A phospholipase A$_2$ of *Daboia siamensis* venom suppressed expression of genes and proteins in MAPK pathway

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ABSTRACT: High mortality rate of Melanoma is due to poor response to radiotherapy and chemotherapy. A phospholipase A$_2$ (PLA$_2$) of *Daboia siamensis* venom has been reported to inhibit melanoma cells. The aim of this study is to evaluate the effects of dssPLA$_2$ on gene and protein expression of MAPKs (Mitrogen-activated protien kinases) in melanoma cells. The mRNA and protein were determined by quantitative real-time PCR and western blotting to detect the expression levels of MEK1, MEK2, BRAF V600E and ERK1/2. Results indicated that 0.25 µg/ml of dssPLA$_2$ reduced the mRNA expression level of these genes in a time dependent manner. Interestingly, at 48 h, the mRNA expression level of all genes was extremely decreased compared with untreated cells in the same condition. Whereas, the protein level of MEK1, MEK2, BRAF V600E and ERK1/2 were clearly reduced after dssPLA$_2$ incubation. Our results show that dssPLA$_2$ is a strong inhibitor to MAPK genes at 48 h.

KEYWORDS: melanoma, MAPK pathway, phospholipase A$_2$, *Daboia siamensis* venom, BRAF V600E

INTRODUCTION

Melanoma is a harmful malignant tumor, having early metastasis and strong invasiveness with poor prognosis and high mortality. The incidence continues to increase in the majority of the population [1–3]. It often shows a poor response to radiotherapy and chemotherapy. Currently, the molecular understanding was vastly progressed for melanoma therapy agents. Treatment of melanoma focuses on the mutated genes instead of the chemotherapeutic drugs. Targeted therapy and immunotherapy are more effective treatments than chemotherapy which can damage normal cells. Several agents with effects on cancer cells, were studied in clinical trial and laboratory treatment. Anticancer agents were approved by the US Food and Drug Administration (FDA) or European Medicines Agency (EMA) such as Dacarbazine (DTIC) [4], Vemurafenib (Zelboraf®, Roche) and Darafenib (Tafinlar®, GlaxoSmithKline). B-Raf inhibitor has been approved by the FDA to inhibit the MAPK pathway. Sorafinib has restricted to eradicate both C-Raf and B-Raf. In addition, MEK inhibitor such as Trametinib (Mekinist®, GlaxoSmithKline) has been studied in clinical trials. However, these agents have disadvantages due to limited activities in resistant malignant melanoma. Nevertheless, no agent has been improved for the effective long-term treatment of this cancer. Therefore, new agents against cancer are needed and should be used to improve survival [5].

Dysregulation of the MAPKs signaling through the RAF-MEK-ERK cascade is a proven for irregular melanoma [6]. MAPK is a protein cascade consisting of rat sarcoma protein (Ras), mitogen-activated protein kinase kinase kinase (Raf or MAP3K), mitogen-activated protein kinase kinase (MEK or MAP2K) and extracellular signal-regulated protein kinase (ERK or MAPK). These proteins act as receptors in the extracellular activator signaling [7]. Expression of MAPK genes concerns cellular activities such as proliferation, differentiation, survival and transformation of the cells. Aberrant expression of Ras/Raf/MEK/ERK genes, especially Ras and Raf causes the reproductive cancer disease in human [6, 8].

dssPLA$_2$; a Phosphoslipase A$_2$ (PLA$_2$) was isolated from *Daboia siamensis* venom. PLA$_2$ hydrolyzed phospholipid membrane producing lysophospholipid and free fatty acid, which caused cell damage [9, 10]. PLA$_2$ has been studied against various cancer cells [11–13]. Previously, dssPLA$_2$ has been shown to inhibit melanoma cells which showed reduced BRAF V600E mRNA expression

level in the MAPKs pathway without affected normal cell [14]. To explore in more detail the action of dssPLA$_2$ on melanoma cells, BRAF V600E and also other MAPK genes were investigated. Since MAPKs is a signaling cascade with cooperation of genes network. Those genes belong to the MAPK pathway concerning with the proliferation and progression of various cancer cells. In the event of gene expression, the effects of dssPLA$_2$ could lead to cell damage or cell death which acts as a potent melanoma inhibitor. In this work, we studied MEK1, MEK2, BRAFV600E and ERK1/2 gene and protein expression of MAPKs pathway.

