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High mobility group box-1 is phosphorylated by protein kinase C zeta and secreted in colon cancer cells

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ABSTRACT

High mobility group box-1 (HMGB1), a nuclear protein, is overexpressed and secreted in cancer cells. Phosphorylation on two different nuclear localization signal regions are known to be important for the nuclear-to-cytoplasmic transport and secretion of HMGB1. However, little is known about the biochemical mechanism of HMGB1 modifications and its subsequent secretion from cancer cells. To identify the specific enzyme and important sites for HMGB1 phosphorylation, we screened the protein kinase C (PKC) family in a colon cancer cell line (HCT116) for HMGB1 binding by pull-down experiments using a 3XFLAG-HMGB1 construct. Strong interactions between atypical PKCs (PKC-ζ, λ, and ι) and cytoplasmic HMGB1 were observed in HCT116 cells. We further identified the most critical PKC isotype that regulates HMGB1 secretion is PKC-ζ by using PKC inhibitors and siRNA experiments. The serine residues at S39, S53 and S181 of HMGB1 were related to enhancing HMGB1 secretion. We also demonstrated overexpression and activation of PKC-ζ in colon cancer tissues. Our findings suggest that PKC-ζ is involved in the phosphorylation of HMGB1, and the phosphorylation of specific serine residues in the nuclear localization signal regions is related to enhanced HMGB1 secretion in colon cancer cells.

1. Introduction

High mobility group (HMG) proteins primarily reside in the nucleus, and regulate gene expression by binding to DNA without any demonstrated sequence specificity [1]. High mobility group box-1 (HMGB1), a member of the HMG protein family, binds to the minor groove of DNA in a non-sequence-specific manner [2], and is involved in structural and transcriptional regulation of DNA. The DNA affinity and extranuclear localization of HMGB1 are reported to be regulated by phosphorylation and acetylation [3,4].

HMGB1 also localizes to the cytoplasm and can be secreted. Secreted HMGB1 functions as a cytokine. Secretion of HMGB1 has been reported in mouse activated immune cells, where it triggers inflammation by endotoxin-related lethality [5]. HMGB1 secretion is mediated by passive diffusion from necrotic cells [6], or by active transport from activated macrophages and dendritic cells [7,8]. Active secretion of HMGB1 from immune cells is known to be mediated by non-conventional vesicular secretion through secretory lysosomes [9]. In monocytes, this unusual secretory pathway is activated by the acetylation of several lysine residues on HMGB1 [9].

HMGB1 secretion has also been reported in many cancer cells, including glioma, colon cancer, lung cancer, and melanoma [10–12]. We have recently reported that HMGB1 is phosphorylated and secreted in colon cancer cells [13]. These observations suggest that phosphorylation of HMGB1 enhances its secretion in cancer cells. Analyses of consensus phosphorylation sites reveal that protein kinase C (PKC) is the most likely candidate kinase family for the phosphorylation of the two nuclear localization signal (NLS) regions of HMGB1.

Cellular PKCs are divided into three groups according to structural differences: calcium- and diacylglycerol (DAG)-dependent conventional PKCs (cPKCs; α, β, and γ), calcium-independent and DAG-dependent novel PKCs (nPKCs; δ, ε, η, and θ), and calcium- and DAG-independent atypical PKCs (aPKCs; ζ, ι, and λ) [14]. In immune cells, the involvement of cPKCs in HMGB1 phosphorylation was previously reported. Nuclear PKC-α phosphorylates HMGB1 and mediates HMGB1 secretion from monocytes [4]. However, the specific PKCs involved in cancer cells and the critical phosphorylation sites required for HMGB1 secretion are still unknown.

In this study, we identified the specific isotype of PKC which binds to HMGB1 in cancer cells. We also identified critical phosphorylation sites related to HMGB1 secretion in colon cancer cells.

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2. Materials and methods

2.1. Cells and media

HCT116 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA; http://www.atcc.org). Cells were grown in RPMI or MEM media supplemented with 10% fetal bovine serum (FBS; Life Technologies, Grand Island, New York, USA), penicillin, and streptomycin at 37 °C, in a humidified 5% CO_2 environment.

