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PKR and HMGB1 expression and function in rheumatoid arthritis

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ABSTRACT. The pathogenesis of rheumatoid arthritis (RA) is characterized by inflammation. We aimed to examine the roles of double-stranded RNA-activated protein kinase (PKR) and high-mobility group box chromosomal protein 1 (HMGB1) in a rat model of RA. Male SD rats were divided into three groups: control, RA model, and intervention (RA model plus treatment). The model of RA was made by injecting Freund's adjuvant into the posterior right limb of the rat and the intervention group received a PKR-specific inhibitor C16 after RA modeling. The degree of limb swelling was measured following RA modeling and intervention. In addition, plasma levels of HMGB1 were determined using ELISA. The mRNA and protein levels of PKR and HMGB1 were detected in rat synovium using quantitative PCR and western blot, respectively. The degree of limb swelling in the RA model was increased compared to control, while it was decreased in the intervention model compared to the RA model. Plasma HMGB1 levels in the model group were significantly higher compared to the control group but were lower in the intervention group compared to the model group. PKR and HMGB1 protein and mRNA levels in the rat synovium were elevated in the model group and markedly reduced in the intervention group. Increased

levels of PKR and HMGB1 in synovium or blood appear to be involved in the occurrence and development of RA in a rat model. Selective inhibition of PKR improves the symptoms of RA, perhaps by inhibiting the release of HMGB1.

Key words: Rheumatoid arthritis; Inflammatory factor; Double-stranded RNA-activated protein kinase; High-mobility group box chromosomal protein 1

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease characterized by cartilage damage and joint synovitis that occurs on both sides of the body (symmetrical effect). Due to its progressive development and high rate of disability, more than half of patients show joint function damage within 1-2 years of diagnosis and some patients may also suffer from joint deformities (Behnes et al., 2015). Several studies have shown that multiple inflammatory and pro-inflammatory factors are significantly elevated in the synovial fluid and synovium of RA patients such as monocyte chemotactic protein 1, monocyte colony-stimulating factor, interleukins-1 and -6 (IL-1, IL-6), and tumor necrosis factor-α. This suggests that inflammation is closely related to RA occurrence and development (Sato et al., 2015). High-mobility group box chromosomal protein 1 (HMGB1) is a type of non-histone nucleoprotein that is ubiquitously expressed. It participates in regulation of gene transcription and promotes chromatin stability (Hong et al., 2015). HMGB1 becomes an inflammatory factor when it leaves the cell and participates in the proliferation of multiple infectious diseases such as sepsis and endotoxin blood disease (Li et al., 2015). Double-stranded RNA-activated protein kinase (PKR) is a serine-threonine kinase and plays an important role in antiviral infection (Ogolla et al., 2013). In this study, we examined the effect of PKR and HMGB1 in synovial tissues using a rat model of RA.

MATERIAL AND METHODS

Animals and RA model development

Thirty male SD rats were provided by the China Medical University Laboratory Animal Center. They had average weight 245 ± 25 g (~220 to 280 g) and age 10-12 weeks. The rats were randomly divided into equal groups: control, model, and intervention. After 1 week of adaptive feeding, rats in the model and intervention groups received an injection of 0.1 mL Freund's complete adjuvant in the right rear footpad to induce RA (Bilasy et al., 2015). Rats in the control group received an injection of the same amount of saline in the right rear footpad. After RA modeling 7 days later, rats in the intervention group received the PKR specific inhibitor C16 (150 μg/kg) by intraperitoneal injection every day for 7 days (Liu et al., 2014), while the rats in the control and model groups received an equal amount of saline.

Swelling degree measurement

The degree of right foot swelling was measured in all groups before modeling, 7 days after modeling, and 7 days after intervention (Koo et al., 2015). In order to do this, the posterior

right limb (0.2 mm) of each rat was immersed in 20 mL water. The amount of water that needed to be removed in order to bring the volume back down to 20 mL was measured (foot volume). The degree of swelling was determined using (foot volume 7 days after modeling - foot volume before modeling) / (foot volume before modeling) x 100%. The degree of swelling after intervention was determined using (foot volume 7 days after intervention - foot volume 7 days after modeling) / (foot volume before modeling) x 100%.

