Non-histone protein HMGB1 inhibits the repair of damaged DNA by cisplatin in NIH-3T3 murine fibroblasts

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INTRODUCTION

The nuclear non-histone protein high mobility group box (HMGB) 1 is known to having an inhibitory effect on the repair of DNA damaged by the antitumor drug cisplatin in vitro. To investigate the role of HMGB1 in living cells, we studied the DNA repair of cisplatin damages in mouse fibroblast cell line, NIH-3T3. We evaluated the effect of the post-synthetic acetylation and C-terminal domain of the protein by overexpression of the parental and mutant GFP fused forms of HMGB1. The results revealed that HMGB1 had also an inhibitory effect on the repair of cisplatin damaged DNA in vivo. The silencing of HMGB1 in NIH-3T3 cells increased the cellular DNA repair potential. The increased levels of repair synthesis could be "rescued" and returned to less than normal levels if the knock-down cells were transfected with plasmids encoding HMGB1 and HMGB1 K2A. In this case, the truncated form of HMGB1 also exhibited a slight inhibitory effect. [BMB Reports 2016; 49(2): 99-104]

Keywords: Acetylation, Cisplatin, HMGB1, NER, Tail-less HMGB1 protein

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**RESULTS**

Over-expression of HMGB1 in NIH-3T3 cells inhibits the repair of damaged DNA with the antitumor drug cisplatin

To evaluate the effect of HMGB1 protein and the role of acetylation and the C-terminal domain on the DNA repair, we created three plasmid constructs: WT-encoding wild type full length protein, K2A-mutated at site of acetylation Lys2 and ΔC: HMGB1 lacking its C-terminal domain. All were cloned in EGFP-C1 vector (Fig. 1A). The transfection level of the three GFP constructs was comparable (25-30%) as well as the amount of the synthesized fusion proteins demonstrated by western blot analysis (Fig. 1B). The relative endogenous HMGB1 level and the three exogenous GFP fusion proteins’ levels were quantified using Odyssey software and normalized to the number of transfected cells. In all cases, the amount of the exogenous GFP fused proteins was approximately 1.4 times greater than the cellular HMGB1. All fused proteins showed nuclear localization (Fig. 1C). The three forms were expressed in NIH-3T3 cell line and their effects on the cellular repair capacity of cis-platinated pDsRedEx2C1 plasmid encoding red fluorescent protein were analysed. Cells expressing GFP alone were used as a control to eliminate the effect, if any, of the green fluorescent protein. The percentage of the successful transfection of both EGFP-C1 and pDsRedEx2C1 vectors was comparable (data not shown). Only double positive cells (red and green) were calculated for the host reactivation (HR) assay. All data were normalized to the reactivation in the control cells. The results presented in Fig. 2 showed that the full length HMGB1 and the mutated HMGB1 K2A had a well defined inhibitory effect on the DNA repair.
whereas the C-terminal lacking form revealed negligible decrease. To optimize the conditions for the HR assay, we did experiments with different levels (1:50 and 1:70) of cis-platinated DsRed plasmid (Fig. S1, A). The repair potential of the cells expectedly depended on the damaged DNA level. The HR inhibition pattern of the three forms of GFP-HMGB1 fusion proteins on the repair of cis-platinated DsRed plasmids (1:50, 1:70) was the same suggesting that the effects of the HMGB1 proteins were independent of damage level (Fig. 1S, B).

Down-regulation of HMGB1 in NIH-3T3 cells enhances the repair of damaged DNA by cisplatin

We generated NIH-3T3 cells with significant down-regulation of HMGB1 using shRNA construct to diminish the effect of the endogenous HMGB1 protein and estimate more precisely the in vivo effect of HMGB1, its mutated, and truncated form. After several transfections and selection with puromycin, we generated a cell line with 60% decreased HMGB1 protein levels analysed by western blot (Fig. 3A). The DNA repair capacity of control 3T3 cells and HMGB1 knocked-down cells was compared. HR assay showed that down-regulation of HMGB1 enhanced the repair of damaged DNA and the effect was well defined 16 h after transfection (Fig. 3B).

We overexpressed the three GFP HMGB1 forms in knocked-down NIH-3T3 cells and analysed their effect on the repair of cis-platinated DNA 16 hours after transfection when the increase of the repair potential of HMGB1 knocked-down 3T3 cells was more prominent. The addition of exogenous GFP HMGB1 inhibited the repair synthesis even less compared to the levels in control cells (Fig. 4). However, K2A mutant form, which inhibitory effect was the most notable in the non-silenced cells (Fig. 2), showed the same tendency in the silenced cells too (Fig. 4). We registered interesting results after overexpression of HMGB1 lacking C-terminal domain. Whereas in non-silenced cells, this form did not have an effect on DNA repair capacity, in silenced cells, it showed slight inhibitory effect (Fig. 4). It is worth mentioning that in the knock-downed NIH-3T3 cells, we observed 5-10% of dead cells measured by staining with trypan blue – the same value as in the “normal” cells after transfection which indicated that the viability of the cells was not affected.

