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Role of PI3K/Akt signal pathway on proliferation of mesangial cell induced by HMGB1

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ABSTRACT

Mesangial cell (MC) proliferation is an important event in LN. Our previous studies have shown that extracellular High Mobility Group Box-1 protein (HMGB1) plays a critical role in pathophysiological mechanism of lupus nephritis (LN) and HMGB1 could induce MC proliferation. The purpose of this study is to investigate the effect of phosphatidylinositide 3-kinase (PI3K)/protein kinase B (Akt) signal pathway activation on mesangial cell proliferation induced by HMGB1 and whether Toll-like receptor 2 (TLR2) plays an important role in this progress. The results showed that HMGB1 induced overexpression of p85, p110 and p-Akt in mouse mesangial cell (MMC) and increased the proliferative level of MMC cells. In addition, HMGB1 induced a physical interaction between TLR2 and p85. The TLR2 neutralization antibody and LY294002 both reduced the MMC proliferation levels induced by HMGB1 and also blocked the HMGB1-dependent phosphorylation of the Akt. Thus, HMGB1 increases interaction between TLR2 with p85 and in sequence phosphorylates Akt at ser473, thereafter mediates MMC proliferation, which contributed significantly to the pathophysiology of MMCs dysfunction.

Short title: PI3K/Akt contributes to HMGB1-induced MMC proliferation

1. Introduction

High Mobility Group Box-1 protein (HMGB1) is a highly conserved nuclear protein that acts as a chromatin-binding factor capable of promoting access of transcriptional complexes to the DNA. In addition to its nuclear role, HMGB1 functions as an extracellular signalling molecule regulating both inflammation and regenerating processes (Thomas and Stott, 2012). Both inflammatory and necrotic cells release HMGB1 and the extracellular HMGB1 protein stimulates monocytes/macrophages and neutrophils to secrete other inflammatory cytokines amplifying the inflammatory response (Limana et al., 2011; Scaffidi et al., 2002). Our previous studies have shown that HMGB1 was located in cytoplasm and extracellular area in glomeruli of mice with lupus nephritis and HMGB1 administration induces mesangial cell proliferation and lipid deposition of mesangial cell (Feng et al., 2012; Hao et al., 2013), which mediated renal injure of lupus nephritis.

As a non-histone nucleoprotein, the display of extracellular HMGB1 function dependents on the recognition of its special receptor (Winter et al., 2009). Toll-like receptor 2 (TLR2) is one of the HMGB1 receptor, and the activated TLR2 triggered up intracellular signalling pathways through recruitment of adaptor protein (Ding and Chang, 2012). Phosphatidylinositide 3-kinase (PI3K) was one of the TLR2-related pathways and the downstream factor of protein kinase B (Akt) was involved in the regulation of proliferation (Eun et al., 2015).

Therefore, in the present study, we investigated the effect of PI3K/Akt signal pathway activation on mesangial cell proliferation induced by HMGB1 and whether TLR2 plays an important role in this progress.

Abbreviations: HMGB1, High Mobility Group Box-1 protein; LN, lupus nephritis; PI3-K, phosphatidylinositide 3-kinase; Akt, protein kinase B; TLR2, Toll-like receptor 2; MMC, mouse mesangial cell; BrdU, 5-bromo-2'-deoxyuridine; ROS, reactive oxygen species; NF-κB, nuclear factor κB.

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2. Materials and methods

2.1. Reagents

5-bromo-2′-deoxyuridine (BrdU) and LY294002 were purchased from Sigma-Aldrich (St. Louis, MO). Rabbit anti-p110, anti-p85, anti-p-Akt-ser473, anti-Akt, anti-BrdU, anti-Cyclin D1 and anti-β-actin antibodies were purchased from Epitomics (Cambridge, UK). Mouse anti-PCNA and rabbit anti-TLR2 antibodies were purchased from Abcam (Cambridge, UK). RNA Prep Pure Micro Kit and TIANScript RT Kit were purchased from Tiangen Biotechnology Co. (Beijing, China). Premix Ex TaqTM Kit was purchased from Takara Co (Mie, Japan).

2.2. Cell culture and groups

Mouse mesangial cells (MMCs) were stored in our lab and obtained from the Chinese Academy of Sciences Shanghai Institute for Biological Sciences Cell Resource Center. The cells were cultured in DMEM/F12 (3:1) medium supplemented with 10% fetal bovine serum (Gibco, BRL). (1) To investigate the effect of HMGB1 on cell proliferation, the cells were randomly divided into a control group and an HMGB1 groups (100 $\mu g\,L^{-1}$) and collected at 8 h. The cells were mixed with BrdU (10 $\mu mol\,L^{-1}$. SIGMA) 2 h before collection. Immunofluorescence staining was used to detect the proliferative level. In addition, PCNA and Cyclin D1 expression were detected by western blot and qRT-PCR. (2) The cells were collected at 10, 20, 30, 40, 50 and 60 min after stimulation with HMGB1 to detect the expression of p85, p110, p-Akt-ser473 and Akt. IP was used to analyze the interaction of TLR2 and p85 at 10 min exposed to HMGB1. (3) To detect the role of PI3 K/Akt pathway on the cell proliferation induced by HMGB1, the cells were randomly divided into three groups (control, HMGB1, HMGB1 + LY294002). The cells in the HMGB1 + LY294002 group were pre-treated with LY294002 (30 $\mu mol\,L^{-1}$) for 1 h and then the cells were collected at 30 min as well as 8 h after stimulation with HMGB1 (100 $\mu g\,L^{-1}$), and the phosphorylation level of Akt (P-Ser473), the expression of PCNA, Cyclin D1 and proliferation level were detected respectively. (4) To further determine the effect of TLR2 on HMGB1-induced cell proliferation, a TLR2 neutralization antibody (5 $\mu g\,mL^{-1}$) was used to block TLR2, and the cells were collected at 8 h after exposure to HMGB1 for the detection of the proliferation level by BrdU incorporation.

