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The PTEN/PI3K/Akt signaling pathway mediates HMGB1-induced cell proliferation by regulating the NF-κB/cyclin D1 pathway in mouse mesangial cells

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Feng XJ, Liu SX, Wu C, Kang PP, Liu QJ, Hao J, Li HB, Li F, Zhang YJ, Fu XH, Zhang SB, Zuo LF. The PTEN/PI3K/Akt signaling pathway mediates HMGB1-induced cell proliferation by regulating the NF-κB/cyclin D1 pathway in mouse mesangial cells. Am J Physiol Cell Physiol 306: C1119–C1128, 2014. First published April 24, 2014; doi:10.1152/ajpcell.00385.2013.—Our previous experiment confirmed that high-mobility group box chromosomal protein 1 (HMGB1) was involved in the pathogenesis of Lupus nephritis (LN) by upregulating the proliferation of the mouse mesangial cell line (MMC) through the cyclin D1/CDK4/p16 system, but the precise mechanism is still unknown. Therefore, in the present study, we demonstrated that HMGB1 induced the proliferation of MMC cells in a time- and concentration-dependent manner, downregulated phosphatase and tensin homolog deleted on chromosome ten (PTEN) expression, increased the level of Akt serine 473 phosphorylation, and induced p65 subunit nuclear translocation. The overexpression of PTEN prevented the upregulation of HMGB1-induced proliferation by blocking the activation of Akt. The knockdown of Akt by siRNA technology and blocking the nuclear factor-κB (NF-κB) pathway using pyrrolidine dithiocarbamate (PDTC) and SN50, inhibitors of NF-κB, both attenuated the HMGB1-induced proliferation by counteracting the activation of the cyclin D1. In addition, while sh-Akt partly blocked the nuclear translocation of the p65 subunit, PDTC did not affect the activation of the Akt induced by HMGB1 in MMC cells. These findings indicate that HMGB1 induced the proliferation of MMC cells by activating the PTEN/phosphoinositide-3-kinase (PI3K)/Akt/NF-κB signaling pathway.

HMGB1; PTEN; Akt; NF-κB; cell proliferation; mouse mesangial cell

SYSTEMIC LUPUS ERYTHEMATOSUS (SLE) is a common systemic autoimmune disease and mainly occurs in young women. The pathogenesis of lupus nephritis (LN), one of the most severe and frequent complications of SLE, is inadequately understood (18). However, the overexpression of cytokines and their interactions seem to be involved in the initiation and progression of LN (12).

Recently, high-mobility group box chromosomal protein 1 (HMGB1) has been demonstrated to be involved in many inflammatory and autoimmune diseases (26). In the cell nucleus, HMGB1 organizes the structure of chromatin by interacting with H1 (21). However, once released, voluntarily or passively, into the cytoplasm and other extracellular spaces, HMGB1 has been confirmed as a novel cytokine mediator and is involved in many biological functions, including mediating the activation of innate immune diseases such as LN (1–2). Excessive mesangial cell proliferation is the main pathological change in LN (19), and the glomerulosclerotic changes could be alleviated effectively by inhibiting the proliferation of mesangial cells (11). Our previous studies have revealed that HMGB1 is an important cytokine in LN pathogenesis and that HMGB1 could induce mesangial cell proliferation in mice (6) and Toll-like receptor-2 (TLR-2) was one of the important receptors of HMGB1 in the mouse mesangial cell line (MMC) (27). However, the precise mechanism and possible signaling pathways are unknown.