MATERIALS AND METHODS

Reagents

Primary-antibody MEK1 was the product of the R&D system (Bio-Tech, USA). Anti-MEK2, Anti-GAPDH, Anti-ERK1/2, and Anti-BRAF V600E were purchased from Bio-Rad (USA) and Biosciences (USA), respectively. Second-antibodies were anti-mouse monoclonal and anti-rabbit polyclonal conjugated by IgG-HRP from Bio-Rad and Sigma-Aldrich (USA). The clarity substrate was provided following the Bio-Rad procedure. All reagents were of analytical grade.

Cell culture

Human skin melanoma cell line, SK-MEL-28 (ATCC® HTB-72™), was purchased from the American type culture collection (ATCC, Manassas, Virginia, USA). The SK-MEL-28 cells were cultured in MEM medium supplemented with 10% FBS (Fetal bovine serum), 1 mM glutamine, 100 U/ml streptomycin and 100 U/ml penicillin and incubated at 37 °C with 5% CO$_2$. The media, FBS, streptomycin and penicillin were purchased from Invitrogen, USA.

Purification of dssPLA$_2$

dssPLA$_2$ was purified from *D. siamensis* venom following the method described by Khunsap et al [14]. Briefly, *D. siamensis* venom in lyophilized form was dissolved with 0.02 M phosphate buffer pH 6.0 and applied to ion-exchange chromatography on HiTrap™ CM FF column. The proteins were eluted with 0–1 M NaCl linear gradient in 0.02 M Phosphate buffer pH 6.0. The highest PLA$_2$ activity peaks were further purified by size exclusion chromatography on a pre-equilibrated Superdex™ 75 10/300 GL column.

cDNA synthesis

The SKMEL-28 cells were treated or untreated with 0.25 µg/ml dssPLA$_2$ for 24, 48 and 72 h. Total RNA was extracted by the Trizol reagent kit. One microgram of RNA from the cells was used to be the cDNA template. The reaction mix was performed, followed the manufacturer manual (ThermoFisher Scientific, USA). Random hexamer primer 1 µl, 5 × reaction buffer 4 µl, RiboLock RNAase Inhibitor 1 µl, 10 mM dNTP Mix 2 µl, and RevertAid M-MuLV Reverse Transcriptase 1 µl, were incubated at 42 °C for 60 min and 70 °C for 5 min. cDNA was kept on −20 °C until use.

Quantitative Real-Time PCR

Quantitative Real-Time PCR was performed by Bio-Rad manufacture kit. One microlitter of cDNA was used as the template for gene expression. The cycling condition was performed under the default of the CFX96 Touch™ Real-Time PCR detection system by using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, USA). The amplification began with an initial denaturation at 95 °C for 3 min, 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Melting analysis was collected by 95 °C for 10 s, 65 °C for 5 s and 95 °C for 50 s. Specific primer sequences were as follow; GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) forward: ACC ACA GTC CAT GCC ATC; GAPDH reverse: TCC ACC ACC CTG TTG CTG; BRAF V600E forward: AGG TGA TTT TGG TCT AGC TAC AGA; BRAF V600E reverse: TAG TAA CTC AGC AGC ATC TCA GGG C; MEK2 forward: GCA GTC GGA CAT CTG GAG CA; MEK2 reverse: CAC CGT TGG GCA GCT TAG GA. The primer of MEK1 (MAP2K1) and ERK (MAPK1) genes were analyzed by using PrimePCR Unique assay ID qHsaCID0011553 and qHsaCED0042738, respectively, (Bio-Rad USA). All data analyses were performed by the CFX manager software detection system under a Bio-Rad license.

Western blot analysis

The SKMEL-28 cells treated or untreated with 0.25 µg/ml dssPLA$_2$ for 24, 48 and 72 h were collected and lysed under cold condition. The amount of total protein was measured by the Qubit kit protein assay (Invitrogen, USA). A thirty micrograms of each sample was separated by 4–12% BIS-Tris SDS-PAGE (Invitrogen, USA) and transferred onto PVDF membrane. The membranes were blocked by 5% BSA in PBST (PBS with 0.05% Tween 20) for 3 h, incubated with primary an-
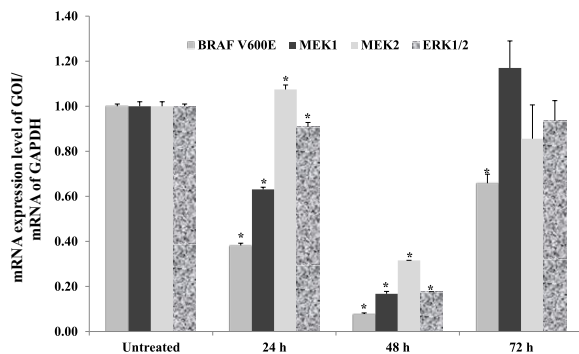
Fig. 1 The mRNA expression level of BRAF V600E, MEK1, MEK2 and ERK1/2 in SKMEL-28 cells treated with 0.25 µg/ml dssPLA$_2$ for 24, 48 and 72 h. $*$ $p < 0.05$ was considered as statistically significant compared with untreated cells in the same experiment. GOI = gene of interest; GAPDH, a housekeeping gene.