2.3. Immunofluorescence

After treated with HMGB1, the cells were fixed in a 4% paraformaldehyde solution and permeabilized with 0.5% Triton X-100 for 30 min at room temperature and re-treated with 2 N HCL for 2 h at 37 °C to denature the DNA. Then, the cells were incubated with an anti-BrdU antibody (1:100) overnight at 4 °C. After conjugation with a rhodamine-labelled affinity purified mouse IgG antibody and DAPI (10 $\mu mol\,L^{-1}$, KPL, USA), the sections were observed using an Olympus microscope (OLYMPUS, BX71, Jap). The image was characterized quantitatively by digital image analysis using the Image Pro-Plus 5.0 software (Media Cybernetics, Silver Spring, MD) by using the method introduced by Feng et al. (Feng et al., 2012).

2.4. Protein extraction and western blotting

MMC cells were collected and the protein extraction was performed as described previously (Feng et al., 2012; Hao et al., 2011). 50 μg protein extracts were separated by 10% SDS-PAGE and then transferred to PVDF membranes. The membranes were incubated overnight at 4 °C with anti-p85, p110, Akt, p-Akt (P-S473), PCNA, cyclin D1 or β-actin (1:1000;) antibodies. On the second day, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG antibody (1:5000) and then the image was observed using the LI-COR Odyssey Infrared Imaging System. All experiments were repeated at least three times.

2.5. Immunoprecipitation

Total protein extracts were used for IP assays. Briefly, 1 mg of extract was incubated for 4 h at 4 °C with 1 μg of antibody (anti-mouse TLR2) (Anderson, 2010; Gruszka et al., 2010) and subsequently for overnight at 4 °C with Dynabeads Protein G (Invitrogen, USA). Samples were then blotted with anti-p85 polyclonal antibodies (1:1000; Epitomics) as described above.

2.6. Real-time PCR

The cells were collected and total RNA was extracted with TRIzol (Invitrogen, USA) according to the manufacturer's instructions. A reverse transcription kit was used to obtain cDNA. SYBR Premix EX Taq was used for the amplification with the Real-Time QPCR System (PCNA: 5′-TTG CAC GTA TAT GCC GAG ACC -3′ and 5′-GGT GAA CAG GCT CAT TCA TCT CT-3'; Cyclin D1:5′-GCG TAC CCT GAC ACC AAT CTC-3′ and 5′-ACT TGA AGT AAG ATA CGG ACG GC-3′; β-actin: 5′-AGA TGT GGA TCA GCA G-3′ and 5'-GCG CAA GTT AGG TTT TGT CA-3′). The $2^{-\Delta\Delta CT}$ method was used to normalize the cDNA for quantitative real-time PCR. $\Delta\Delta CT = (CT._{Target} - CT._{Actin})\ Time_x - (CT._{Target} - CT._{Actin})\ Time_0$. All experiments were repeated at least three times.

2.7. Statistical Analysis

The quantitative data are presented as the mean ± s.d. Statistical analyses were performed using one-way analysis of variance (ANOVA) with the Student-Newman-Keuls test. Correlations were calculated by using Spearman's rank correlation. *P* values of less than 0.05 were considered statistically significant.

3. Results

3.1. HMGB1 upregulated cell proliferative level and PCNA, Cyclin D1 expression in vitro

The results of BrdU incorporation showed that the proliferation level of MMC cells was significantly increased in the HMGB1 group compared with that of the control group. The positive signal was mainly located in nuclei of mesangial cell and the positive ratio was greater than 70% in HMGB1 group, but it was only 21.6% in control group (Fig. 1a). Additionally, as shown in Fig. 1b,c, the PCNA and Cyclin D1 mRNA and protein expressions increased in HMGB1 group by Real-time PCR and Western blot.

3.2. Activation of PI3K/Akt signal pathways mediated HMGB1-induced proliferation in MMC cells

As results shown, HMGB1 activated PI3K signal pathway and induced Akt phosphorylation at ser473. The expressions of p85, p110 and p-Akt (ser473) protein were elevated respectively at 10 min, 20 min and 30 min in MMC cells exposed to HMGB1 (Fig. 2a,b). To determine the effect of PI3K activity on cell proliferation and Akt activation, MMC cells were incubated with LY294002, a special inhibitor of PI3K signal pathway, for 1 h before exposure to HMGB1, and then MMC cells were collected at 30 min and 8 h respectively. As shown in Fig. 2c,d, p-Akt-ser473 was markedly higher in MMC cells stimulated with HMGB1 than that in control cells, but was dramatically reduced by LY294002. Importantly, LY294002 absolutely inhibited cell proliferation level induced by