Phosphoinositide-3-kinase (PI3K) plays a crucial role in cell growth and cell survival (7). The PI3K enzyme acts on membrane PI to generate the second messenger lipid PI-3,4,5-triphosphate (PIP3). PIP3 recruits phosphatidylinositol-dependent kinase 1 (PI3K) and Akt kinase to the membrane, and then PI3K/Akt activates Akt. Activated Akt phosphorylates several downstream proteins, including NF-κB, GSK3-β, etc., that control cell growth, cell survival, and protein synthesis. Phosphatase and tensin homolog deleted on chromosome ten (PTEN) has been confirmed to dephosphorylate PIP3 into PI(4,5)P2 (3), thereby downregulating the activation of the PI3K/Akt pathway, which results in the relieving of the biological process (9, 15). Research has shown that the activation of Akt induced the overexpression of RIO to mediate the cell proliferation of glioblastomas (17). As a trans-acting factor, transcription factors of the nuclear factor NF-κB play a pivotal role in the development of proliferation; Li et al. (14) reported the proliferative effect of NF-κB as downstream of PI3K/Akt. Our previous study revealed the activation of Akt and NF-κB nuclear translocation, following with overexpression of HMGB1 and cell proliferation in the renal tissues of patients and LN and BXS B mice (a model of LN). In addition, HMGB1
induced the proliferation of MMC cells in vitro (6). Therefore, we speculate that the Akt/NF-κB signaling pathway might mediate the HMGB1-induced proliferation of MMC cells.

To confirm the hypothesis, we first further ascertained the relationship between HMGB1 and the proliferation of MMC cells by the 5-bromo-2′-deoxyuridine (BrDU) incorporation method. Furthermore, an expression vector, siRNA technology, and two inhibitors of the NF-κB pathway were used to investigate the effect of the PTEN/Pi3K/Akt/NF-κB pathway, which might contribute to understanding the specific mechanisms of HMGB1-induced proliferation and provide an experimental basis for target gene therapy for LN.

MATERIALS AND METHODS

Cell culture and groups. MMC cells (SV40 MES 130) were 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 (GIBCO) supplemented with 10% fetal bovine serum (GIBCO). The cells were synchronized by culturing in serum-free medium for 24 h.

To detect the effect of HMGB1 on cell proliferation, the cells were randomly divided into a control group and the HMGB1 groups (containing 50, 100, and 200 μg/l mouse recombinant HMGB1 protein or not; Sigma). The cells were collected at 2, 4, 8, and 12 h after stimulation or not. The cells were mixed with BrDU (10 μmol/l; Sigma) 2 h before collection.

The cells were collected at 10, 20, 30, 40, 50, and 60 min after stimulation with HMGB1 (100 μg/l) to detect the expression of PTEN, p-Akt (P-S473 and P-T308), Akt, p-IsBo, IsBo, and the nuclear translocation of p65.

In the RNA interference (RNAi) experiment, the cells were divided randomly into four groups (control, HMGB1, HMGB1 + pYr-3.1-shNC, and HMGB1 + pYr-3.1-shNC) and collected at 8 h after stimulation with HMGB1 (100 μg/l). Then, the expression of proliferating cell nuclear antigen (PCNA), cyclin D1, CDK4, p16, proliferation level, and nuclear translocation of p65 were detected.

In the expression vector experiment, the cells were divided randomly into four groups (control, HMGB1, HMGB1 + pcDNA3.1-PTEN-IRE5-EGFP, and HMGB1 + pcDNA3.1-NC-IRE5-EGFP) and collected at 10, 20, 30, 40, and 50 min and 8 h after stimulation with HMGB1 (100 μg/l). Then, the activation level of Akt (P-S473 and P-T308) and the expression of PCNA, cyclin D1, CDK4, p16, and proliferation level were detected.

For the PDTC inhibitor of the NF-κB pathway, the cells were randomly divided into four groups (control, HMGB1, HMGB1 + PDTC, and HMGB1 + DMSO) to explore the effect of PDTC on the proliferation and nuclear translocation of p65 and the expression of PCNA, cyclin D1, CDK4, and p16. The cells in the HMGB1 + PDTC group were pretreated with PDTC (5 μmol/l; Sigma) for 30 min. The cells were collected at 20 and 50 min as well as 8 h after stimulation with HMGB1 (100 μg/l), and the phosphorylation level of Akt (P-S473 and P-T308) and proliferation level were detected, respectively.

Cells were randomly divided into three groups (control group, DMSO group, and HMGB1 group) and collected after stimulated by DMSO or HMGB1 for 90 min, chromatin immunoprecipitation was performed.

