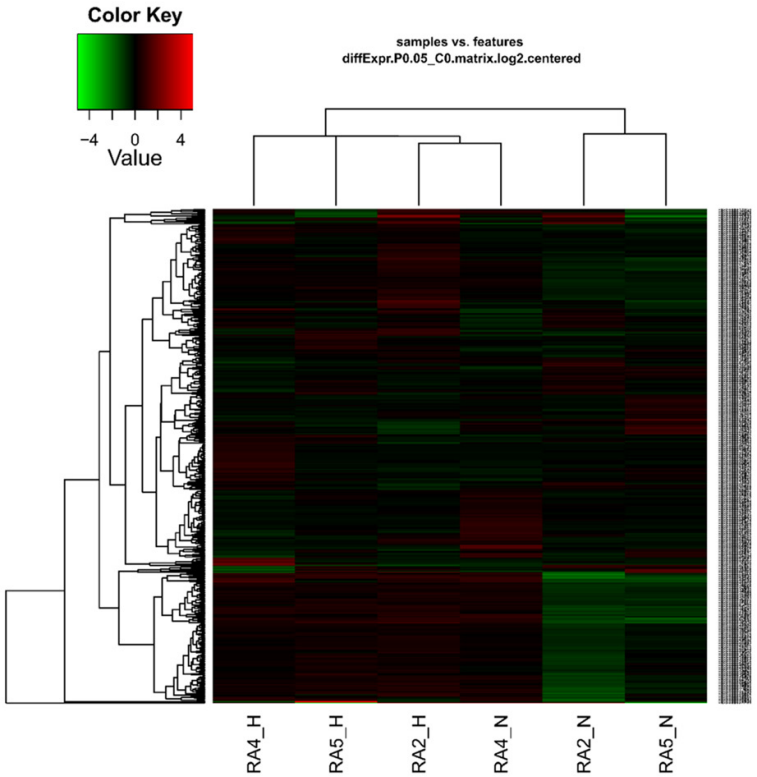

Figure 1. Heatmap plot of differentially expressed mRNAs in RASFs treated or not with HMGB1.

RNA-Seq

Total RNA from cell pellets of RASFs was isolated with TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA). Total RNA was then supplied to Sangon Biotech Co., Ltd. (Shanghai, China) for downstream processing. Paired-end (PE) libraries were prepared according to the Illumina paired-end library preparation protocol (Illumina, San Diego, CA, USA) and were sequenced on an Illumina Hiseq 2500 sequencing system. Raw reads from each sequencing library were assessed using FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to remove adaptor sequences, reads with unknown sequences "N", and low-quality sequences.

Quantitative real-time PCR (qRT-PCR)

Total RNA was purified from RASFs with TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA) and cDNA was synthesized using PrimeScript reverse transcription reagents (TaKaRa, Dalian, China). Real-time PCR was performed on a QuantStudio 3 using SYBR Premix EX Taq reagents. The cycling conditions for qRT-PCR was: denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 3 s and 60°C for 60 s. Primer sequences of GBA1 and GAPDH are listed in **Table 2** (Shanghai Sangon, Co., Shanghai, China). The GAPDH gene was used to normalize all tested genes, and quantification was performed using the ΔΔCT method.

Western blotting

RASFs were lysed in a buffer containing 1.0% (vol/vol) Nonidet-P40, 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, and a protease inhibitor mixture (Roche, Basel, Switzerland). Proteins (10 μg) from each sample were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked with anti-GBA1 antibody (1:500; ab55080; Abcam, Cambridge, MA, USA). Proteins were visualized with an ECL Western Blotting System (Pierce Protein Research Products, Rockford, IL, USA) using a HRP-linked anti-rabbit IgG (Cell Signaling, Danvers, MA, USA).

Statistical analysis

Results are presented as mean ± standard deviation. Statistical analyses were performed using one-way analysis of variance followed by independent-samples T test or satterthwaite t' test. Two-sided *P*-values <0.05 were considered statistically significant. All statistics were performed using SPSS 20 for Windows (IBM, Armonk, NY, USA).

