Tanshinone IIA Induces Autophagic Cell Death via Activation of AMPK and ERK and Inhibition of mTOR and p70 S6K in KBM-5 Leukemia Cells

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Although tanshinone IIA (Tan IIA) from Salviae miltiorrhizae was known to induce apoptosis in various cancers, its underlying mechanism of autophagic cell death was not reported yet. Thus, in the present study, the molecular mechanism of autophagic cell death by Tan IIA was investigated in KBM-5 leukemia cells. Tan IIA significantly increased the expression of microtubule-associated protein light chain 3 (LC3) II as a hallmark of autophagy in western blotting and immunofluorescence staining. Tan IIA augmented the phosphorylation of adenosine monophosphate-activated protein kinase (AMPK) and attenuated the phosphorylation of mammalian target of rapamycin (mTOR) and p70 S6K in a dose-dependent manner. Conversely, autophagy inhibitor 3-methyladenine partly reversed the cytotoxicity and the phosphorylation of AMPK, mTOR and p70 S6K induced by Tan IIA in KBM-5 leukemia cells. In addition, Tan IIA dramatically activated the extracellular signal regulated kinase (ERK) signaling pathway including Raf, ERK and p90 RSK in a dose-dependent and time-dependent manner. Consistently, ERK inhibitor PD184352 suppressed LC3-II activation induced by Tan IIA, whereas PD184352 and PD98059 did not affect poly (ADP-ribose) polymerase cleavage and sub-G1 accumulation induced by Tan IIA in KBM-5 leukemia cells. Furthermore, Tan IIA could induce autophagy via LC3-II activation in various cancer cells such as prostate (PC-3), multiple myeloma (U266), lung (NCI-H460), and breast (MDA-MB-231) cells. Overall, these findings suggest that Tan IIA induces autophagic cell death via activation of AMPK and ERK and inhibition of mTOR and p70 S6K in KBM-5 cells as a potent natural compound for leukemia treatment. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: tanshinone IIA; autophagy; LC3 II; AMPK; ERK; mTOR; p70 S6K.

INTRODUCTION

Because cancer cells avoid programmed cell death and enhance cellular proliferation, induction of cancer cell death has been considered as a major target of cancer therapy (Hanahan and Weinberg, 2000). During the last decade, apoptosis (type I programmed cell death) has been focused as the broad concept of programmed cell death. In contrast, recent studies revealed the role of autophagy, a type II programmed cell death, in tumorigenesis, malignant tumor progression, and resistance of cancer therapy (Okada and Mak, 2004). Autophagy is a cellular process of self-digestion that involves lysosome-dependent recycling of intracellular components, whereas apoptosis is a process of self-killing that allows multicellular organisms to eliminate damaged cells (Maiuri et al., 2010; Mizushima et al., 2008). Autophagy can be activated by adenosine monophosphate-activated protein kinase (AMPK) (Poels et al., 2009; Wang and Guan, 2009) and negatively regulated by mammalian target of rapamycin (mTOR) (Kondo and Kondo, 2006) and mitogen-activated protein kinase (MAPK) signaling (Corcelle et al., 2007).

Salviae miltiorrhizae has been traditionally utilized for the treatment of angina pectoris (Zhou et al., 2005), hyperlipidemia (Wu et al., 2007), acute ischemic stroke (Cheng, 2007), and chronic renal failure (Wang et al., 2010) in Korea, China, and Japan. Several bioactive compounds such as tanshinone I, tanshinone IIA, cryptotanshinone, and dihydrotanshinone were isolated from the root of S. miltiorrhizae (Kim et al., 2008). Especially, tanshinone IIA (Tan IIA) has been reported to possess anticancer activity by inducing apoptosis and inhibiting angiogenesis and metastasis (Dong et al., 2007; Kim et al., 2011; Sung et al., 1999; Xu et al., 2012; Zhang et al., 2012). Nonetheless, the underlying mechanism of autophagic cell death by Tan IIA was not elucidated until now. Thus, in the present study, the molecular mechanism of autophagic cell death by Tan IIA was examined in KBM-5 leukemia cells mainly in association of AMPK/extracellular signal regulated kinase (ERK), mTOR and p70 S6K signaling pathways in KBM-5 leukemia cells.

MATERIALS AND METHODS

Chemicals. Tanshinone IIA (Tan IIA) with high purity (over 98%) was isolated from S. miltiorrhizae Bunge
and identified using several spectroscopy and mass spectrometry as previously described (Choi et al., 2004), and 3-methyladenine (3-MA) was obtained from Sigma Chemicals Co. (St. Louis, MO). PD98059 and PD184352 were purchased from Enzo Life Sciences (Plymouth Meeting, PA).

