MOLECULAR ONCOLOGY 6 (2012) 392–404

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Co-administration phenoxodiol with doxorubicin synergistically inhibit the activity of sphingosine kinase-1 (SphK1), a potential oncogene of osteosarcoma, to suppress osteosarcoma cell growth both *in vivo* and *in vitro*

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ARTICLE INFO

Article history:
Received 21 January 2012
Received in revised form
23 April 2012
Accepted 27 April 2012
Available online 5 May 2012

Keywords:
Sphingosine kinase-1
Osteosarcoma
Doxorubicin
Phenoxodiol
Chemotherapy
Signal transduction

ABSTRACT

Elucidation of the mechanisms of chemo-resistance and implementation of strategies to overcome it will be pivotal to improve the survival for osteosarcoma (OS) patients. We here suggest that sphingosine kinase-1 (SphK1) might be the key factor contributing to chemo-resistance in OS. Our Western-blots and immunohistochemistry results showed that SphK1 is over-expressed in multiple clinical OS tissues. Over-expression of SphK1 in OS cell line U2OS promoted its growth and endorsed its resistance against doxorubicin, while knocking-down of SphK1 by shRNA inhibited U2OS cell growth and increased its sensitivity to doxorubicin. Co-administration phenoxodiol with doxorubicin synergistically inhibited SphK1 activity to trigger cellular ceramide accumulation, and achieved synergistic anti-OS growth effect, accompanied with a significant increased of apoptosis and cytotoxicity. Increased cellular level of ceramide by the co-administration induced the association between Akt and Protein Phosphatase 1 (PP1) to dephosphorylate Akt, and to introduce a constitutively active Akt (CA-Akt) restored Akt activation and diminished cell growth inhibition. Further, phenoxodiol and doxorubicin synergistically activated apoptosis signal-regulating kinase 1(ASK1)/c-jun-NH2-kinase (JNK) signaling, which also contributed to cell growth inhibition. Significantly, the role of SphK1 in OS cell growth and the synergistic anti-OS effect of phenoxodiol and doxorubicin were also seen in a mice OS xenograft model. In conclusion, our data suggest that SphK1 might be a critical oncogene of OS and co-administration phenoxodiol with doxorubicin synergistically inhibited the activity of SphK1 to suppress osteosarcoma cell growth both *in vivo* and *in vitro*.

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Abbreviation: SphK1, sphingosine kinase-1; SKI-II, SphK1 inhibitor II; PARP, poly ADP ribose polymerase; OS, osteosarcoma; ASK1, apoptosis signal-regulating kinase 1; JNK, c-jun-NH2-kinase; PP1, protein phosphatase 1.

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doi:10.1016/j.molonc.2012.04.002

1. Introduction

For patients with osteosarcoma (OS), the use of chemotherapy has significantly improved survival from 11% with surgical resection alone in the 1960s, to 70% by the mid-1980s (Chou and Gorlick, 2006). Since then, despite significant advances in chemotherapies against OS in the past a few years, the survival of OS patients has not been much improved due to chemoresistance and other factors. As such, elucidation of the mechanisms of chemoresistance and implementation of strategies to overcome chemoresistance will be pivotal to improving survival for these patients.

The principal plant hormones flavonoids are known to possess diverse functional roles and to regulate plant cell apoptosis and cell cycle (Kandaswami et al., 2005). Many plant flavonoids show similar functional effects in animal and human cells, and are shown to be effective in inducing mitotic arrest and cell apoptosis in a number of cancer cells. Genistein, among all the flavonoids, has been proved to exert many anti-tumor effects (Kamsteeg et al., 2003; Lamartiniere et al., 1998), arguing that this molecule may offer novel approaches to cancer therapy (Banerjee et al., 2008; Sarkar and Li, 2003). Genistein is well tolerated in human, however, the clinical development of this agent has been compromised by the fact of its inability to achieve adequate plasma concentrations (Miltyk et al., 2003; Takimoto et al., 2003). As such, synthetic analogs of genistein are being developed for better anti-tumor efficacy. Of these genistein analogs, phenoxodiol is being assessed in several clinical studies against a range of cancer types and was shown to have a much better anticancer potency and efficacy in preclinical models and is less susceptible to metabolism compared with Genistein (Mor et al., 2006; Silasi et al., 2009).