Results

HMGB1 regulated mRNA expression in RASFs

We used HMGB1 to treat RASFs for 24 h and RNA-seq to screen the changes of transcriptional factors at the transcriptome level. The results showed that a number of mRNAs in RASFs treated with HMGB1 were differentially

Table 3. Genes and related proteins changed after HMGB1 treatment in RASFs (partial results)

Genes	Proteins	Control	HMGB1	Log 2-Fold change	P
Down-regulated					
GBA1	Glucosidase beta acid 1	5.01	0.31	-3.99	<0.001
FN1	Fibronectin 1	6110.94	5366.12	-0.19	0.0450
Up-regulated					
RGPD5	RANBP2-like and GRIP domain containing 5	0.24	1.71	2.86	0.0012
MRPS36	Mitochondrial ribosomal protein S36	0.39	2.42	2.61	0.0055
SRP9	Signal recognition particle 9	11.84	47.86	2.02	0.0067
KPNA5	Karyopherin subunit alpha 5	1.44	4.41	1.62	0.0045
ZNF117	Zinc finger protein 117	2.11	5.53	1.39	0.0065
TMEM106B	Transmembrane protein 106B	8.80	20.74	1.24	0.0065

HMGB1: high mobility group box protein 1.

expressed compared with RASFs without HMGB1 pretreatment. GBA1 was downregulated significantly (-3.99 folds, P<0.001) (**Figure 1** and **Table 3**). Fibronectin 1 (FN1) also was downregulated (-0.19 fold, P=0.045), while RANBP2-like and GRIP domain containing 5 (RGPD5), mitochondrial ribosomal protein S36 (MRPS36), signal recognition particle 9 (SRP9), Karyopherin subunit alpha 5 (KPNA5), zinc finger protein 117 (ZNF117), and transmembrane protein 106B (TMEM106B) were upregulated (all P<0.01) by HMGB1 (**Table 3**). These results suggest that HMGB1 decreases GBA1 transcription in RASFs.

HMGB1 downregulated the GBA1 mRNA expression in RASFs

We validated the RNA-seq results using qRT-PCR. RASFs were treated with 100 ng/ml HMGB1 for different time periods. GBA1 mRNA in RASFs decreased significantly after treatment with HMGB1 for 16 or 24 h compared with RASFs without HMGB1 treatment (P<0.05, **Figure 2C**, **2D**). There was no difference with controls for GBA1 mRNA in RASFs using HMGB1 treatment for 2 or 4 h (P>0.05, **Figure 2A**, **2B**).

HMGB1 downregulated the GBA1 protein in RASFs

Since the GBA1 mRNA was suppressed after HMGB1 treatment for 16 or 24 h, we further investigated whether the expression of the GBA1 protein was also decreased. We used 100 ng/ml HMGB1 to treat RASFs for 24 h. Western blot showed that the GBA1 protein expression was downregulated compared with cells without HMGB1 treatment (**Figure 3**).

Discussion

HMGB1 plays an important role in the pathogenesis of RA [5-9], but the pathogenic mechanisms of HMGB1 in RA and the involvement of GBA1 are not fully elucidated. Therefore, the aim of the present study was to use HMGB1 to treat RASFs and to examine the changes in transcriptional factors. The results suggest that the late-stage inflammatory mediator HMGB1 probably exerts its pathogenic role in RA by downregulating GBA1.

HMGB1 has been demonstrated to play an important role in the pathogenesis of RA [2, 5]. Further evidence of the role of HMGB1 in joint inflammation comes from studies indicating disease attenuation by agents (including monoclonal and polyclonal anti-HMGB1 antibodies, recombinant A box domain of HMGB1, soluble RAGE, recombinant thrombomodulin, corticosteroids, and gold salts) that antagonize the expression or the activity of HMGB1 [6, 26-31]. In addition, in patients with RA, HMGB1-nucleosome complexes lead to the formation of autoantibodies against DNA and nucleosome [2]. HMGB1 also promotes proteolytic enzymes and the osteoclasts maturation requires HMGB1 [2]. Importantly, in experimental animal models, neutralization of HMGB1 can protect against the characteristic cartilage and bone destruction observed in RA [32]. Nevertheless, the mechanisms are still unclear.

The present study suggests that HMGB1 influences the transcription of a number of mRNAs.

