Biochemical and Biophysical Research Communications 441 (2013) 53–58

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

MST1 activation by curcumin mediates JNK activation, Foxo3a nuclear translocation and apoptosis in melanoma cells

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ARTICLE INFO

Article history:
Received 28 September 2013
Available online 14 October 2013

Keywords:
Curcumin
Melanoma
MST1
Apoptosis
JNK and Foxo3a

ABSTRACT

Different groups including ours have shown that curcumin induces melanoma cell apoptosis, here we focused the role of mammalian Sterile 20-like kinase 1 (MST1) in it. We observed that curcumin activated MST1-dependent apoptosis in cultured melanoma cells. MST1 silencing by RNA interference (RNAi) suppressed curcumin-induced cell apoptosis, while MST1 over-expressing increased curcumin sensitivity. Meanwhile, curcumin induced reactive oxygen species (ROS) production in melanoma cells, and the ROS scavenger, N-acetyl-cysteine (NAC), almost blocked MST1 activation to suggest that ROS might be required for MST1 activation by curcumin. c-Jun N-terminal protein kinase (JNK) activation by curcumin was dependent on MST1, since MST1 inhibition by RNAi or NAC largely inhibited curcumin-induced JNK activation. Further, curcumin induced Foxo3 nuclear translocation and Bim-1 (Foxo3 target gene) expression in melanoma cells, such an effect by curcumin was inhibited by MST1 RNAi. In conclusion, we suggested that MST1 activation by curcumin mediates JNK activation, Foxo3a nuclear translocation and apoptosis in melanoma cells.

1. Introduction

The incidence of malignant melanoma increases over the last 30 years in Caucasians, and it continues to be one of the leading causes of cancer-related deaths in the US and around the world [1,2]. Due to the fact that malignant melanoma cells are generally less response to traditional chemo-drugs [3–5], long-term survival of metastatic melanoma patients is dismal [1,3–5].

Curcumin, 1,7-bis(4-hydroxy-3methoxyphenyl)-1,6-heptadien-3,5-dione, is the primary bioactive component of turmeric, the dietary spice made from the rhizome of Curcuma longa [6]. It possesses wide-range anti-tumor properties [6], which are mainly attributed to its abilities to induce cancer cell apoptosis, and to inhibit cancer-related angiogenesis and/or inflammations [6]. Preclinical studies (including ours [7,8]) have confirmed that curcumin's dramatic anti-melanoma efficiency both *in vivo* and *in vitro* [6,9–11]. Several phase I and phase II clinical trials indicate that curcumin is quite safe and may exhibit therapeutic efficacy in patients with melanoma [9].

The underlying mechanisms of curcumin-induced tumor cell apoptosis remain to be explored [6,12]. It has been shown that curcumin induces tumor cell growth inhibition and apoptosis through regulation of multiple signaling pathways, including cell proliferation/survival pathways, apoptosis pathways, tumor suppressor pathway, and multiple protein kinase pathways [12]. Our previous studies have been focusing on how curcumin kills melanoma cells, and several mechanisms including c-Jun N-terminal protein kinase (JNK) activation [7], ceramide and reactive oxygen species (ROS) production [8,9,11] have been proposed.

MST1 (mammalian STE20-like kinase 1) is a serine/threonine kinase that is activated during apoptosis [13–15], which in turn activates its downstream pro-apoptotic targets including JNK, histone H2B and Foxo [13–15]. A number of stress inducers including chemo-drugs activates MST1-dependent cell apoptosis [16]. In the current study, we found that curcumin-induced melanoma cell apoptosis was also dependent on MST1 activation.

2. Materials and methods

2.1. Chemicals and reagents

N-acetyl-cysteine (NAC), antibodies of MST1 and Foxo3a were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Abbreviations: MST1, mammalian Sterile 20-like kinase 1; JNK, c-Jun N-terminal protein kinase; NAC, N-acetyl-cysteine; ROS, reactive oxygen species; IP, immunoprecipitation; RNAi, RNA interference; MTT, 3-(4,5-dimethyl-thiazol-2-yl)2,5-diphenyl tetrazolium bromide.

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http://dx.doi.org/10.1016/j.bbrc.2013.10.008

Curcumin and mouse monoclonal anti-tubulin antibody were obtained from Sigma (St. Louis, MO). Antibodies of phospho-MST1 (Thr 183), JNK1, c-Jun, phospho-JNK1/2 (Thr 183/Tyr 185), Bim-1, cleaved-caspase 3 and cleaved-caspase 9 were purchased from Cell Signaling Technology (Bevery, MA).

2.2. Cell culture

Melanoma cell lines B16 and WM-115 [7,8], as well as HEK-293 cells were maintained in a DMEM medium, supplemented with a 10% FBS (Sigma, St. Louis, MO), Penicillin/Streptomycin (1:100, Sigma) and 4 mmol/L L-glutamine (Sigma, St. Louis, MO), in a CO_2 incubator at 37 °C .

2.3. Cell survival assay

Cell survival was measured by 3-(4,5-dimethyl-thiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) dye assay as described [7,8]. Value of treatment group was expressed as percentage change vs. untreated control group.

2.4. Apoptosis assay

After treatment, melanoma cells were washed with cold PBS and incubated with 0.5 ml of Binding Buffer (10 mM HEPES, pH 7.4, 150 NaCl, 2.5 $CaCl_2$, and 1 mM $MgCl_2$, and 4% BSA), containing 3 μg/ml annexin V-FITC for 10 min. Cells were then washed with PBS and resuspended. A total of 20,000 cells of each sample were analyzed by flow cytometry in a FACS (Beckton Dickinson FACScan, Taibei, China). The percentage of Annexin V was recorded as apoptosis rate.