2.2. Vector construction

To facilitate immunoprecipitation of HMGB1, 3XFLAG-HMGB1 expression vectors containing the complete sequence of HMGB1, encoding the full-length 214-amino acid protein, were constructed as previously described [13].

2.3. Site directed-mutagenesis

We have constructed mutant vector sets containing various mutations in the NLS 1 and 2 regions: NLS-AT (all serine residues replaced by alanine), NLS-ET (all serine residues replaced by glutamic acids), and NLS-WT (vector containing wild-type NLS regions). An additional six new vectors were constructed that retained one serine residue, while the other five serine residues in the NLS1 and NLS2 regions were substituted to alanine; these were designated as NLS-S35, NLS-S39, NLS-S42, NLS-S46, NLS-S53, and NLS-S181, where the number denotes the position of the intact serine. These vectors were made using the 3XFLAG-HMGB1 expression vector. All vectors were constructed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), and sequences were confirmed by direct sequencing analyses.

2.4. Selection of tissue samples

For the analysis of PKC-ζ and phosphorylated PKC-ζ expression in human tissue samples, 30 paired fresh frozen colon cancer tissues and matched normal mucosa tissues were selected. The fresh frozen human tissue specimens were supplied from the Liver Cancer Specimen Bank supported by the National Research Resource Bank Program of the Korea Science and Engineering Foundation in the Ministry of Science and Technology. Consents to use the tissue specimens for research purposes were obtained from each of the patients, and the utilization of the specimens for this research was authorized by the Institutional Review Board of the College of Medicine, Yonsei University.

2.5. Immunoblotting

Proteins from immunoprecipitation were resolved by SDS–PAGE, transferred to nitrocellulose membranes, and blocked by incubating at room temperature with TBS-T containing 5% skim milk. Antisera used were anti-FLAG (Sigma–Aldrich), anti-JL-8 (Clontech, Mountain View, CA, USA), anti-PKC-α (Cell Signaling, Danvers, MA, USA), anti-PKC-β1 (Santa Cruz Biotechnology Santa Cruz, CA, USA), anti-PKC-β2 (Santa Cruz Biotechnology), anti-PKC-δ (Cell Signaling), anti-PKC-θ (Cell Signaling), anti-PKC-ζ (Cell signaling), anti-PKC-ι (Cell Signaling), and anti-PKC-λ (BD Biosciences, Franklin Lakes, NJ, USA), all diluted at 1:1000 in blocking buffer, and applied to membranes and incubated overnight at 4 °C. Membranes were washed, incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology), washed again, and developed with ECL-Plus (Amersham Pharmacia Biotech, Uppsala, Sweden). Results were quantified by densitometry using TINA software (version 2.0).

2.6. PKC inhibitor treatments

For the pan-PKC inhibitor, Gö6983 (Calbiochem, Merck, Darmstadt, Germany), ATG (Sigma–Aldrich), and rottlerin (Sigma–Aldrich) were used. Cells were treated with Gö6983 at 7 or 60 nM, ATG at 1, 5, or 10 μM, and rottlerin at 1, 5, 10, 20, or 40 μM. All drugs were dissolved in either distilled water or dimethyl sulfoxide (DMSO) and were added to the culture medium in volumes not exceeding 0.5% of the total culture media volume.

2.7. siRNA treatment for PKC-ζ

HCT116 cells were transiently transfected using Lipofectamine 2000 (Invitrogen) with siGENOME PRKCZ M-003526-04 siRNA (Dharmacon, Lafayette, CO, USA) in 100 mm plates for the knockdown of PKC-ζ mRNA. Culture media were exchanged for serum-free RPMI at 4–6 h after transfection. Cells were harvested 3 days later and culture media were collected for concentration. Total cell lysates were prepared for the confirmation of knockdown efficiency by immunoblotting.

3. Results

3.1. PKC is the candidate kinase for HMGB1 phosphorylation

Analyses of the possible phosphorylation sites in the HMGB1 protein sequence was done by use of the web program NetPhos 2.1. We identified 14 suspected residues as phosphorylation sites, six of which were located in NLS regions, which are important for HMGB1 translocation. The suspected kinases responsible for phosphorylation of HMGB1 were, protein kinase A (PKA), protein kinase G (PKG), PKC, casein kinase (CK)I, CKII, ribosomal s6 kinase (RSK), and the insulin receptor (InsR). Among these enzymes, PKC was the most likely candidate for the phosphorylation of the serine residues in the NLSs. All of the analyzed data are depicted in Supplementary Fig. S1, and the representative scores are statistically significant.

