EBioMedicine 19 (2017) 49–59

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Research Paper

Fluvastatin Prevents Lung Adenocarcinoma Bone Metastasis by Triggering Autophagy

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ARTICLE INFO

Article history:
Received 11 December 2016
Received in revised form 6 April 2017
Accepted 10 April 2017
Available online 11 April 2017

Keywords:
Fluvastatin
Autophagy
Lung adenocarcinoma bone metastasis
Statins
p53

ABSTRACT

Bone is one of the most preferred sites of metastasis in lung cancer. Currently, bisphosphonates and denosumab are major agents for controlling tumor-associated skeletal-related events (SREs). However, both bisphosphonates and denosumab significantly increase the risk for jaw osteonecrosis. Statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors and the most frequently prescribed cholesterol-lowering agents, have been reported to inhibit tumor progression and induce autophagy in cancer cells. However, the effects of statin and role of autophagy by statin on bone metastasis are unknown. In this study, we report that fluvastatin effectively prevented lung adenocarcinoma bone metastasis in a nude mouse model. We further reveal that fluvastatin-induced anti-bone metastatic property was largely dependent on its ability to induce autophagy in lung adenocarcinoma cells. *Atg5* or *Atg7* deletion, or 3-methyadenine (3-MA) or Bafilomycin A1 (Baf A1) treatment prevented the fluvastatin-induced suppression of bone metastasis. Furthermore, we reveal that fluvastatin stimulation increased the nuclear p53 expression, and fluvastatin-induced autophagy and anti-bone metastatic activity were mostly dependent on p53.

1. Introduction

Lung cancer is one of the most common cancers worldwide (Siegel et al., 2011; Kuo et al., 2013). Metastasis, a critical step in cancer progression, indicates a more advanced cancer stage and poorer prognosis, and bone is a frequently preferred site of metastasis for lung cancer. Around 30–40% of patients with advanced lung cancer may develop bone metastasis leading to a dramatic increase in mortality and rapid reduction in quality of life (Rove and Crawford, 2009).

For patients with bone metastases, current treatments are designed to decrease tumor burden, prevent further progression and metastasis and inhibit tumor-associated skeletal-related events (SREs), such as pathologic fracture and spinal cord compression. Individual lesions can be surgically excised or irradiated either before or after the surgery (Suva et al., 2011). Moreover, bisphosphonates are most frequently used agents for cancer patients with bone metastases. Bisphosphonates, synthetic analogs of pyrophosphate, bind to hydroxyapatite and trigger apoptosis in osteoclasts after internalization (Lopez-Olivo et al., 2012). Denosumab, a fully human anti-RANKL monoclonal antibody, is an FDA-approved agent (2010) for the prevention of SREs in patients with solid tumors and bone metastases. Compared with bisphosphonates, denosumab may longer delay the onset of a first SRE (Scagliotti et al., 2012; Fizazi et al., 2011). However, the worst side effect of either treatment is significant increase in the risk of osteonecrosis in the jaw. Other side effects may include pyrexia, joint and muscle pain, increased risk of infections, such as cellulitis and hypocalcemia (Taylor et al., 2010; McLeod et al., 2012; Bamias et al., 2005). These side effects, especially the risk for jaw osteonecrosis, greatly restrict the use of

Abbreviations: SREs, skeletal-related events; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; 3-MA, 3-methyadenine; Baf A1, Bafilomycin A1; MICA, MHC class I chain-related protein A; CQ, chloroquine; PFTα, pifithrin-α; ACC, acetyl-CoA carboxylase; ECM, extracellular matrix; BLI, bioluminescence imaging.

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http://dx.doi.org/10.1016/j.ebiom.2017.04.017

these therapeutic agents to control bone metastasis. Therefore, there is an urgent need to develop novel anti-bone metastasis agents with high efficacy and minimal side effects.

Statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, include lovastatin, simvastatin, mevastatin, fluvastatin, pravastatin, atorvastatin, rosuvastatin and cerivastatin. Statins have been widely used for lowering cholesterol, but recent research reveal other roles, such as anti-inflammation, immunomodulation, neuroprotection and antitumor effects (Zhang et al., 2013).

During the last decade, the role of statins in cancer prevention or therapy has been controversial, but accumulating evidence based on retrospective analyses support that statins could be beneficial to patients with certain types of tumors (Nielsen et al., 2012; Singh et al., 2013; Hamilton et al., 2014). In addition, numerous experimental studies have revealed that statins have a role in inhibiting tumor growth and metastasis (Campbell et al., 2006; Clendening and Penn, 2012; Hindler et al., 2006). However, the underlying mechanisms are complex, and the working hypothesis can be divided into Rho GTPase dependent and independent mechanisms. Rho GTPases are considered to be important signaling molecules that drive tumor growth and metastasis. Statins may inhibit Rho GTPase activation by interfering with mevalonate pathway and Rho geranylgeranylation, a key posttranslational modification for Rho GTPase biological activities (Collisson et al., 2003; Kusama et al., 2001; Tsubaki et al., 2015). Furthermore, statins involve the innate immune response against human metastatic melanoma cells by inducing MHC class I Chain-related protein A (MICA) membrane expression. As a result, independent of Ras and Rho GTPase signaling pathways, melanoma cells are more sensitive to lysis by NK cells (Pich et al., 2013). In addition, simvastatin may prevent triple-negative breast cancer metastasis via regulation of FOXO3a (Wolfe et al., 2015).