![Fig. 3](http://bmbreports.org)

**Fig. 3.** Down-regulation of HMGB1 enhances the repair of damaged DNA by cisplatin. (A) Western blot analysis showed a decrease in the intracellular levels of HMGB1 with 60% in stable knock-down cell line in comparison to the control cells. The relative HMGB1 levels were quantified using Odyssey software and were normalized to β-actin expression. (B) Host cell reactivation assay of cis-platinated DsRedExC1 in normal and knock-downed cells. *P < 0.05.

![Fig. 4](http://bmbreports.org)

**Fig. 4.** Host cell reactivation assay in NIH-3T3 HMGB1 knock-down line in the presence of GFP-HMGB1 forms. The cells were transfected with cis-platinated pDsRedExC1 (1:50) and HMGB1 forms fused with EGFP (WT, mutated K2A and lacking C-terminal domain-ΔC). The reactivation in control (3T3 cells, expressing GFP alone) is used for the normalization of data (given in percentage with SEM). Asterisks indicate differences between the reactivation in HMGB1 forms expressing cells and the control with *P < 0.05. Student t-test for comparisons between groups was used.
DISCUSSION

HMGB1 protein specifically recognizes and binds to UV lesions (3) and DNA damaged by the anticancer drug cisplatin (4-6, 21), and the affinity of HMGB1 to the lesions is modulated by acetylation (21) and the acidic C-tail (6). Several published data (7, 8) demonstrated that HMGB1 protein is involved in NER pathway. The protein inhibits the repair of cis-platinated DNA in vitro (6, 9). One possible mechanism for the explanation of this finding is the protection of cisplatin lesions from the repair machinery. However, the inhibitory effect of HMGB1 does not correlate with the binding affinity of the protein to the lesion, but is modulated by its C-terminal domain (6). Our results demonstrated that HMGB1 also inhibited the DNA repair in vivo in NIH-3T3 cells. Furthermore, we confirmed the previous findings in cell free extracts (6) that HMGB1 lacking the acidic tail did not inhibit the repair in presence of endogenous HMGB1. The mutated form (K2A) that cannot undergo acetylation, had higher capacity to inhibit the repair than the wild type form in non-silenced cells. Our previously stated hypothesis was (6) that the C terminus was responsible for the inhibitory effect by protein/protein interactions. In case of acetylation or mutation of the lysine at position 2, the C tail becomes “free” and functional as its physical contact with Lys2 is disrupted (23).

One experimental approach to clarify the role of HMGB1 protein and its C terminus in vivo is to knock-down HMGB1 in NIH-3T3 cells in order to eliminate the impact of the endogenous protein. We succeeded in diminishing the cellular HMGB1 level up to 60%. The first important result that we observed, was the stimulation of the DNA repair capacity of the cell line. That finding was an indirect indication that the protein exerted an inhibitory effect on the repair process of cis-platinated DNA. That finding was in agreement with our previous findings in cell free extracts (6) that HMGB1 lacking the acidic C-tail did not inhibit the repair in presence of endogenous HMGB1. The mutated form (K2A) that cannot undergo acetylation, had higher capacity to inhibit the repair than the wild type form in non-silenced cells. Our previously stated hypothesis was (6) that the C terminus was responsible for the inhibitory effect by protein/protein interactions. In case of acetylation or mutation of the lysine at position 2, the C tail becomes “free” and functional as its physical contact with Lys2 is disrupted (23).

We may speculate that the inhibitory effect of HMGB1 in the DNA damage response is accomplished through capturing factors from the repair machinery away from the damages or suppressing their function. The binding partners of HMGB1 include numerous proteins including NER proteins RPA, XPA and XPC (7, 8). The inhibitory effect of HMGB1 in our study was detectable only in the early hours after transfections of normal cells and was compensated at the 36th hour. One possible explanation is the activation of the transcription of the repair genes and their accumulation in the cell at the late hours. The biosynthesis of DNA damage sensors directly dictates the capacity of DNA lesion removal by NER. A critical threshold regulatory function has been ascribed to p53, which governs transcription and thus maintains basal-level expression of rate-limiting human DDB2 and XPC damage sensors under normal growth conditions (34). p53-deficient cells contain reduced levels of DDB2 and XPC, and display diminished GG-NER activity (34-36). So it is possible for HMGB1 to inhibit the DNA repair by binding the repair proteins when they are at basal levels but not at up-regulated levels. Studies revealed that more than 24 hours are needed for the maximum accumulation of proteins whose transcription is induced by p53 after DNA damage (34). Our findings suggest that when estimating the involvement of HMGB1 protein in the DNA repair response and the role of the C terminus in the living cells, we should consider it as a complex phenomenon affecting numerous nuclear processes as repair, remodelling, transcription etc.