3.2. Atypical PKCs bind to HMGB1 in HCT116 cells

To identify which PKC is involved in the phosphorylation of cytoplasmic HMGB1 and in its secretion in cancer cells, we screened PKCs that were bound to HMGB1, using HCT116 cell line. Immune complexes containing proteins generated from 3XFLAG-HMGB1 vectors were precipitated from nuclear, cytoplasm, and total cell lysates using anti-FLAG antibody, which were then immunoblotted with different anti-PKCs. We found that the nPKC (PKC-δ), and the aPKCs (PKC-ζ, ι, and λ), were bound to HMGB1 both in the nucleus and cytoplasm (Fig. 1A). A small amount of the cPKCs, PKC-α and PKC-β1 were bound to cytoplasmic HMGB1, while no binding was observed in nuclear HMGB1. A larger amount of PKC-δ was bound to nuclear HMGB1 than to cytoplasmic HMGB1. The aPKCs, including PKC- ζ, ι, and λ, were bound to cytoplasmic HMGB1 to a greater extent than to nuclear HMGB1. To verify these results, we performed co-immunoprecipitation assays using anti-PKC-δ, ζ, and λ with nuclear, cytoplasmic, and whole-cell extracts from HCT116 cells. As shown in Fig. 1B, HMGB1 was bound to PKC-δ, ζ, and λ in the nuclear and cytoplasmic fractions. We also performed co-localization assays for HMGB1 and PKC-ζ using immunofluorescent microscopy. The cytoplasmic HMGB1 was selectively stained using 0.04 mg/ml saponin, which enables only cytoplasmic membrane permeabilization without nuclear