Although statins may trigger autophagy in a few cancer cells through inhibition of geranylgeranylation (Araki et al., 2012; Yang et al., 2010; Parikh et al., 2010), whether statin-induced autophagy plays a role in promotion or suppression of cancer metastasis is largely unknown. Only few in vitro studies showed that inhibition of autophagy may enhance statin-induced apoptosis or cytotoxicity in some cancer cell lines (Yang et al., 2010; Misirkic et al., 2012; Kang et al., 2014). The role of autophagy in tumorigenesis and progression is quite complicated, because induction of autophagy could either accelerate the tumor progression or suppression (Zhang et al., 2013; Su et al., 2015a). Many studies emphasize cancer stages, cancer types and tumor microenvironments as the determining factors. In our current study, we validate that statins prevent lung adenocarcinoma bone metastasis though triggering autophagy. Moreover, we propose a mechanism by which autophagy serves a positive role in suppression of cancer bone metastasis.

p53, the most well-known tumor suppressor, serves a critical role in safeguarding the integrity of the genome. After activation by intracellular stresses, such as DNA damage, hypoxia and oncogene activation, p53 initiates a series of cell events including apoptosis, cell cycle arrest and DNA repair to suppress tumorigenesis (Jin, 2005). In recent years, p53 has been revealed to play an important role in either inducing or suppressing autophagy depending on its subcellular localization. Specifically, nuclear p53 acts as a transcription factor to activate a series of pro-autophagic genes; cytoplasmic p53 serves a role in repressing autophagy via unclear mechanisms (Maiuri et al., 2010; Tasdemir et al., 2008). In this study, we investigate the involvement of p53 in both fluvastatin-induced autophagy and suppression of lung adenocarcinoma bone metastasis and determine the signaling pathway bridging p53 to autophagy.

2. Materials & Methods

2.1. Reagents

Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotech, China); Lipofectmin 2000 (Invitrogen, USA); Entranster™-in vivo transfection reagent (Engreen Biosystem, China); pifithrin-α (Sigma, USA); fluvastatin (Novartis Pharma, Switzerland); 3-methyadenine (Sigma, USA); Bafilomycin A1 (Santa Cruz, CA, USA), chloroquine (MedChemExpress, USA), denosumab (Prolia, Amgen Inc., USA); mouse-anti-AMPKα1/2 (sc-74461, Santa Cruz, USA); rabbit-anti-pAMPKα1/2 (Thr 172) (sc-33524, Santa Cruz, USA); rabbit-anti-ACCα (sc-30212, Santa Cruz); rabbit-anti-ACCα (sc-271965, Santa Cruz); mouse-anti-PTEN (sc-7974, Santa Cruz); rabbit-anti-AKT (sc-8312, Santa Cruz); rabbit-anti-pAKT (Ser 473) (sc-33437, Santa Cruz); rabbit-anti-Histone H3 (sc-10809, Santa Cruz); mouse-anti-p53 (sc-126, Santa Cruz); rabbit-anti-LC3 (sc-28266, Santa Cruz); rabbit-anti-mTOR (sc-8319, Santa Cruz); rabbit-anti-mTOR (Ser 2448) (sc-101738, Santa Cruz); mouse-anti-β-Actin (sc-47778, Santa Cruz).

2.2. Cells

293 T cells and the human lung adenocarcinoma cell line A549 were obtained from the ATCC and cultured with DMEM (Invitrogen, USA) supplemented with 10% fetal bovine serum (Hyclone, USA). Human lung adenocarcinoma cell line SPC-A-1 was purchased from the Cell Resource Centre of Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences and cultured with RPMI 1640 medium (Invitrogen, USA) supplemented with 10% fetal bovine serum. Luciferase-expressing SPC-A-1 cells were established in our lab by a stable transfection with pCMV-G Luc 2 plasmid (New England Biolabs, USA). *Atg5*$^{-/-}$ and *Atg7*$^{-/-}$ SPC-A-1 cells were established by CRISPR/Cas9 system (Inovogen, Beijing, China). Exon 2 of *Atg5* and exon 1 of *Atg7* were selected for design of guide RNA. Guide RNA sequences: Atg5: 5′-TGCTTCGAGATGTGTGGTT-3′; Atg7: 5′-AAGCTGAACGAGTATCGGC-3′.

2.3. Wound Healing Assay

Briefly, after seeding A549 or SPC-A-1 cells in 12-well plates, media was changed to 0.1% FBS DMEM before scratching. Scratch wound was generated with a 10 μl pipette tip, and then cells were incubated with 10 μM fluvastatin or combined with other drugs at different time points. Photographs were taken at indicated time points. The percentage of wound healing was calculated using the Image J software. Wound healing rate (%) = (initial wound area − non-healing area)/initial wound area.

2.4. Matrigel Invasion Assay

The Matrigel invasion assay was done using the BD Biocoat Matrigel Invasion Chamber (pore size: 8 μm, 24-well; BD Biosciences) and following the manufacturer's protocol. Cells (5×10^4) were plated in the upper chamber in serum-free medium with 10 μM fluvastatin or other drugs. The bottom chamber contained medium with 10% FBS. After indicated times, the cells that had invaded through the membrane to the lower surface were fixed, stained with 0.1% Crystal Violet and counted by microscopy.

2.5. Bioluminescence Imaging (BLI)

Mice were inoculated with 5×10^6 luciferase-transfected SPC-A-1 cells and then treated with different drugs for 3 weeks. At the end of treatment, mice were injected (i.p.) with 100 μl of the D-luciferin solution (150 mg/kg) (Gold Biotechnology, St. Louis, MO). During the image acquisition, mice were anesthetized with 50 mg/kg pentobarbital sodium (i.p.) and then imaged in a dorsal position using the Xenogen IVIS Spectrum system (Caliper Life Sciences, USA). Signal intensity in both left and right hind limbs was quantified as photons flux (photons/s/cm^2/sr) using Living Image software 4.2 (Caliper Life Sciences) as described previously (Hu et al., 2011).