The cells were randomly divided into four groups (control, HMGB1, HMGB1 + SN50, and HMGB1 + DMSO) to explore the effect of SN50 on the proliferation and the activation of P-S473. The cells in the HMGB1 + SN50 group were pretreated with SN50 (18 μmol/l; Sigma) for 1 h. The cells were collected at 50 and 80 min after stimulation with HMGB1 (100 μg/l), and the proliferation level was detected.

ELISA for BrDU incorporation. MMC cell proliferation was evaluated by measuring the incorporation of BrdU (Sigma) using the BrdU Cell Proliferation Kit (Merck Millipore) according to the manufacturer’s instructions. Cells were incubated with different concentrations of HMGB1 (50, 100, and 200 μg/l) in 100 μl/well culture medium for 2, 4, 8, and 12 h. BrdU (10 μmol/l) was added to the culture medium 2 h before the cells were collected. Then, the cells were incubated for 1 h with diluted a peroxidase-conjugated anti-BrdU antibody at 37°C. The absorbance values were measured at 450 nm using a Microplate reader (BioTek Instruments.).

Transfection. The expression vector of PTEN (pcDNA3.1-PTEN-IRE5-EGFP) and the control vector (pcDNA3.1-IRE5-EGFP) were designed and produced by Beijing Fungecome Technology (Beijing, China). The sh-Akt vector (pYr-3.1-shAkt) and the negative control sh-Scramble vector (pYr-3.1-shNC) were designed and produced by Yingrun Biotechnologies (Changsha, China). Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used for transient transfections according to the manufacturer’s instructions. At approximately 50–70% confluence, the cells were transfected with 4.0 μg of vector DNA with 10 μl Lipofectamine 2,000 in 2 ml of serum-free DMEM-F12 medium. Six hours after the transfection, the cells were incubated for 48 h with normal DMEM/F12 medium containing 10% fetal bovine serum. Then, 100 μg/l HMGB1 were added to the medium, and the cells were collected at different time points.

Immunofluorescence. After the HMGB1 treatment, the cells were fixed in a 4% paraformaldehyde solution and permeabilized with 0.5% Triton X-100. Then, the cells were incubated with an anti-BrdU antibody (1:100; Abcam) and a p65 antibody (1:100; Epitomics) overnight at 4°C. After conjugation with a rhodamine-labeled affinity purified mouse IgG antibody and DAPI (10 μmol/l; KPL), the sections were observed using an Olympus microscope (Olympus BX71). In the BrdU incorporation experiment, the cells were treated with 2 N HCl for 2 h at 37°C to denature the DNA.

Protein extraction and Western blotting. MMC cells were collected, and the protein extraction was performed as described previously (10). All operations were in strict compliance with the specifications. The protein extracts were separated by 10% SDS-PAGE and then transferred to PVDF membranes. The membranes were incubated overnight at 4°C with Akt (1:1,000; Epitomics), p-Akt (P-T308 and P-S473: 1:1,000; Epitomics), PTEN (1:1,000; Epitomics), p-IκBα (1:1,000; Epitomics), IsBo (1:1,000; Epitomics), p65 (1:1,000; Epitomics), PCNA (1:1,000; Abcam), cyclin D1 (1:1,000; Epitomics), CDK4 (1:1,000; Epitomics), p16 (1:1,000; Epitomics), and β-actin (1:1,000; Epitomics) antibodies. On the second day, the membranes were incubated with a horseradish peroxidase-conjugated goat anti- rabbit IgG antibody (1:5,000; ZSGB-BIO) and then the image was observed using the LI-COR Odyssey Infrared Imaging System.

Chromatin immunoprecipitation assay. Cells were collected at 90 min stimulated by DMSO or HMGB1. Chromatin immunoprecipitation (CHIP) was performed with the CHIP (Millipore) according to the manufacturer’s instructions (2 ul antibody/IgG), DNA was recovered with E.Z.N.A tissue DNA kit (Omega). The DNA was amplified with PCR master mix (Promega) using a specific primer spanning the κB2 site of cyclin D1 (forward 5′-CCAGCTTCTTCCAAGTGGCC-3′ and reverse 5′-AGGGACGGCGTATTCA-3′). The PCR products were subjected to 1% agarose gel electrophoresis and analyzed with GDS-8000 Bioimaging System (UVP) and LabWorks 4.5 software.