tibodies at 4 °C overnight, then incubated with horseradish peroxidase-conjugated secondary antibodies for 45 min. The protein complex was detected by the ECL (Enhanced chemiluminescence) substrate kit (Bio-Rad, USA).

Statistical analysis

mRNA expression level of the interested gene was normalized to the GAPDH housekeeping gene and quantified with untreated cells in the same experiment. Statistical differences were evaluated by Primer program version 3.02. $p < 0.05$ was considered statistically significant.

RESULTS

Inhibition of MAPKs gene expression

Mutation and dysregulation of protein kinases play causal roles in human diseases. They were used as the basis for developing antagonists of these proteins for human therapy [15]. In this study, we found that 0.25 µg/ml of dssPLA$_2$ could reduce BRAF V600E, MEK1 and ERK mRNA expression level of SK-MEL-28 cells at 24 h, but not the MEK2 gene. Interestingly, after 48 h of incubation time, dssPLA$_2$ could decrease the expression of all genes. Although the mRNA expression level of genes has slightly increased at 72 h, all were still expressed at lower level than the untreated, except for the MEK1 gene (Fig. 1).

Inhibition of MAPK protein expression

The expression levels of BRAF V600E, MEK1, MEK2 and ERK1/2 proteins in the SKMEL-28 cells induced

by dssPLA$_2$ were determined by Western blot analysis. The results showed that dssPLA$_2$ at 0.25 µg/ml could inhibit the level of BRAF V600E, MEK1, MEK2 and ERK1/2 proteins (Fig. 2). The levels of BRAF V600E and MEK2 proteins were clearly decreased with time after treating with dssPLA$_2$. The level of MEK1 protein was decreased after 24 h period and increased slightly up to 48 h. However, the ERK1/2 protein level showed a small decrease at 24 h but extremely decreased after 48 h of incubation.

DISCUSSION

In previous study, we investigated the dssPLA$_2$ properties as a melanoma inhibitor by inhibiting migration and inducing apoptosis cell death [14]. In the present study, we focused on the effect of dssPLA$_2$ on the genes and proteins in MAPK pathways which are important in broad cellular activities and physiological processes. RAF (MAP3K)/MEK (MAP2K)/ERK (MAPK) interaction cascades usually play an important role in proliferation, cell division and differentiation. Mutations of MAPK kinase lead to several diseases and also cancers [8, 16, 17]. Whereas dssPLA$_2$ inhibited the mutated MAPK kinase, thus blocked proliferation and caused poor progression of cancer.

BRAF V600E mutation has been identified by about 66% in malignant melanomas [13]. The constitutive activation of BRAF V600E caused overexpression of the MEK-ERK signaling pathway which led to the step of tumor development and progression. Numerous BRAF inhibitors for melanoma treatments have been approved by the FDA many years ago. Unfortunately, no patients had progression free-survival more than 6 months after treatment with both single and combined therapy [16]. Resistance to the treatment is a main problem for the clinician. The approved drugs and new promising agents still have to empirically develop. Our results indicated that 0.25 µg/ml of dssPLA$_2$ inhibited the BRAF V600E gene, especially at 48 h. Although at 72 h, the mRNA expression level of the gene was slightly increased, it was still a down-regulation response. However, the BRAF V600E protein level has serially decreased from 24–72 h. Incubation at 48 h could be considered as a good protocol for the action of dssPLA$_2$ against melanoma. At present, the FDA approved many BRAF inhibitors for the mutant BRAF kinases such as Vemurafenib, Dabrafenib and LGX818. Alone and a variety of combinations are currently being studied in clinical trials. However, the progression-free survival of patients is slowly increasing, but tumors do not
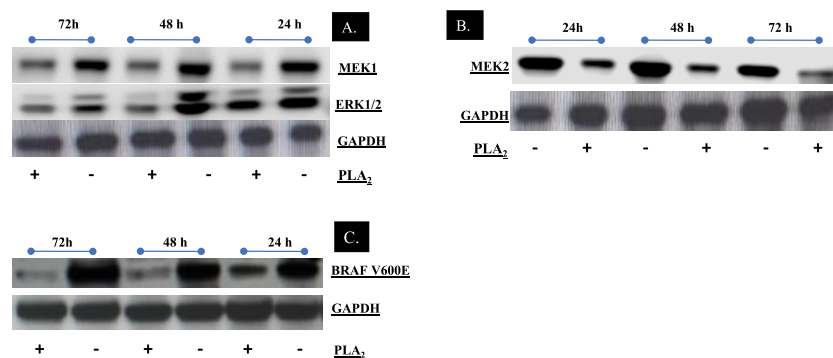
Fig. 2 The level of BRAF V600E, MEK1, MEK2 and ERK1/2 protein expressed in SKMEL-28 cells treated (+) or untreated (-) with 0.25 µg/ml dssPLA$_2$. (A) MEK1 and ERK1/2, (B) MEK2 and (C) BRAF V600E. GAPDH, a housekeeping gene.