Cell culture. KBM-5 cells (human chronic myelogenous leukemia) were kindly provided by Dr Bharat B. Aggarwal (University of Texas MD Anderson Cancer Center, Houston, TX). U266 (multiple myeloma), PC-3 (prostate cancer), MBA-MB231 (breast cancer), and NCL-H460 (lung cancer) cells were purchased from American Type Culture Collection. The cells were maintained in Dulbecco’s modified Eagle’s media (Sigma Chemicals Co.) supplemented with 15% fetal bovine serum, 2-μM L-glutamine, and penicillin/streptomycin.

Immunofluorescence staining. KBM-5 cells were exposed to 80 μM of Tan IIA, plated onto poly-L-lysine coated slide glass, fixed in 4% (v/v) methanol-free formaldehyde solution (pH 7.4) at 4°C for 25 min and permeabilized in 0.2% (w/v) triton X-100, blocked in 5% (w/v) BSA, 0.5% (v/v) tween-20 in humidified chamber. The slides were incubated with anti-light chain 3(LC3) antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:100) followed by incubation with fluorescein isothiocyanate (FITC)-conjugated secondary antibody. LC3 expression was observed under an Olympus Fluoview FV10i confocal microscopy (Olympus, Tokyo, Japan).

Western blotting. Western blot analysis was performed as previously described (Won et al., 2012). KBM-5 cells were lysed in lysis buffer (50mM Tris–HCl, pH 7.4, 150-mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate 1-mM EDTA, 1-mM Na3VO4, 1-mM NaF, and protease inhibitors cocktail). The extracts were incubated on ice for 30 min, and supernatants were collected by centrifugation at 14,000 g at 4°C. Protein contents in the supernatant were measured by a Bio-Rad DC protein assay kit II (Bio rad, Hercules, USA). Proteins were separated by electrophoresis on 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrotransferred onto a Hybond enhanced chemiluminescence (ECL) transfer membrane (GE healthcare Biosciences, PA,USA) with transfer buffer (25-mM Tris, 250-mM glycine, and 20% methanol) at 300 mA for 90 min. The membrane was blocked in 5% nonfat skim milk and probed with primary antibodies for LC3-I/II (Santa Cruz Biotechnology), LC3-II, phospho-mTOR, phospho-p70 S6K, phospho-AMPKα, phosphor-ERK, phosphor-Raf, phosphor-p90 RSK (Cell Signaling, Danvers, MA), and β-actin (Sigma Chemicals Co.) followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Protein expression was detected by using enhanced chemiluminescence system (Amersham Pharmacia, Arlington Heights, IL).

Cytotoxicity assay. Cytotoxicity of Tan IIA was evaluated by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay as previously described (Kim et al., 2012). Cells were seeded onto 96-well microplates at a density of 2 × 10^4 cells per well, treated with various concentrations of Tan IIA (0, 10, 20, 40, or 80 μM) for 24 h. The cells were incubated with MTT (1 mg/mL) (Sigma Chemicals Co.) for 2 h. At the completion of incubation, cytotoxicity was assessed by adding 10 μL of MTT labeling reagent into each well and incubating at 37°C for 4 h. Reaction was stopped by adding solubilization solution and incubating the plates at 37°C overnight. Optical density was measured using a microplate reader ( Molecular Devices Co., Sunnyvale, CA) at 570 nm. Cell viability was calculated as a percentage of viable cells in Tan IIA-treated group versus untreated control by the following equation.

\[ \text{Cell viability} (\%) = \frac{\text{OD (Tan IIA)} - \text{OD(Blank)}}{\text{OD(Control)} - \text{OD(Blank)}} \times 100 \]

Cell cycle analysis. Cell cycle was analyzed as previously described (Ahn et al., 2010). KBM-5 cells were treated with Tan IIA for 24 h, collected and fixed in 70% ethanol. The fixed cells were then incubated at 37°C with 0.1% RNase A in phosphate-buffered saline (PBS) for 30 min and suspended in PBS containing propidium iodide (25 μg/mL) for 30 min at room temperature. The stained cells were analyzed for DNA content in FACS Calibur (Becton Dickinson, Franklin Lakes, NJ) using the CellQuest program (Becton Dickinson, Franklin Lakes, NJ).