Real-time PCR. Total RNA was extracted from TRizol (Invitrogen) according to the manufacturer’s instructions. A reverse transcription kit (Promega) was used to obtain cDNA. SYBR Premix EX Taq (Takara Biotechnology) was used for the amplification with the Real-time QPCR System (Agilent Stratagene Mx3005p) (PCNA: 5′-TTGACGTATATGC CGGAC-3′ and 5′-GTTGACACGCTTACACATCC-3′; Akt: 5′- TCTACCGTGGCAGATTG-3′ and 5′-TCCGGCATACACACCTTGC-3′; cyclin D1: 5′-GGTCCTCTGACAACTCCC-3′ and 5′-CTCTGAGAGATACCCG-3′; CDK4: 5′-ATGAGTGGCCA CTGTGATTGAAACCGCA-3′ and 5′-GCTTCTCCTTCAATGAACATCTG-3′; p16: 5′-CTCGTGACCTG AGGAGC-3′ and 5′-GGAGCAGTGGCA-3′). The PCR products were sequenced by a 25% agarose gel electrophoresis and analyzed with GDS-8000 Bioimaging System (UVP) and LabWorks 4.5 software.
The 2−ΔΔCT method was used to normalize the cDNA for quantitative real-time PCR. $\Delta\Delta CT = (C_{T,\text{Target}} - C_{T,\text{Actin}})$ time 1 − $(C_{T,\text{Target}} - C_{T,\text{Actin}})$ time 0 (25).

Statistical analysis. The quantitative data are presented as the means ± SD. Statistical analyses were performed using one-way ANOVA with the Student-Newman-Keuls test. Spearman’s test was performed to assess the correlation between two sets of data. A $P$ value of < 0.05 was considered statistically significant.

RESULTS

The PTEN/PI3K/Akt/NF-κB signaling pathway is activated by HMGB1 in MMC cells followed by cell proliferation. As anticipated, the BrdU assay showed that the proliferation level

Fig. 1. Proliferation measurement of mouse mesangial cell line (MMC) cells by the 5-bromo-2'-deoxyuridine (BrdU) incorporation method. A: ELISA for the quantification of cell proliferation, compared with control group; the proliferation level of MMC cells in all high-mobility group chromosomal protein 1 (HMGB1)-treated groups increased from 2 h and the growth became slower at 12 h; there was a noticeable promote of the proliferation level in MMC cells stimulated by HMGB1 for 8 and 12 h in the 100 and 200 μg/l groups, but there was no significant difference neither between the 100 and 200 μg/l groups nor between the 8- and 12-h groups. *$P < 0.01$ vs. control. #$P > 0.01$ vs. 100 μg/l (8 h). B: measurement of BrdU incorporation detected by immunocytochemistry (×100). The expression of BrdU is located in the nuclei of the cells (red), and the BrdU-positive cell ratio shows the proliferation level of MMC. IOD, integrated optical density.
of MMC cells in the HMGB1-stimulated group increased from 2 h and reached a peak at 8 h; the proliferation level then gradually decreased to a lower level at 12 h. Compared with the proliferation level at 8 h, the difference was not statistically significant. Moreover, the level of proliferation in the 100 and 200 μg/l HMGB1-stimulated groups was significantly higher than that in 50 μg/l group, but there was no significant difference between the 100 and 200 μg/l group \((P > 0.05)\); Fig. 1A). The immunocytochemistry analysis revealed that the percentage of BrdU-positive cells in the HMGB1 group was increased noticeably compared with the control group at 8 h (Fig. 1B).