completely disappear [16, 18, 19]. It seems likely that dssPLA$_2$ induced down-regulation of mRNA expression and protein level of BRAF V600E on SK-MEL-28 cells. After 24 and 48 h periods, dssPLA$_2$ had the potential for the treatment of this cancer. The data might support and contribute to treatment among cancer patients.

MEK family consists of 2 isomers, MEK1 and MEK2 which belong to the MAPKs pathway. MEK signaling has played the role of ERK1/2 activator in MAPKs interaction cascade, as a checkpoint of the MAPKs signaling cascade. Activation of MEK1 and MEK2 of intestinal epithelial cells could induce the formation of metastatic intestinal tumors in mice [20]. Therefore, MEK1/2 inhibition has been specifically targeted for therapeutic agents of various cancers [8] and also melanoma [19]. In our study, dssPLA$_2$ reduced only MEK1 gene expression, but did not affect MEK2 at 24 h incubation. In contrast, MEK1 was slightly up-regulated at 72 h which might be a recovery point of the cell damage. The level of MEK1 protein was clearly reduced after 24 h of dssPLA$_2$ incubation, whereas MEK2 had a slower rate of decreased expression (Fig. 2B). This means dssPLA$_2$ has a greater specific binding to MEK1 isomer than MEK2. Li et al [21] reported that MEK1-YAP protein interaction led to critical human liver cancer both *in vitro* and *in vivo*. Interaction of dssPLA$_2$ and MEK1 might be a way of damage leading to cell death.

ERK1/2 is a downstream effector of MEK1/2 signaling protein. They have distinct roles in physiological and developmental processes. Activation of ERK1/2 by any stimuli leads to inhibition of apoptosis, whereas inhibition of ERK1/2 promotes apoptosis. Our results showed that dssPLA$_2$ slightly inhibited ERK1/2 gene expression at 24 and 72 h but

its expression was greatly decreased at 48 h (Fig. 1). This suggested that 48 h incubation was optimal for dssPLA$_2$ to show its effect on ERK1/2 genes damage in the SKMEL-28 cells. The result of the ERK1/2 protein expression at 48 h (Fig. 2A) was in accord with that of the ERK1/2 gene expression (Fig. 1). In contrast, there was an inverse correlation of mRNA expression level and protein level of ERK1/2 at 72 h. The level of mRNA of ERK1/2 was higher than at 48 h while the protein level was down-regulated. One possible reason is ERK1/2 activity can be down-regulated by dephosphorylation or degradation of ERK1/2 by long-term stimulation [15]. However, the changes in mRNA level and protein level do not correlate which might be due to the regulation control at different levels. The relationship between protein and mRNA levels of MAPK cascade was explained under various scenarios such as steady-state, long-term state changes and short-term adaptation, demonstrating the complexity of gene and protein expression regulation [22].

In summary, our results demonstrate that dssPLA$_2$ decreased gene and protein expression of MAPKs family, especially after 48 h exposure. The expression level of genes had a trendy increase after 48 h which may be attributed to resistance mechanisms to dssPLA$_2$. To sum up, dssPLA$_2$ can act as a suppressor of the MAPKs family by sequentially downstream signaling. Thus, suppression by dssPLA$_2$, both at mRNA and protein levels of MAPK cascade, could enhance the susceptibility of SK-MEL-28 cells to synergistic therapeutic application.

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