2.5. Reactive oxygen species (ROS) assay

ROS level was determined by concomitant increase in dichlorofluorescein fluorescence (DCFH-DA), according to protocol (Gene Research Lab, Taibei, China), as previous reported [17]. After indicated treatments, melanoma cells were stained with 10 μM of DCFH-DA for 30 min at 37 °C. Fluorescently stained cells were transferred to polystyrene tubes with cell-strainer caps (Falcon, Shanghai, China) and subjected to FACS, using Cell Quest 3.2 (Beckton Dickinson) software for acquisition and analysis. In each analysis, 20,000 events were recorded. Induction of ROS generation was expressed in arbitrary units (vs. Control).

2.6. Immunoblotting

Immunoblotting was performed according to previous protocol [7,8]. For detecting nuclear proteins, nuclear fractions of attached cells were isolated based on protocols of [18]. The band intensity was quantified through ImageJ software. The intensity of each phosphorylated kinase was normalized to the intensity of non-phosphorylated kinase. The value was expressed as folds vs. control group.

2.7. Immunoprecipitation (IP)

After treatment, 800 μg of cell lysates were pre-cleared with 20 μl of protein A/G PLUS-agarose (Santa Cruz Biotech) for 1 h. The supernatant was then rotated overnight with 2 μg of anti-MST1 (Santa Cruz Biotech). Next, lysates were centrifuged for 5 min at 4 °C in a micro-centrifuge to remove nonspecific aggregates. Protein A/G PLUS-agarose (35 μl) was then added to the supernatants for 4 h at 4 °C. Pellets were washed six times with PBS, resuspended in lysis buffer, and then assayed by immunoblotting.

2.8. Expression constructs

As previously reported [19], FLAG-tagged MST1 was cloned into a pCMV5 expression vector, and transfection was performed by Lipofectamine 2000 protocol according to instructions provided by supplier. The MST1 shRNA targeting sequence (5′-GGGCACTG TCCGAGTAGCAGC-3′ [19]) was commercially synthesized by Nanjing Genetech company (Nanjing, China), and was cloned into pSuper puro RNAi system (Addgene). Transfection was performed through Lipofectamine 2000, and stable cells was selected by puromycin. Control cells were transfected with scramble shRNA (Ctrl shRNA, addgene).

2.9. MST1 siRNA knockdown

MST1 expression was silenced using a pool of two small interfering RNAs (siRNAs) directed against the coding region of MST1

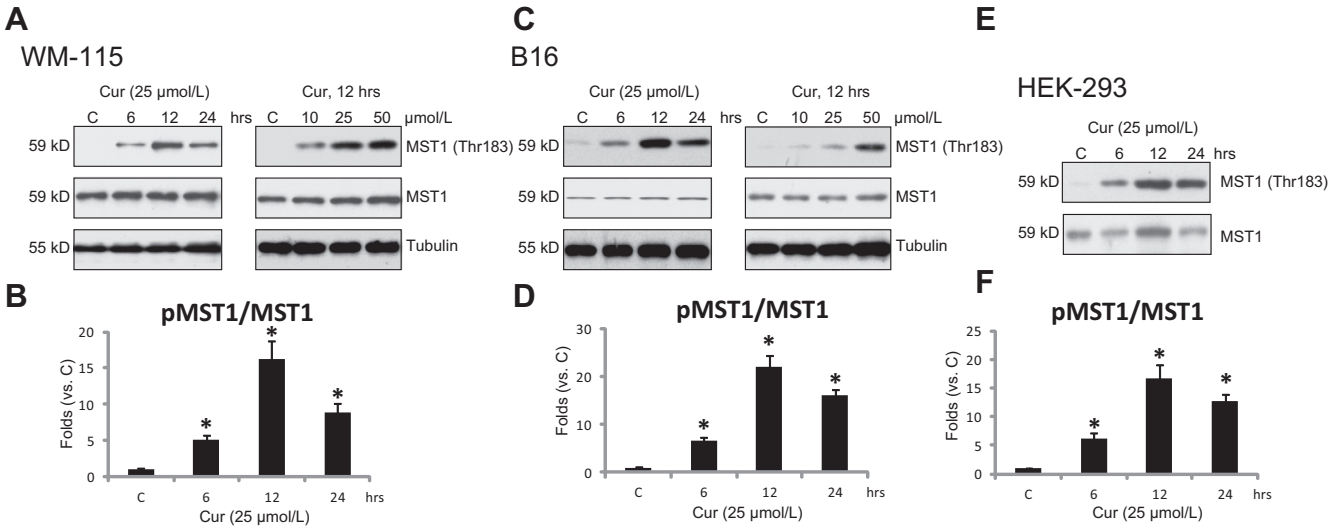

Fig. 1. Curcumin induces MST1 activation in melanoma cells. Representative immunoblots showing the expressions of phospho-MST1 (Thr 183), regular MST1 and tubulin in WM-115 (A), B16 (C) and HEK-293 (E) cells stimulated with curcumin (Cur) as indicated. Phospho-MST1 was quantified, and mean ± S.E. of three independent experiments were shown (B, D and E). *$p < 0.05$ vs. control group ("C").