We determined the effect of HMGB1 on the expression of PTEN and activation of the Akt and NF-κB by Western blot and immunofluorescent analysis in MMC cells. As shown in Fig. 2A, the expression of PTEN protein significantly decreased at 10 min in MMC cells exposed to HMGB1. At 20 min, P-S473 (the phosphorylation of Akt Ser473) was elevated \((P < 0.01);\) Fig. 2A), but HMGB1 had no effect on the P-T308 expression (the phosphorylation of Akt Thr308; \(P > 0.05);\) Fig. 2A). Additionally, the expression of IκBα was downregulated and accompanied with upregulated expression of p-IκBα 30 min after HMGB1 stimulation \((P < 0.01);\) Fig. 2B). The positive expression of p65 was located in the cytoplasm, and...
no positive staining was observed in the nuclei of the control MMC cells (Fig. 2C). However, the positive expression of the p65 subunit was located in the nuclei and significantly increased 50 min after HMGB1 treatment. The ChIP experiment showed that, amplified by a pair primer spanning the κB2 site in promoter of cyclin D1, no PCR signal was found in control group and DMSO group; an obvious fragment was obtained from chromatin that immunoprecipitated with p65 antibody in MMC cells that stimulated by HMGB1 (P < 0.01; Fig. 3).

Overexpression of PTEN prevents the activation of Akt and, thereby, blocks the upregulation of HMGB1-induced proliferation. The transient transfection experiments were performed to further confirm the precise effect of PTEN on Akt phosphorylation and cell proliferation. Compared with the control group, pcDNA3.1-PTEN-IRESGFP effectively upregulated the PTEN protein and mRNA levels of MMC cells by 2.4 and 2.6 times (P < 0.01; Fig. 4A). Correspondingly, p-S473Akt decreased in the MMC cells transfected with specific PTEN expression vector 10 min after HMGB1 stimulation (P < 0.01), but no obvious changes in p-Akt expression were detected at the other time points (Fig. 4B).

By BrdU incorporation and immunocytochemistry, the ratio of positive MMC cells significantly decreased in the HMGB1 + PTEN vector group compared with the HMGB1 group, but there was no effect of pcDNA3.1-NC-IRESGFP on HMGB1-induced cell proliferation (Fig. 1B).

The expression level of the PCNA protein showed a similar result. HMGB1 upregulated the PCNA protein expression (P < 0.01); however, the PCNA protein expression in the HMGB1 + PTEN vector group decreased compared with the PCNA expression in the HMGB1 group by Western blot (Fig. 4C).

The upregulation of cyclin D1 and CDK4 and the down-regulation of p16 in the HMGB1 group were all prevented in HMGB1 + PTEN vector group (P < 0.01). However, the expression levels of PCNA, cyclin D1, CDK4, and p16 in the HMGB1 + pYr-3.1-shNC group displayed no obvious changes compared with the HMGB1 group (Fig. 4C). The real-time PCR analysis revealed similar results (Fig. 4D).

Sh-Akt attenuates the HMGB1-induced proliferation of MMC cells and decreases the activation of the cyclin D1/CDK4/p16 system. RNA interference was used to silence the expression of Akt and to assess its effects on HMGB1-induced MMC cell proliferation. Measurements of protein expression showed that the targeted Akt shRNA knocked down ~80% of Akt that was induced by HMGB1 (Fig. 5A). Compared with HMGB1-stimulated untransfected cells and control vector-transfected cells, the BrdU-positive cell ratio presented down-regulation in the HMGB1 + pYr-3.1-shAkt group by immunocytochemistry (Fig. 1B), and the expression levels of PCNA protein and mRNA decreased (P < 0.01, Fig. 5, B and C). Moreover, compared with the HMGB1 group, the expression of cyclin D1 and CDK4 protein and mRNA in the HMGB1 + shAkt vector group decreased; in contrast, the p16 protein expression increased (P < 0.01; Fig. 5, B and C). However, the expression of PCNA, cyclin D1, CDK4, and p16 in the HMGB1 + shNC vector group demonstrated no obvious changes compared with the HMGB1 group (P > 0.05; Fig. 5, B and C).