The lipid kinase sphingosine kinase 1 (SphK1) catalyzes the phosphorylation of sphingosine to sphingosine-1-phosphate (S1P) (Shida et al., 2008; Vadas et al., 2008). *In vivo* and *in vitro* studies have proven that SphK1 is associated with cancer cell survival, proliferation, transformation, and prevention of apoptosis, the chemoresistance and angiogenesis (Shida et al., 2008; Vadas et al., 2008). Evidence from clinical samples demonstrates that SphK1 is over-expressed in many tumor types and that inhibitors of SphK1 may sensitize tumors to chemotherapeutic agents (Shida et al., 2008; Vadas et al., 2008). However, at least to our knowledge, the potential role of SphK1 in OS is largely missing. Though phenoxodiol is generally not known as a SphK1 specific inhibitor, phenoxodiol's major action, however, is believed to be blocking the activation of SphK1 (Gamble et al., 2006) (also see discussion in Shida et al., 2008). Our study here suggests that SphK1 might be a critical oncogene of OS and co-administration phenoxodiol with doxorubicin synergistically inhibited the activity of SphK1 to suppress osteosarcoma cell growth.

2. Materials and methods

2.1. Reagents

Phenoxodiol, doxorubicin, fumonisin B1, N-dimethylsphingosine, SKI-II and SP 600125 were obtained from Sigma (Sigma, St. Louis, MO); Anti-SphK1 (M-209, sc-48825), AKT1, tubulin, rabbit IgG-HRP and mouse IgG-HRP antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). p-SphK1 (Ser 225) antibody was obtained from Antibodies Online (ABIN265165, Shanghai, China). All other antibodies used in this study were purchased from Cell Signaling Technology (Beverly, MA).

2.2. Cell culture

Human osteosarcoma cell lines U2OS, MG-63, and SaOs-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) containing 10% fetal calf serum (Sigma, St. Louis, MO), 2 mmol/L L-glutamine, and 100 mg/mL penicillin/streptomycin (Sigma, St. Louis, MO).

2.3. Live cell counting by trypan blue staining

Live OS cells after indicated treatment/s were determined by trypan blue staining assay and the % of live cell was calculated by the number of the trypan blue stained cells of treatment group divided by that of untreated control group.

2.4. Cell viability assay (MTT assay)

Cell viability was measured by the 3-[4,5-dimethylthylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) method. Briefly, cells were collected and seeded in 96-well plates at a density of 4×10^5 cells/ml. 20 μl of MTT tetrazolium salt (Sigma, St. Louis, MO) dissolved in PBS at a concentration of 5 mg/ml was added to each well with indicated treatment and incubated in CO_2 incubator for 3 h at 37 °C. 150 μl of DMSO (Sigma, St. Louis, MO) was added to dissolve formazan crystals and the absorbance of each well was observed by a plate reader at a test wavelength of 490 nm.

2.5. Clonogenicity assay

U2OS cells (5×10^4) were suspended in 1 ml of DMEM containing 1% agar (Sigma, St. Louis, MO), 10% FBS and with indicated treatment/s or vehicle controls. The cell suspension was then added on top of a presolidified 1% agar in a 100 mm culture dish. The medium was replaced every 2 days. After 8 days of incubation, colonies were photographed at 4×. Colonies larger than 50 μm in diameter were quantified for number using Image J Software.

2.6. Western blotting

Cells were washed with ice-cold PBS, scraped into PBS, and collected by centrifugation. Pellets were re-suspended in a lysis buffer containing 50 mmol/L HEPES, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 10% glycerol, 0.5% NP-40, 0.5% Tween 20, 1 mmol/L dithiothreitol, and protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and vortexed for 20 min at 4 °C; insoluble material was removed by centrifugation. Proteins (30 μg) were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated sequentially in TBS containing 0.05% Tween-20

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