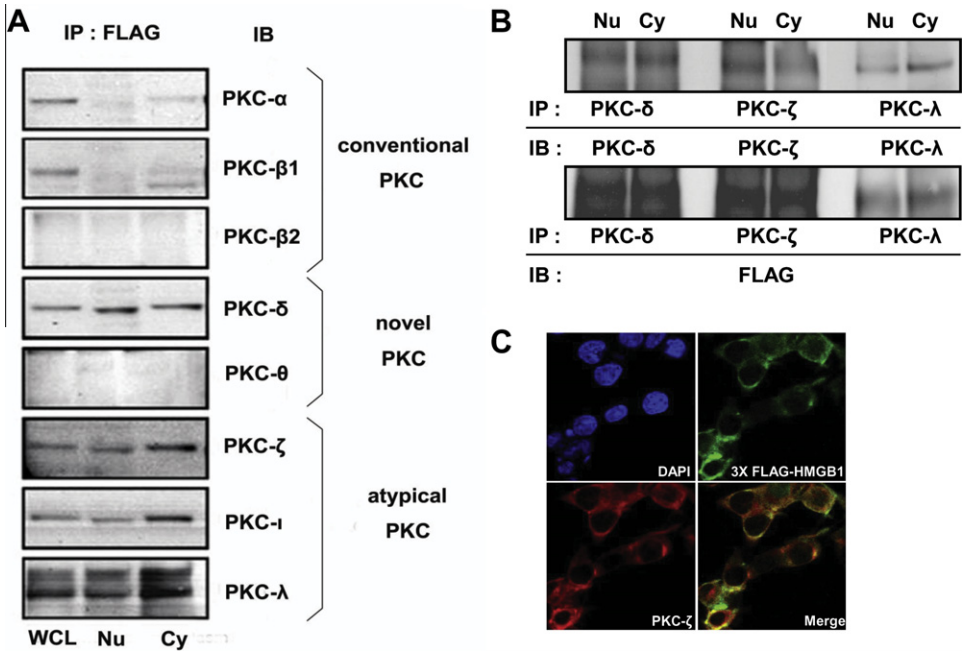

Fig. 1. Atypical PKCs are bound to HMGB1 in HCT116 cells. (A) Immunoprecipitation analysis of 3XFLAG-HMGB1 was performed to screen bound PKC isotypes in HCT116 cell lines. The conventional PKCs, PKC-α, β, γ, and the novel PKC, PKC-θ, were not bound to HMGB1 strongly in the nucleus and cytoplasm. The novel PKC, PKC-δ was bound to HMGB1 in both the nucleus and the cytoplasm. All of the three atypical PKCs (PKC-ζ, ι, and λ) were bound to HMGB1 in both the nucleus and cytoplasm, especially in the cytoplasm. (B) Immunoprecipitation analyses of PKC-δ, ζ, and λ, to detect the binding of specific PKCs to HMGB1. Anti-PKC- δ, ζ, and λ were used to detect the immunoprecipitated PKCs and the anti-FLAG antibody was used to detect PKCs bound to HMGB1 in HCT116 cells. (C) Immunofluorescence assay for PKC-ζ demonstrates the co-localization of cytoplasmic HMGB1 and PKC-ζ.

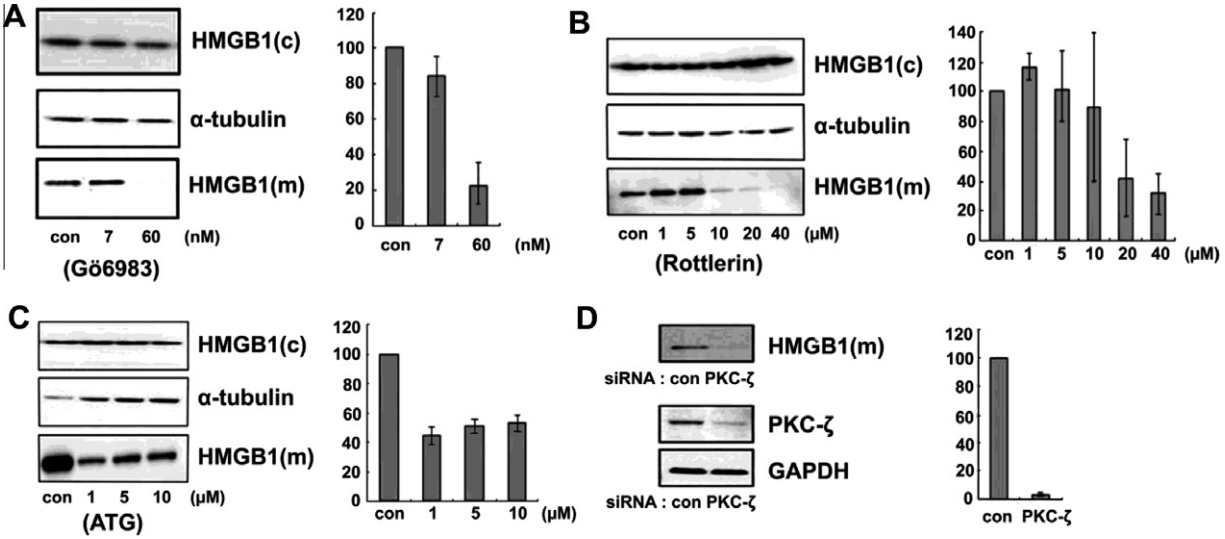

Fig. 2. PKC-ζ is involved in HMGB1 secretion in HCT116 cells. (A) HCT116 cells were treated with Gö6983 to inhibit PKCs at 7 nM (cPKC) and 60 nM (aPKC). Secreted HMGB1 was significantly decreased at the 60 nM concentration, but was unchanged at 7 nM. (B) HCT116 cells were treated with rottlerin to inhibit PKCs at low dose (cPKC) and high dose (nPKC and aPKC). Secreted HMGB1 was significantly decreased at the high concentration of inhibitor (10 μM). (C) HCT116 cells were treated with ATG to inhibit PKC-ι. Secreted HMGB1 was not changed with drug treatment. (D) siRNA knockdown of PKC-ζ in HCT116 cells. PKC-ζ was significantly knocked down by siRNA treatment and secreted HMGB1 was decreased by PKC-ζ knockdown in HCT116 cells. In all panels, the bar graphs depict values from densitometry obtained using TINA software. (c) denotes cytoplasmic protein, and (m) denotes the secreted protein obtained from the media.

membrane permeabilization [15]. As expected, co-localization of PKC-ζ and HMGB1 in the cytoplasm of HCT116 cells was observed (Fig. 1C). These results indicate that (1) HMGB1 is bound to different subclasses of PKCs in colon cancer cells, (2) PKC-δ is the major PKC that binds to nuclear HMGB1, and (3) aPKCs (PKC-ζ, ι, and λ) are predominantly bound to cytoplasmic HMGB1.