PDTC blocks the HMGB1-induced proliferation of MMC cells by regulating the cyclin D1/CDK4/p16 system. PDTC is an inhibitor of the NF-κB signaling pathway. To determine the effect of NF-κB activity in the HMGB1-induced proliferation of MMC cells, MMC cells were incubated with PDTC for 30 min before exposure to HMGB1, and the MMC cells were collected at 50 min and 8 h stimulated by HMGB1. We then assessed p65 subunit, PCNA, and cyclin D1/CDK4/p16 expression. The nuclear p65 subunit was markedly higher in the MMC cells exposed to HMGB1 compared with the control cells, but PDTC dramatically reduced the nuclei expression of p65 (Fig. 2C).

In addition, compared with the HMGB1 group, the PDTC treatment attenuated HMGB1-induced cell proliferation, and the BrdU-positive cell ratio clearly decreased in the HMGB1 + PDTC group (Fig. 1B). PDTC also inhibited PCNA expression (P < 0.01; Fig. 6, A and B). The cyclin D1 and CDK4 protein and mRNA expression was decreased, and the p16 protein and mRNA expression increased in the HMGB1 + PDTC group compared with the HMGB1 group (P < 0.01; Fig. 6, A and B).

PDTC was first used to detect the role NF-κB. Since PDTC is not the specific inhibitor of NF-κB, it may abolish the proliferation through other ways like antioxidant or metal chelator, SN50 was used to verify the role of NF-κB. The BrdU incorporation method showed that the positive cell ratio in HMGB1 + SN50 group was evidently reduced compared with HMGB1 group. Western blot showed the same result (Fig. 1B), and the expression of PCNA in HMGB1 + SN50 was significantly decreased compared with HMGB1 group (P < 0.01; Fig. 6, C and D).

Sh-Akt partially blocks the nuclear translocation of the p65 subunit; however, PDTC did not affect the activation of the Akt phosphorylation induced by HMGB1. The expression of the nuclear p65 subunit was higher in the HMGB1 group compared with the control group (Fig. 2C). As illustrated in Fig. 2C, the transfection with Akt shRNA reduced the nuclear p65

**Fig. 3.** Recruitment of p65 to the κB2 site of the cyclin D1 promoter in MMC cells pretreated with HMGB1 for 90 min. Chromatin immunoprecipitation (ChIP) analysis was performed with chromatin prepared from cells deprived of serum (lane 1) or stimulated by DMSO or HMGB1 for 90 min (lanes 2 and 3). The chromatin was precipitated with p65 or normal rabbit IgG. PCR was performed from these immunoprecipitates or with input chromatin using a primer covering the κB2 site of the cyclin D1 promoter. PCR products (171 bp) were resolved by agarose gel ethidium bromide staining (means ± SD; n = 3; *P < 0.01).
subunit protein level in MMC cells stimulated by HMGB1. In addition, HMGB1 induced Akt phosphorylation in MMC cells, which peaked at 20 min, but the pretreatment with PDTC did not affect the Akt-P-S473 phosphorylation (P < 0.05; Fig. 7).

**DISCUSSION**

Although first established as a nonhistone nuclear protein, HMGB1 acts as a significant cytokine in many biological behaviors when released into the cytoplasm and extracellular spaces (24). The proliferation effect of HMGB1 was verified in many cell lines (5). By combining with RAGE, HMGB1 partly regulated the proliferation of mesangioblasts (16); Chitanuwat et al. provided evidence pertaining to the important role of HMGB1 in the tissue repair of periodontal cells by inducing the proliferation and migration of hepatocyte growth factor (HGF) and human periodontal ligament fibroblasts (HPDLF) (5). Our previous research also demonstrated that HMGB1 mediates the IFN-γ-induced cell proliferation in MMC cells by regulating cyclin D1/CDK4/p16 signaling and promoting the transition from the G0/G1 to S phase (6).