Plasma HMGB1 level measurement

Blood was extracted from the inferior vena cava (3 mL) and centrifuged at 2500 rpm (Heraeus Multifuge, Thermo Scientific, USA) for 20 min. The supernatant (1 mL) was analyzed by ELISA according to the manufacturer protocol (Lengton Biological Technology Co. Ltd., Shanghai, China).

Western blot

The synovium was digested with lysis buffer (Roche Diagnostics, USA). Total protein was separated by denaturing 10% SDS-PAGE. After incubation with anti-PKR or -HMGB1 (Sigma, USA) primary antibodies, and in turn, the secondary antibody (KPL, USA), the membrane was treated with chemiluminescence (Bio-Rad, USA) and densitometry was calculated with Quantity One (Bio-Rad, USA). Protein levels were normalized to GAPDH.

Real-time PCR

The cDNA was synthesized from 1 µg RNA from the synovium samples with Trizol reagent (Invitrogen, USA) and Reverse Transcript Kit (Invitrogen, USA). The primers used were: PKR forward primer (5'-GAGGATGAAAGAAACAGCCAGCT-3'); PKR reverse primer (5'-CCCGCTATG AAATTAGATTCACGT-3'); HMGB1 forward primer (5'-GAGGATGAAAGAAACAGCCAGCT-3'); HMGB1 reverse primer (5'-CAGATCCCATTGATTTCCAC-3'); GAPDH: forward primer (5'-AACCTG CCAAGTATGATGACATCA-3'); and GAPDH reverse primer (5'-TGTTTGAAGTCACAGGAGAC AACCT-3'). The cycling conditions consisted of an initial, single cycle of 1 min at 90°C, followed by 35 cycles of 10 s at 90°C and 30 s at 70°C. PCR amplifications were performed in triplicate for each sample. Gene expression levels were quantified relative to the expression of GAPDH using an optimized comparative Ct ($\Delta\Delta^{Ct}$) value method $2^{-\Delta\Delta Ct}$.

Statistical analysis

All statistical analyses were performed using the SPSS20.0 software (Chicago, IL, USA). Differences between multiple groups were analyzed using the Bonferroni method. Numerical data are reported as means ± standard deviation. $P < 0.05$ was considered to be statistically significant.

RESULTS

Confirmation of rat RA model and posterior limb swelling

There was no mortality in any of the treatment groups. The rats in the control group were not averse to activity, responded to external stimuli well, had bright eyes, and had light red claws

and auricles. The rats in the RA model group did not move well, had loss of reaction to stimuli, had dull eyes, and their posterior limbs showed evidence of swelling. In the intervention group, rats enjoyed activities, were responsive to external stimuli compared to those in RA model group, had bright eyes, and the posterior limb swell was obviously diminished (Figure 1). Quantitatively, the degree of swelling in the right limbs of the model group increased significantly compared to control ($P < 0.05$) while the swelling was significantly decreased in the intervention group compared to the model group ($P < 0.05$; Table 1).

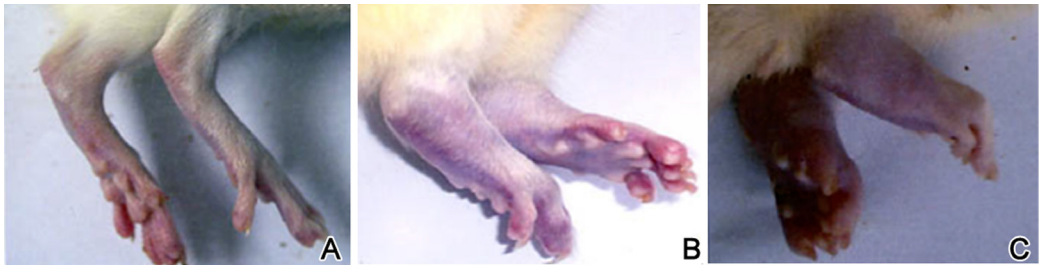

Figure 1. Posterior limb manifestation. Shown are the posterior limbs in the rats 1 week after intervention in the control group (**A**), model group (**B**), and intervention group (**C**).