3.3. PKC-ζ is critical for HMGB1 secretion

Unlike immune cells, cPKCs play no significant role in HMGB1 secretion in cancer cells. We therefore evaluated the role of nPKCs and aPKCs in HMGB1 secretion in cancer cells. We used various concentrations of PKC inhibitors. Gö6983 is a general PKC inhibitor

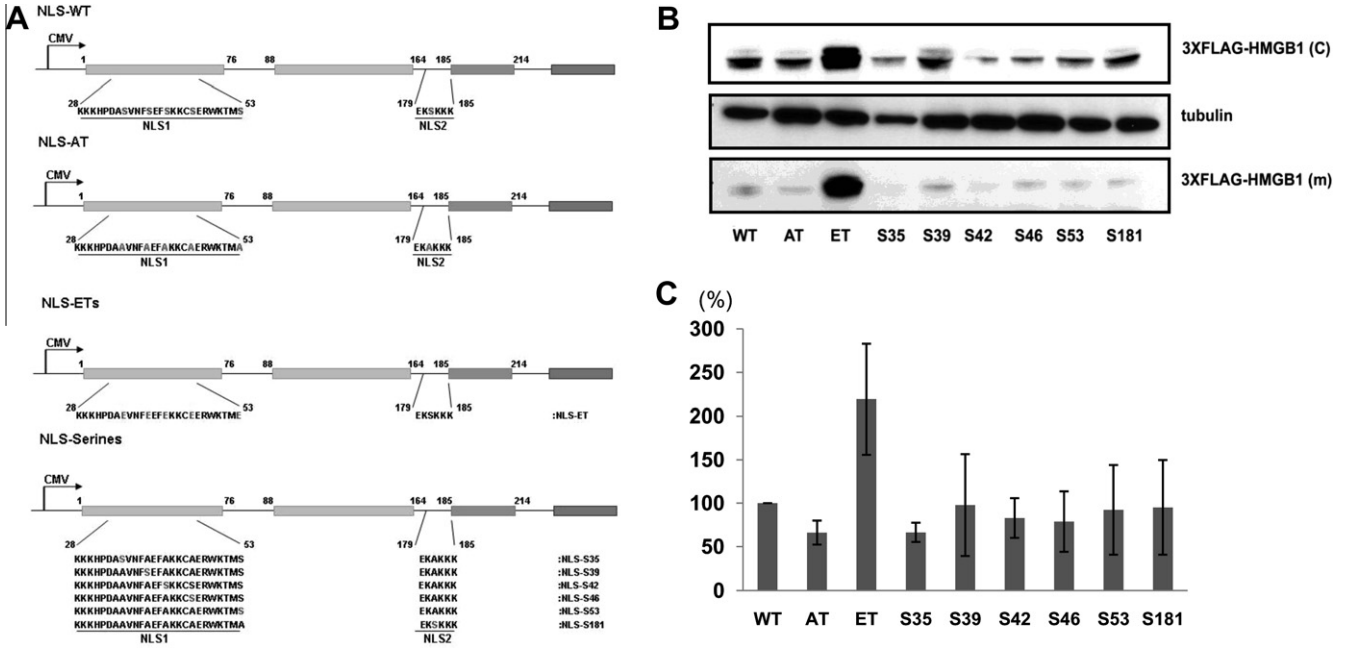

Fig. 3. Phosphorylation of several serine residues is important for HMGB1 secretion. (A) Vectors were cloned into the 3XFLAG tagged vectors. NLS-WT represents wild-type HMGB1, NLS-AT and NLS-ET represents HMGB1 with 6 serine residues were substituted by alanine and by glutamic acid, respectively. NLS-S retained one serine residue, while the other five serine residues in the NLS1 and NLS2 regions were substituted to alanine. These were designated NLS-S35, NLS-S39, NLS-S42, NLS-S46, NLS-S53, and NLS-S181, where the number denotes the position of the intact serine. (B) HCT116 cells were transfected with NLS-S constructs. Secreted HMGB1 was detected by anti-FLAG, and Coomassie blue stained blots were used as loading controls. S39, S46, S53 and S181 mutants showed increased secretion. (C) Average values from four independent experiments were calculated using TINA software. Among the residues, S39, S53 and S181 were related to the increased secretion.