In this study, the BrdU incorporation method was used to reconfirm the effect of HMGB1 on the proliferation of MMC cells. The results revealed that the proportion of positive BrdU-expressing cells noticeably increased after HMGB1 treatment in a time- and concentration-dependent manner, which peaked at 8 h, suggesting that HMGB1 upregulated the proliferation of MMC cells. However, the precise mechanism is unknown.
The MARK/ERK (23), PI3K/Akt (15), Wnt/β-catenin (13), and NF-κB (1) signaling pathways are all common pathways proved to be related with proliferation. As a classic signaling pathway, the PI3K/Akt pathway upregulates cell proliferation in cancer via mediating the expression of PTEN (3). Leptin induces human NP cell proliferation by upregulating cyclin D1 expression partially through the PI3K/Akt signaling pathway (4). The PI3K/Akt pathway-dependent proliferation of some cell lines was based on the activation of the downstream factor NF-κB (20). To investigate whether PI3K/Akt pathway signaling pathway mediates HMGB1-induced proliferation, we detected the activation of several factors. The results showed that HMGB1 decreased the expression of PTEN and induced the phosphorylation of Akt at the Ser473 site and promoted the nuclear translocation of NF-κB in MMC cells. Therefore, the PI3K/Akt pathway is activated by HMGB1 in MMC cells, which is accompanied with MMC cell proliferation. However, it is unknown whether there is significant correlation or not.

From our results and previous studies, we presume that the downregulation of PTEN triggers the phosphorylation of Akt Ser473 but had no effect on Thr308, which results in the activation of the PI3K/Akt pathway. Next, the biological significance and downstream consequences of Akt activation by HMGB1 were evaluated. p-Akt targets NF-κB, which promotes the cytoplasmic to nuclear translocation of NF-κB and is associated with cell proliferation induced by HMGB1. Interestingly, a vector expressing PTEN blocked or attenuated the phosphorylation of Akt and NF-κB nuclear translocation and inhibited the cyclin D1/CDK4/p16 signaling activation and MMC cell proliferation, which suggests that HMGB1 affects key Akt targets. Moreover, to further elucidate the role of Akt in mediating HMGB1-induced cell proliferation, we transfected MMC cells with a specific shRNA vector against mouse Akt. The BrdU-incorporation method showed that HMGB1-induced cell proliferation was decreased by Akt knockdown and that PCNA protein and cyclin D1/CDK4/p16 signal expression were also downregulated. Therefore, HMGB1 promotes Akt phosphorylation and accelerates cell cycle progression, which eventually leads to cell proliferation by inhibiting PTEN. Moreover, the HMGB1-induced nuclear localization of NF-κB was due presumably to Akt activation.

Next, the role of NF-κB in HMGB1-induced proliferation was confirmed. HMGB1 upregulated the expression of p-IκBα (Ser36) at 30 min after stimulation, while the expression of...
IκBa decreased. After another 20 min later, the nuclear expression of the p65 subunit increased. Conversely, PDTC, inhibitor of the NF-κB pathway, noticeably prevented the upregulation of HMGB1-induced cell proliferation. However, PDTC is not a specific inhibitor of NF-κB; it may abolish the proliferation through other mechanisms but NF-κB signal pathway, so another specific inhibitor of NF-κB, SN50, was used to verify the role of NF-κB. Results showed that the upregulation of PCNA and proliferation level of MMC induced by HMGB1 were also significantly reduced by SN50, which suggests that the HMGB1-induced phosphorylation of IκBa triggered the degradation of IκB, which resulted in the escape of NF-κB from the inactive NF-κB-IκBa complex. With the exposure to nuclear localization signals, p65 translocated to the nucleus and induced the proliferation of MMC cells by regulating the transcription of cyclin D1. As a transcriptional factor, NF-κB regulates many genes by recognition and binds to their consensus binding sequence, GGG (G/A) NNYYCC, and finally, mediates the expression of protein. It has been a concern that NF-κB activated the transcription of cyclin D1 by binding to the κB2 sites in cyclin D1 promoter, thereby advancing the reenter to cell cycle in C2C12 cells (8). Toualbi-Abed K et al. (22) demonstrated the role of NF-κB in activation of cyclin D1 in immortalized human hepatocytes (IHH). Our previous study