Statistical analysis. All data were presented as means ± SD. Statistical significance was verified by Student’s t-test or analysis of variance using SigmaPlot software (Systat Software Inc., San Jose, CA).

RESULTS AND DISCUSSION

It is well documented that Tan IIA, as one of the most pharmacologically active phytochemical from S. miltiorrhiza (Yin et al., 2008; You et al., 2010), induces apoptosis in various cancer cells such as gastric (Dong et al., 2007), liver(Yuan et al., 2004), leukemia (Sung et al., 1999), and lung (Hu et al., 2005) cancers. Also, Tan IIA inhibits angiogenesis (Tsai et al., 2011) and metastasis (Jin et al., 2008) in cancer development. Recently, although Su et al. (2012) reported that Tan IIA inhibited human breast cancer MDA-MB-231 cells by decreasing LC3-II, Erb-B2, and NF-κBp65 in breast cancer cells, there was no report on the autophagic cell death of Tan IIA until now. Thus, in the current study, the underlying mechanism of Tan IIA to mediate autophagic cell death was examined in KBM-5 leukemia cells.

Autophagy is one of cell death phenomena (Mizushima et al., 2008). During autophagy, autophagosomes engulf cytoplasmic components, including cytosolic proteins and organelles. Concomitantly, LC3-I is conjugated to phosphatidyethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II), which is recruited to autophagosomal membranes(Tanida et al., 2008). Hence, we examined whether Tan IIA can induce autophagy in KBM-5 leukemia cells via conversion of LC3-I into LC3-II, a crucial step in autophagy (Kuma et al., 2007).
As shown in Fig. 1(A), Tan IIA increased the expression level of LC3-II that is involved in mature autophagosomes in a concentration-dependent manner, whereas LC3-I that is conjugated to phosphatidylethanolamine to form LC3-II was slightly decreased in our study, which was different from the results of Su et al. (2012) study. However, it needs further study to confirm the different effect of Tan IIA on LC3-I and LC3-II in different cell types and conditions in the future. To further confirm that Tan IIA induces autophagy, immunofluorescence staining was performed with LC3 antibody. Fluorescence microscopy showed a diffuse localization of FITC-labeled LC3 in the cytoplasm of untreated control (Fig. 1(B), left). In contrast, Tan IIA-treated cells revealed a punctuate pattern of FITC-LC3 fluorescence (Fig. 1(B), right). Conversely, 3-MA, an autophagy inhibitor, suppressed the cytotoxicity induced by Tan IIA (Fig. 2(A)), implying that the cytotoxicity by Tan IIA can be mediated through autophagy in KBM-5 cells.

Autophagy is stimulated by AMPK that regulates cellular metabolism to maintain energy homeostasis (Poels et al., 2009; Wang and Guan, 2009). Wang et al. (2010) described in their review paper that AMPK may act to inhibit tumorigenesis through regulation of autophagy as well as cell growth, cell proliferation, stress responses, and cell polarity (Wang and Guan, 2009). Also, mTOR, a key regulator of cell growth, is a negative inducer of autophagy (Kondo and Kondo, 2006). In this regard, Iwamaru et al. (2007) reported that...
rapamycin, an mTOR inhibitor, induced autophagy in U87-MG human malignant glioma cells. In addition, silencing of mTOR using siRNA transfection was known to enhance rapamycin-induced autophagy (Ravikumar et al., 2004). Likewise, Ravikumar et al. also reported that inhibition of mTOR induced autophagy and reduced the toxicity of polyglutamine expansions in fly and mouse models of Huntington disease (Iwamaru et al., 2007). Similarly, AMPK activator metformin blocked lymphoma cell growth via inhibition of mTOR pathway and induction of autophagy, indicating the co-relationship between AMPK and mTOR signaling in autophagy pathway (Shi et al., 2012). In this respect, western blotting revealed that Tan IIA increased the phosphorylation of AMPK and suppressed the phosphorylation of mTOR in KBM-5 cells (Fig. 2(B)). Furthermore, Tan IIA also decreased the phosphorylation of p70 S6K, a substrate of mTOR, in a concentration-dependent manner (Fig. 2(B)). Similarly, a natural compound fisetin has been reported to induce autophagy through the suppression of mTOR signaling pathway in prostate cancer cells (Suh et al., 2010). Here, we found that autophagy inhibitor 3-MA treatment blocked the phosphorylation of AMPK and dephosphorylation of mTOR and p70 S6K.