which inhibits different PKC isotypes at different concentrations. We used this inhibitor at a concentration of 7 nM to inhibit cPKCs, and at 60 nM to inhibit aPKCs. HMGB1 secretion was significantly decreased when 60 nM of Gö6983 was used, suggesting that aPKCs are critical for HMGB1 secretion (Fig. 2A). Rottlerin and ATG also inhibit different PKC isotypes at different concentrations. When the cells were treated with rottlerin, which inhibits cPKCs at low concentrations and aPKCs at higher concentrations (1–40 μM), secretion of HMGB1 was significantly decreased at thehigher concentrations (Fig. 2B). ATG, which inhibits PKC-ι, significantly decreased HMGB1 secretion from HCT116 cells at 1–10 μM concentrations (Fig. 2C). These data suggest that aPKCs primarily regulate cytoplasmic HMGB1, and are critical for HMGB1 secretion in HCT116 cells.

We confirmed these results using siRNAs directed against PKC-ζ which were reported to play important roles in colon cancer [16,17]. The secretion of HMGB1 in HCT116 cells was significantly decreased by treating with siRNA for PKC-ζ, indicating that HMGB1 secretion is mediated by PKC-ζ (Fig. 2D).

Serine residues S39, S53, and S181 in the NLS regions are critical for HMGB1 secretion in HCT116 cells.

To address which specific serine residues that are critical for HMGB1 secretion, we used constructs in which six serine residues within the NLS region were changed to glutamic acid, to mimic phosphorylation, or alanine, to prevent phosphorylation. HCT116 cells were transfected with each construct, and the culture medium was changed after 6 h of transfection. Cytoplasmic extracts and concentrated culture media were further analyzed by western blot. To demonstrate the effects of each serine phosphorylation, we tested a set of constructs containing one serine residue, while the other five serine residues in the NLS1 and NLS2 regions were substituted to alanine. The details of all constructs are depicted in Fig. 3A. We found that S39, S53 and S181 were critical residues involved in the translocation and secretion of HMGB1 (Figs. 3B, C and 4).

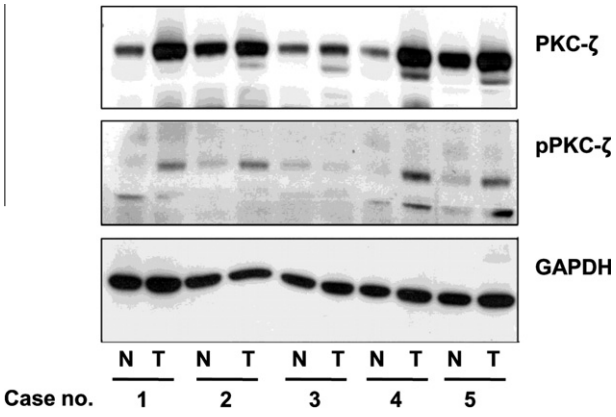

Fig. 4. Expression and activation of PKC-ζ in cancer patients. Expression of PKC-ζ and phospho-PKC-ζ in paired normal and tumor tissue samples. Overexpressions of PKC-ζ and phospho-PKC-ζ were evident in tumor tissues. N denote normal mucosa, T denote colon cancer tissue.

3.4. PKC-ζ expression in tumor tissues

We hypothesized that PKC-ζ is differentially expressed in tumor tissues compared to the adjacent normal mucosa, because it regulates HMGB1 secretion in tumor tissues. To evaluate the expression level of PKC-ζ in colon cancers, we collected specimens from 30 cancer patients with matched normal mucosae. Tumor tissues showed higher expression of PKC-ζ compared to normal tissues. When we evaluated the expression of 30 cases by densitometry, the expression level of tumor was 1.61-fold higher than normal mucosa ($P < .008$).

