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Enhanced mitochondrial biogenesis contributes to Wnt induced osteoblastic differentiation of C3H10T1/2 cells

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ABSTRACT

Mitochondria play a key role in cell physiology including cell differentiation and proliferation. We investigated the changes of mitochondrial biogenesis during Wnt-induced osteoblastic differentiation of murine mesenchymal C3H10T1/2 cells. Scanning electron microscopy demonstrated that activation of Wnt signaling by Wnt-3A conditioned medicum (CM) resulted in significant increase in the number of mitochondria in C3H10T1/2 cells. In addition, the induction of alkaline phosphatase (ALP) activities by Wnt-3A CM was accompanied by significant increase in mitochondrial mass ($p<0.05$), mitochondrial membrane potential ($p<0.05$), intracellular reactive oxygen species production ($p<0.05$), resting oxygen consumption rate ($p<0.05$), cellular ATP content ($p\leq 0.05$) and mtDNA copy number ($p<0.05$) compared to the cells with control CM (L292-CM) treatment. Moreover, co-treatment with Dkk-1 or WIF-1, both of which are Wnt inhibitors, abrogated the Wnt-3A-induced ALP activities as well as mitochondrial biogenesis markers. Upregulation of mitochondrial biogenesis by overexpression of mitochondrial transcription factor A (Tfam) significantly enhanced Wnt-induced osteogenesis as measured by ALP activities. In contrast, inhibition of mitochondrial biogenesis by treatment with Zidovudine (AZT) resulted in significant inhibition of ALP activities. Finally, ALP activities in human osteosarcoma cell line devoid of mitochondrial DNA (ρ^0 cells) was significantly suppressed both in basal and Wnt-3A stimulated state compared to those from mitochondria-intact cells (ρ^+ cells). As a mechanism for Wnt-mediated mitochondrial biogenesis, we found that Wnt increased the expression of PGC-1α, a critical molecules in mitochondrial biogenesis, through Erk and p38 MAPK pathway independent of β-catenin signaling. We also found that increased mitochondrial biogenesis is in turn positively regulating TOPflash reporter activity as well as β-catenin levels. To summarize, mitochodrial biogenesis is upregulated by Wnt signaling and this upregulation contributes to the osteoblastic differentiation of mouse mesenchymal C3H10T1/2 cells.

Introduction

Wnt signaling plays an important role in development and maintenance of many tissues and organs, including osteogenesis. Wnt promotes osteoblastic differentiation, proliferation, and mineralization activities and reciprocally repress alternative mesenchymal differentiation pathways such as adipocyte [1] or chondrocyte differentiation [2]. Binding of Wnt to the frizzled receptor and LRP5/6 coreceptor of cell membrane, inhibits glycogen synthase kinase-3β (GSK-3β) activity, which leads to accumulation of β-catenin in the cytosol with subsequent translocation into the nucleus (for review see ref. [3]). Translocated β-catenin binds to Tcf/Lef transcription factors to activate target genes [4]. A gain-of-function mutation at the LRP5 (G171V) has been shown to result in high bone mass phenotype [5], whereas non-sense or frame shift mutation of LRP5 leads to autosomal recessive osteoporosis–pseudoglioma syndrome with increased risk of fracture [6]. In genetically modified mice, same pattern of high or low bone mass phenotype has been reproduced, using transgenic mice expressing LRP5 (G171V) [7] or knock-out model that lacks LRP5 gene [8]. Recently, it has been demonstrated that β-catenin together with TCF proteins also regulates osteoclast differentiation by regulating the expression of osteoprotegerin, a major inhibitor of osteoclast differentiation [9].

Mitochondria are organelles often regarded as the cell's energy powerhouses, because they generate a majority of ATPs required for cell metabolism using specialized electron transport system. In addition, mitochondria play an essential role in cell proliferation,

Abbreviations: ALP, alkaline phosphatase; CM, conditioned medium; DCF, dichlorofluorescein; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; GSK-3β, glycogen synthase kinase-3β; mtDNA, mitochondrial DNA; Tfam, mitochondrial transcription factor A; NAO, nonyl acridine orange; HIV, human immunodeficiency virus; ROS, reactive oxygen species; TMRE, tetramethylrhodamine ethyl ester; TEM, transmission electron microscopy; AZT, Zidovudine.