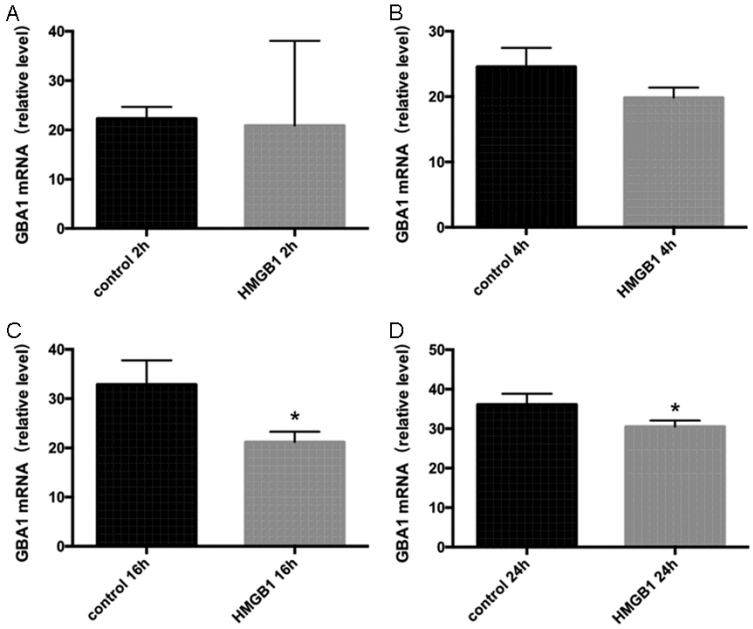

Figure 2. mRNA expression of GBA1 in RASFs with HMGB1 treatment for different periods. A, B. The mRNA expression of GBA1 in RASFs was not different after HMGB1 treatment for 2 or 4 h compared with cells without HMGB1 treatment. C, D. GBA1 mRNA in RASFs was decreased significantly after treatment with HMGB1 for 16 or 24 h compared with cells without HMGB1 treatment.

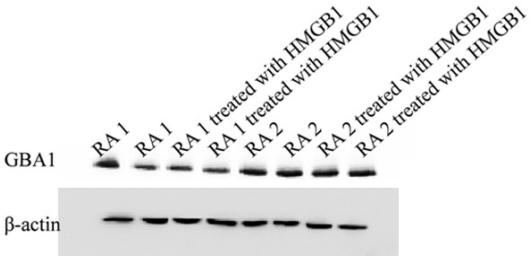

Figure 3. Protein expression of GBA1 in RASFs from two RA patients (RA1 and RA2) with or without HMGB1 treatment for 24 h.

Among them, GBA1 was down-regulated by 4 folds. GBA1 is a widely expressed lysosomal enzyme that cleaves glucosylceramide into ceramides. Ceramides are central in the sphingolipids metabolism and mainly involved in the salvage pathway of sphingolipids formation [33]. Meanwhile, ceramides are also a crucial lipid second messenger. When the extracellular receptors combine with ligands, ceramides mediate signal transduction in a concentration-dependent manner [34]. Mutations of GBA1 gene lead to imbalance of glucosylceramide and ceramides. The abnormal accumulation of glucosylceramide in various organs is one of the major mechanisms in Gaucher's disease and Parkinson's disease [35]. Besides, it has been reported that GBA1-ceramide can inhibit inflammation through regulating different inflammatory signal pathways in various cells deficiency of GBA1-ceramide leads to inflammation [36]. GBA1-ceramide can suppress the MAPK signal pathways (mainly p38, JNK, and ERK1/2) by activating the ceramide-activated protein phosphatase (CAPP), thus inhibiting cytokines (including IL-6) and inflammation [15, 16]. Blocking GBA1 transcription or applying fumonisin B1 to inhibit ceramide formation directly can both significantly promote the p38/IL-6 pathways. Conversely, the increase of intracellular ceramide concentrations suppresses phosphorylation of p38 (primarily p38δ) and IL-6 expression prominently [15, 16]. Furthermore, an animal study showed that knockout of GBA1 in mice led to the production of IgG autoantibodies, increased C5a with antibody induction, release of proinflammatory cytokines, and tissue inflammation [27]. Therefore, taken together, the literature suggests that downregulation or deficiency of GBA1 promotes inflammation. In the present study, HMGB1-treated RASFs showed decreased mRNA and protein GBA1 levels when compared with controls without HMGB1 treatment, suggesting that HMGB1 possibly promoted inflammation of RASFs by downregulating GBA1. Accordingly, a study showed that HMGB1-stimulated macrophages derived from the synovial fluid release proinflammatory cytokines (including IL-6) [5]. Moreover, Kitatani et al. [15, 16] showed that the GBA1-ceramide pathway plays an important role in terminating p38δ activation, which is responsible for IL-6 biosynthesis. Thus, it can be speculated that HMGB1 possibly increased IL-6 production by downregulating GBA1 in RASFs, resulting in the aggravated synovial inflammation observed in RA.

Of course, the present study is not without limitations. This study was performed in cultured

cells that could lose RA characteristics over time due to their isolation from the proinflammatory systemic state found in RA. In addition, this study examined only GBA1 and did not comprehensively examine cytokines and pathways (such as p38, ERK, and JNK) that could be involved in an increased inflammatory state. Future studies will have to address these issues.

Conclusion

Taken together, the results suggest that after treatment with HMGB1, RASFs exhibited decreased GBA1 levels, as shown by RNA-seq, qRT-PCT, and western blot. We may conclude that the late-stage inflammatory mediator HMGB1 may exert its pathogenic effects in RA by downregulating GBA1, but these results will have to be validated in more comprehensive studies.

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Disclosure of conflict of interest

None.

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