PKC-ζ is usually activated by phosphorylation; therefore, we also tested the expression levels of phosphorylated PKC-ζ in the same patients. Phosphorylated PKC-ζ was also 1.19-fold higher in

tumor tissues than normal mucosa ($P < 0.001$), indicating that PKC-ζ was overexpressed and activated in colorectal carcinomas. These data strongly support our hypothesis that cytoplasmic HMGB1 in colon cancer cells are easily phosphorylated through PKC-ζ activation, and redirected toward its secretion from colon cancer cells.

4. Discussion

In this study, we demonstrated that PKC-ζ, one of the atypical PKCs, is involved in the phosphorylation of HMGB1, resulting in the secretion of this protein from HCT116 colon cancer cells. We identified several critical phosphorylation sites on HMGB1 that are important for secretion. We also found that PKC-ζ is overexpressed and activated in tumor tissues. This is the first report that identifies the specific kinase involved in regulating HMGB1 secretion in cancer cells.

There are several differences in HMGB1 secretion mechanisms between immune cells and cancer cells. In immune cells, HMGB1 is passively released from necrotic cells or actively secreted in response to TNF-α, interleukin (IL)-1β, or LPS [6], upon extensive acetylation on lysine residues throughout the length of the protein [5]. The protein is concentrated within a subpopulation of secretory lysosomes by lysophosphatidylcholine in myeloid cells [9], and by single methylation of HMGB1 on lysine 42, in neutrophils [18]. Although many clinical and experimental results report roles for HMGB1 in cancer; few studies report the mechanisms of its cytoplasmic translocation and extracellular secretion. Previously, we have reported that HMGB1 can be translocated and secreted even during the resting state of cancer cells [15]. In this study we report that PKC-ζ is involved in HMGB1 secretion in cancer cells, in contrast to immune cells. Furthermore, unlike immune cells, we demonstrated that PKC-α is not involved in HMGB1 phosphorylation in cancer cells. It had been reported that PKC-α was significantly downregulated during the carcinogenesis of colon cancer [19], implying the involvement of another PKC isotype for HMGB1 phosphorylation and secretion in cancer cells. The biological significance of HMGB1 phosphorylation by a different isotype of PKC should be studied in the future, to determine if other differences are found between monocytes and cancer cells in HMGB1 secretion.

Post-translational modification of the NLS regions is thought to be one of the most critical factors for HMGB1 translocation and secretion. Several groups have reported the modification of NLS regions for its translocation [4,13,20–24]. In this study, we identified the critical region for PKC-ζ-mediated phosphorylation of HMGB1, which is necessary for secretion from HCT116 cells. Among the six serine residues, three (NLS-S39, -S53 and -S181) are thought to be important for HMGB1 secretion. Additionally, the possibility for multiphosphorylation in NLS regions could enhance the HMGB1 secretion should be elucidated. Further functional studies of HMGB1 after phosphorylation at specific serine residues are necessary.

PKCs are involved in many functions of cancer cells, including cell proliferation and angiogenesis [25]. PKCs regulate multiple signaling pathways including phosphoinositide 3-kinase (PI3-K)/Akt, mitogen-activated protein kinase (MAPK), and glycogen synthase kinase 3 (GSK3)-β. PKCs are commonly dysregulated in prostate, breast, colon, pancreatic, liver, and kidney cancers. For these reasons, several PKC inhibitors are being evaluated in clinical trials for cancer prevention and therapy. Recently, PKC-ζ was reported to have diverse roles in cancer, including breast and lung cancer cell chemotaxis [26,27], and migration and invasion in glioblastoma [28]. In this study, we demonstrate that phosphorylated PKC-ζ is strongly associated with cytoplasmic HMGB1 and its secretion in colon cancer cells. Secreted HMGB1 is expected to be involved in critical functions of cancer cells including cell proliferation, migration, and invasion [29]. Inhibition of HMGB1 secretion from cancer cells by modulation of PKC-ζ might provide a novel therapy for preventing colon cancer progression in the future.

In conclusion, we have identified that PKC-ζ is bound to cytoplasmic HMGB1, resulting in the phosphorylation of several key serine residues in NLSs. Additionally, PKC-ζ is overexpressed and activated in tumor tissues. Our findings suggest that PKC-ζ might be an important candidate as a target to control colon cancer progression, through the inhibition of HMGB1 secretion.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.116.

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