Fig. 6. Both pyrrolidine dithiocarbamate (PDTC) and SN50 page HMGB1-induced cell proliferation. A: expression of PCNA, cyclin D1, CDK4, and p16 protein was determined by Western blot analysis and quantified by densitometry (means ± SD; n = 3; P < 0.01). B: expression of PCNA, cyclin D1, CDK4, and p16 mRNA was determined by real-time PCR analysis and quantified by densitometry (means ± SD; n = 3; P < 0.01). C: expression of PCNA was determined by Western blot analysis and quantified by densitometry (means ± SD; P < 0.01). D: expression of PCNA was determined by real-time PCR analysis and quantified by densitometry (means ± SD; P < 0.01). The multiple target protein bands in Figs. 4C, 5B, and 6B came from different blots.

Fig. 7. PDTC does not affect the activation of the PI3K/Akt pathway induced by HMGB1. Expression of p-Akt and Akt protein was determined by Western blot analysis and quantified by densitometry (means ± SD; n = 3), P < 0.01.
had confirmed the important role of cyclin D1/CDK4/p16 system in HMGB1-induced proliferation. To explore the relationship of NF-κB and cyclin D1, ChIP was used. The result revealed the recruitment of p65 on the promoter of cyclin D1 in MMC cells stimulated by HMGB1, which indicated that the nuclear translocation of p65 induced by HMGB1 triggers the transcription and translation of cyclin D1, leads to the activation of cyclin D1/CDK4/p16 system, and in the end results in the upregulation of proliferative level. Therefore, the NF-κB pathway was involved in the cell proliferation induced by HMGB1.

To investigate the interaction of the PTEN/PI3K/Akt pathway and the NF-κB pathway, we inhibited each of them, respectively, to observe the activation of another. The results showed that silencing Akt in MMC cells clearly blocked the HMGB1-induced activation of the NF-κB pathway, but neither PTDC nor SN50 affected the activation of Akt phosphorylation induced by HMGB1. These results indicate that the NF-κB pathway is involved in the HMGB1-induced cell proliferation as the downstream factor of the PTEN/PI3K/Akt pathway.

In summary, our results indicate that HMGB1 triggered the activation of the PI3K/Akt pathway by downregulating the expression level of PTEN, thereby activating the downstream factor NF-κB, which led to the nuclear translocation of p65 subunit and promoted cyclin D1 activation and the proliferation of the MMC cells. However, despite the preliminary exploration of cell proliferation and the possible mechanism by which this occurs in HMGB1-induced mesangial cells, there are many interesting questions that remain to be discussed. For example, it remains unknown whether the PI3K/Akt pathway mediates the cell proliferation of glomeruli or whether the knockdown of HMGB1 attenuates kidney injury and decreases the activation of the PI3K/Akt pathway in lupus mice, etc. Therefore, to determine the precise mechanism of cell proliferation in LN pathogenesis and the exact roles of PI3K/Akt signaling and HMGB1, we plan to conduct an in vivo study in transgenic or gene-knockout mice to evaluate whether altering signaling and HMGB1, we plan to conduct an in vivo study in the activation of the PI3K/Akt pathway in lupus mice, etc.

knockdown of HMGB1 attenuates kidney injury and decreases expression level of PTEN, thereby activating the downstream factor of the PTEN/PI3K/Akt pathway. However, despite the preliminary exploration of cell proliferation and the possible mechanism by which this occurs in HMGB1-induced mesangial cells, there are many interesting questions that remain to be discussed. For example, it remains unknown whether the PI3K/Akt pathway mediates the cell proliferation of glomeruli or whether the knockdown of HMGB1 attenuates kidney injury and decreases the activation of the PI3K/Akt pathway in lupus mice, etc. Therefore, to determine the precise mechanism of cell proliferation in LN pathogenesis and the exact roles of PI3K/Akt signaling and HMGB1, we plan to conduct an in vivo study in transgenic or gene-knockout mice to evaluate whether altering signaling and HMGB1, we plan to conduct an in vivo study in the activation of the PI3K/Akt pathway in lupus mice, etc.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