Table 1. Comparison of degree of right foot swelling (means ± SD, %).

Group	Cases	Before model	After model	After intervention
Control	10	0	7.2 ± 3.1	14.9 ± 5.8
Model	10	0	65.2 ± 19.6*	71.3 ± 24.7*
Intervention	10	0	66.1 ± 20.4*	42.5 ± 15.4*#

*P < 0.05, compared with control group; #P < 0.05, compared with model group.

Plasma HMGB1 level comparison

Plasma HMGB1 levels in the model group were upregulated significantly compared to the control group ($P < 0.05$), and were significantly lower in the intervention group compared to the model group ($P < 0.05$; Table 2).

Table 2. Comparison of HMGB1 plasma levels (means ± SD, μg/L).

Group	Cases	Before model	After model	After intervention
Control	10	1.29 ± 0.17	1.33 ± 0.26	1.34 ± 0.29
Model	10	1.26 ± 0.15	2.29 ± 0.47*	2.54 ± 0.51*
Intervention	10	1.31 ± 0.19	2.17 ± 0.44*	1.68 ± 0.37*#

*P < 0.05, compared with control group; #P < 0.05, compared with model group.

PKR and HMGB1 protein and gene expression

After 1 week of intervention, we found that the levels of PKR and HMGB1 protein in the rat synovium were significantly elevated in the model and intervention groups compared to control ($P < 0.05$); however, the protein levels in the intervention group were lower than those of the model group ($P < 0.05$; Figure 2 and Table 3). The mRNA levels of PKR and HMBG1 were

elevated in the model group compared to control ($P < 0.05$) while they were reduced (although not back to control levels) in the intervention group compared to the model group ($P < 0.05$; Table 4).

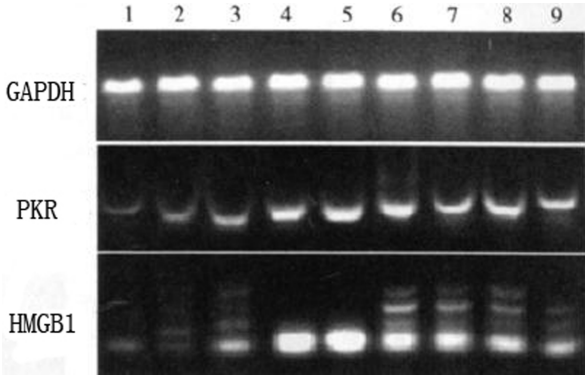

Figure 2. Western blot of PKR and HMGB1 protein expression. *Lanes 1-3*: control group; *lanes 4-6*: model group; and *lanes 7-9*: intervention group. GAPDH was used as an internal control.

Table 3. Levels of PKR and HMGB1 protein.

Group	PKR	HMGB1
Control	0.05 ± 0.01	0.03 ± 0.01
Model	0.52 ± 0.12$^{\Delta}$	1.53 ± 0.72$^{\Delta}$
Intervention	0.26 ± 0.09$^{\Delta\blacktriangle}$	0.62 ± 0.23$^{\Delta\blacktriangle}$

Densitometry was calculated with Quantity One. $^{\Delta}P < 0.05$, compared with control group; $^{\blacktriangle}P < 0.05$, compared with model group.

Table 4. Comparison of PKR and HMGB1 gene expression (means ± SD).