Figure 3. Tanshinone IIA (Tan IIA)-induced autophagy is induced by the activation of the extracellular signal regulated kinase (ERK)/Raf/p90 RSK signaling in KBM-5 cells. (A) Cells were treated with Tan IIA (0, 40, or 80 μM) for 24 h. Cell lysates were prepared and subjected to western blotting to detect phosphorylation of ERK, Raf, and p90 RSK. (B) Cells were treated with Tan IIA (80 μM) for 0, 6, 12, 18, and 24 h. Cell lysates were prepared and subjected to western blotting to detect phosphorylation of ERK, Raf, and p90 RSK. (C and D) Cells were treated with Tan IIA (80 μM) in the absence or presence of (MAPK/ERK) kinase (MEK) inhibitor PD184352 for 24 h. (C) Cell lysates were subjected to western blotting for light chain 3 (LC3)-II. (D) Cell lysates were subjected to western blotting to detect poly (ADP-ribose) polymerase (PARP) and phospho-ERK. (E) Cell cycle analysis was performed to determine the sub-G1 apoptotic DNA contents. Cells were treated with Tan IIA (80 μM) in the absence or presence of MEK inhibitors PD184352 or PD98059 for 24 h. After fixation in 75% ethanol, cells were stained with Propidium Iodide (PI) and analyzed by flow cytometry. Data represent means ± SD. **, p < 0.01 versus control.
induced by Tan IIA in KBM-5 cells (Fig. 2(C)), implying that Tan IIA induces autophagy through the AMPK/mTOR/p70 S6k signaling in KBM-5 cells.

The ERK pathway plays an important role to regulate cell proliferation, differentiation, survival, and cell death (Seger and Krebs, 1995; Yang et al., 2006). Regarding the cell death in cancer, ERK activation can mediate apoptosis and/or autophagy depending on the cell types and stimuli (Subramaniam and Unsicker, 2006). For instance, Cha et al. (2012) reported that allicin inhibits cell growth and induces apoptosis in U87MG human glioblastoma cells through an ERK-dependent pathway. Likewise, Chen et al. (2012) reported that targeting cathepsin S induced autophagy via the epidermal growth factor receptor-ERK signaling pathway. Also, Choi et al. (2012) reported that p38 MAPK/ERK signaling regulated autophagy and apoptosis through the dual phosphorylation of glycogen synthase kinase 3β. Interestingly, there are accumulating evidences that the ERK pathway mediates autophagy induced by several natural compounds such as β-carboline alkaloids (Abe et al., 2011), pheophorbide-a (Bui-Xuan et al., 2010), concanavalin A (Li et al., 2011), and aloe emodin (Mijatovic et al., 2005) in cancer cells.

In the current study, a dramatic increase in the phosphorylation of ERK was observed in Tan IIA-treated KBM-5 cells in concentration-dependent and time-dependent manners (Fig. 3(A) and (B)). Tan IIA also significantly increased the phosphorylation of Raf as an upstream of ERK and p90RSK as a downstream of ERK, in concentration-dependent and time-dependent manners (Fig. 3(A) and (B)). MAPK/ERK kinase inhibitor PD184352 suppressed LC3-II activation induced by Tan IIA (Figure 3(C)), whereas PD184352 and PD98059 did not affect poly (ADP-ribose) polymerase cleavage (Fig. 3(D)) and sub-G1 accumulation (Fig. 3(E)) induced by Tan IIA in KBM-5 leukemia cells, indicating that Tan IIA induces autophagic cell death through the ERK signaling pathway in KBM-5 cells. Finally, we examined whether Tan IIA can mediate autophagy in other types of cancer cells such as PC-3 (prostate cancer), U266 (multiple myeloma), NCI-H460 (lung cancer), and MDA-MB-231 (breast cancer). As shown in Fig. 4, LC3-II level was increased in PC-3 (A), U266 (B), and NCI-H460 (C) cells by Tan IIA in a concentration-dependent manner, whereas LC3-II was slightly increased in MDA-MB-231 cells at 40 μM of Tan IIA (Fig. 4(D)).

In summary, our findings demonstrate that Tan IIA can induce autophagic cell death by the phosphorylation of AMPK and dephosphorylation of mTOR in KBM-5 leukemia cells. Furthermore, Tan IIA-induced autophagy is associated with the activation of the Raf/ERK/p90 RSK signaling. To date, although the predominant type of Tan IIA-induced programmed cell death is apoptosis in leukemia (Sung et al., 1999), we suggest that autophagic cell death can be partly induced by Tan IIA in KBM-5 leukemia cells.

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The authors declare no competing interests.