MATERIALS AND METHODS

Generation of stable HMGB1 knockdown cell lines

Mouse fibroblast (NIH-3T3) cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and Penicillin/Streptomycin (Invitrogen) in 5% CO2 at 37°C. A short hairpin RNA construct was made in pBabeH1 vector (pBabeH1-HMGB1) using the sequence: 5’-UGCUGC CUACAGGCCUAAGGAA-3’, targeting mouse HMGB1 was kindly provided by Dr. E. Abraham (37). Stable NIH3T3 cell lines with down-regulation of HMGB1 were generated by several transfections with pBabeH1-HMGB1 plasmid DNA using Lipofectamine 2,000 reagent (Invitrogen) followed by selection with puromycin (2 mg/ml).

Plasmid treatment with cisplatin

Plasmid pDsRedEx2C1 (Clontech) containing reporter geneDsRed-Express2 for the red fluorescent protein was propagated in E. coli strain DH5α. Plasmid DNA in TE buffer was incubated overnight on room temperature with cisplatin (Sigma) in different molar ratios. As previously described in (6) cisplatin treated plasmid DNA using Lipofectamine 2,000 reagent (Invitrogen) followed by selection with puromycin (2 mg/ml).

Plasmid constructions

Full length, mutant (K2A) and truncated (ΔC) forms of HMGB1 were cloned by PCR-based sub-cloning using primers containing BglII and EcoRI restriction sites for full length (214 aa), mutant K2A and ΔC truncated (184 aa) in pET28a (Novagen) plasmid as a template. The PCR was carried out using Vent DNA polymerase (NEB) at 95°C/5 min; 30 cycles (95°C/30 s,
57°C/40 s, 72°C/60 s; 72°C/5 min. The amplicons were inserted between BglII and EcoRI sites of pEGFP-C1 (Clontech), ligated, propagated in E. coli strain DH5α and purified.

**Host cell reactivation (HCR) assay**
Fifty percent confluent NIH-3T3 cells in 96 well plates were transfected with 0.4 μl LipofectaminTM 2000 (Invitrogen) and 0.15 μg damaged and undamaged plasmid pDsRedEx2C1 (Clontech) plasmid DNA encoding red fluorescent protein. Cells were also co-transfected with equal quantity GFP plasmids. For each experiment, one well from 96 wells plate per DNA mixture was transfected containing pDsRedExC1 (damaged or undamaged) and pEGFP-C1 (GFP alone or fused with HMGB1 forms). After 18 or 36 hours, cells were imaged at magnification 10x and recorded with differential interference contrast (DIC) and filters for green and red fluorescence. Five regions per well were photographed and fluorescent and nonfluorescent cells were counted (~1,500 cells per experiment). The average percentage of fluorescent cells was used for calculation of the reactivation (R) by the formula: R = red cells with damaged plasmid/ red cells with undamaged plasmid × 100.

**Microscopy**
Fibroblasts were seeded on coverslips placed in 24 well plates and transfected with 2 μl LipofectaminTM 2000 (Invitrogen) and 0.75 μg plasmid DNA (pEGFP-C1 or pEGFPC1-HMGB1). After 24 hours, cells were washed with PBS and fixed in paraformaldehyde. Coverslips were inverted onto the drop of mounting media containing DAPI (UltraCruzTM Mounting Medium, Santa Cruz). Cells were observed with Axiovert 200M inverted fluorescent microscope (Carl Zeiss). Images were acquired and processed by Carl Zeiss AxioVision Rel.4.7 software.

**Western blot analysis**
NIH-3T3 cells were harvested, lysed in Nonidet-P40 buffer and denatured in sample buffer (50 mM Tris HCl pH 6.8; 2% SDS; 10% glycerol; 1% β-mercaptoethanol; 12.5 mM EDTA, 0.02% bromphenol blue). Proteins were run on a 12% sodium dodecyl sulfate-polyacrylamide electrophoresis gel and transferred to nitrocellulose membranes. HMGB1 quantities were detected on a protein blot using polyclonal anti-HMGB1 (Millipore 1:1,000) and goat anti-rabbit IRDye800CW secondary antibody (LI-COR; 1:10,000). β-actin was detected with monoclonal β-actin (Thermoscientific 1:2,000) and goat anti-mouse IRDye 680 secondary antibody (LI-COR; 1:10,000). The bands were visualized using the Odyssey Infrared Imaging System (LI-COR). The relative HMGB1 levels were quantified using Odyssey software and were normalized to β-actin expression.

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