Group	Cases	PKR		HMGB1	
		Δ^{Ct}	Relative expression level	Δ^{Ct}	Relative expression level
Control	5	0.412 ± 0.079	0.417 ± 0.086	0.023 ± 0.009	0.022 ± 0.006
Model	5	0.671 ± 0.162*	0.684 ± 0.183*	0.331 ± 0.023*	0.337 ± 0.026*
Intervention	5	0.672 ± 0.159*	0.514 ± 0.147*$^{\#}$	0.334 ± 0.025*	0.179 ± 0.014*$^{\#}$

*$P < 0.05$, compared with control group; $^{\#}P < 0.05$, compared with model group.

DISCUSSION

While much is known about the phenotype of RA, its mechanism of pathogenesis is not fully elucidated. A survey conducted in the 1990s focused on teenagers with RA and found that an antibody against HMGB1 was present in 45% of the patients (Rabasseda, 2014). Pullerits et al. (2006) found that HMGB1 levels were high in the joint fluid and serum of patients with RA and in the plasma of an RA model in rats. More recent studies have shown that plasma and protein levels of HMGB1 are significantly higher in RA patients and closely linked with RA pathological staging (Ke et al., 2015). HMGB1 is passively released into the extracellular space during cell death, leading to

an increase in the mRNA levels of the cytokines IL-1α, IL-1β, and IL-6. HMGB1 is also implicated in other diseases, such as pancreatitis and cancer. Xiang et al. (2014) suggested that inhibition of HMGB1 activity in patients with acute pancreatitis could improve clinical outcomes. Another study revealed that HMGB1 plays an important role in the process of malignant tumorigenesis by dysregulating cancer-related genes, promoting metastasis, and facilitating tumor growth and survival (Inoue et al., 2015). Our results found that HMGB1 mRNA levels and protein expression in plasma were both significantly elevated in a rat model of RA, which is consistent with clinical results (Bobek et al., 2014).

The human PKR gene is located on chromosome 2p21-22 and contains 17 exons. The PKR protein is ubiquitously expressed and has a strong affinity for double-stranded RNA. Its primary functions are in signal transduction and in immunity as an antiviral agent (Yoshida et al., 2012). PKR can induce apoptosis, inhibit proliferation, and act as an anti-viral agent by regulating protein translation, which could also make it an ideal marker of viral infection. Clinical studies have demonstrated that PKR is involved in multiple signaling pathways such as transcription factor 1, transcription activation factor 3, mitogen activated protein kinases, p53, interferon regulatory factor (IFN), and NF-κB (Couturier et al., 2011). From basic research and clinical studies (Lu et al., 2012; Kapil et al., 2014), it has been found that PKR has the following functions: inhibiting protein translation, inhibiting viral replication, inducing cell apoptosis, and inducing production of cytokines. Recent research has shown that PKR mRNA and protein levels increase significantly in RA (Yu et al., 2014). PKR may also participate in the occurrence and development of systemic lupus erythematosus, thereby increasing damage to the circulatory system. Some studies have found that HMGB1 acetylation is mediated by NF-κB in the nucleus, and it is activated by PKR to induce inflammation after release into the blood (Ito et al., 2015). In this study, PKR protein and mRNA levels were significantly higher in the RA model and intervention groups than in the control group, while they were lower in the intervention group compared to the model group. Furthermore, HMGB1 mRNA and protein levels in plasma were lower after intervention with a PKR inhibitor, suggesting that overexpression of PKR is involved in RA. Our results also demonstrate that selective inhibition of PKR could block HMGB1 release.

In conclusion, overexpression of PKR was indicative of the occurrence and development of RA in a rat model. PKR-mediated release of HMGB1 is the primary mechanism of inflammation in RA. Selective inhibition of PKR could block HMGB1 release, thereby significantly improving the symptoms of RA. In follow-up studies, we will further explore the mechanism of PKR inhibition of HMGB1 activity in RA patients to provide new insight into the mechanism of RA and potentially identify new therapeutic targets.

Conflicts of interest

The authors declare no conflict of interest.

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