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differentiation, and apoptosis [10]. Mitochondria have their own genomic system, mitochondrial DNA (mtDNA), a 16.5 kb closed-circular double stranded DNA. Mitochondrial biogenesis is highly orchestrated by the transcriptional regulatory circuits between this mtDNA and the genes encoded by the nucleus [11,12]. Dynamic changes in mitochondrial biogenesis were observed during spontaneous differentiation of human embryonic stem cells by us [13] and other groups [14,15].

A number of studies suggested the possibility of interaction between Wnt signaling pathway and mitochondria. First of all, GSK-3β has been shown to be localized to mitochondria [16,17]. In addition, treatment with lithium chloride, an inhibitor of GSK-3β enzymatic activity, not only activated mesenchymal precursors to differentiate into osteoblast [18–21], but also increased mitochondrial biogenesis [22]. It has been also shown that the activation of the Wnt/β-catenin signaling pathway inhibits mitochondria-mediated apoptosis in colorectal cancer cell line [23].

Since Wnt signaling is one of the key pathways in osteoblastic differentiation, we hypothesized that mitochondria and its biogenesis may play a role in Wnt-induced osteoblastic differentiation of mesenchymal progenitors. In support of our notion are a number of epidemiological studies showing a high prevalence of osteopenia and osteoporosis in Human Immunodeficiency Virus (HIV)-infected patients receiving anti-retroviral therapy, which has toxic side effect on mitochondria [24,25]. Moreover, osteoblastic differentiation from fetal rat calvarial cells has been shown to be associated with increased mitochondrial biogenesis [26]. In this study, we investigated the changes of mitochondrial biogenesis during Wnt-induced osteoblastic differentiation from mesenchymal progenitor cells using a murine mesenchymal C3H10T1/2 cell line.

Materials and methods

Materials

The nonyl acridine orange (NAO), tetramethylrhodamine ethyl ester (TMRE) and 20, 70-dichlorofluorescein diacetate (DCFDA) dye which are the fluorescent probe to measure mitochondrial biogenesis were purchased from Invitrogen Corp (Carlsbad, CA, USA). Random priming kit and reagents for the luciferase assay were obtained from Promega Corp. (Madison, WI, USA) and Lipofectamine Plus Reagent were from Invitrogen Corp (Carlsbad, CA, USA). Antibody against mitochondrial transcription factor A (Tfam) was purchased form Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti β-catenin, Erk, phospho-Erk, p38, and phospho-p38 antibodies were purchased form Cell Signaling Technology (Beverly, CA, USA). Zidovudine (AZT), an antiretroviral agent that specifically blocks mitochondrial biogenesis was purchased from Biotrend Chemical (Cologne, Germany). MAPK inhibitors SB203580 (p38 inhibitor) and PD98059 (Erk inhibitor) were purchased from Calbiochem (Boston, MA, USA). All other chemicals, including cell culture media, were from Sigma Chemical (St Louis, MO, USA), unless otherwise indicated.

Cell culture and preparation of conditioned medium

Murine embryonic mesenchymal C3H10T1/2 (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C. A human osteosarcoma cells (143B.TK$^-$, ρ^+) and cells completely devoid of mitochondrial DNA (mtDNA) by exposure to 10 ng/ml ethidium bromide for 2 weeks (143B.206ρ^0) [27] were kindly provided by Dr. Yau-Huei Wei (National Yang-Ming University, Taiwan) and were cultured in DMEM with 10% FBS and 50 μg/ml uridin was additionally supplemented to ρ^0 cell. Wnt-3A conditioned medium (CM) were collected from L Wnt-3A cells [28] which secrete biologically active mouse Wnt-3A. L Wnt-3A cells were cultured in DMEM with 10% FBS, 4 mM L-glutamine supplemented with 0.4 mg/ml G-418, upon reaching 80% confluence. Then, cells were transferred into DMEM containing 2% FBS, 1× penicillin/streptomycin, and 4 mM L-glutamine, and 1-day past confluence, supernatant was collected. It has been previously shown that 1× Wnt-3A CM obtained from these cells are approximately 5 nM [29]. For osteoblastic differentiation, cells were cultured with 0.25× Wnt-3A CM for 72 h. As a control, cells were cultured with 0.25× L 292 CM which was collected from L 292 cells. Dkk-1 or WIF-1 CM was collected from CHO cells that were transduced with retrovirus encoding Dkk-1 (pMSCV-Dkk-1-IRES-GFP) or WIF-1 (pMSCV-WIF-1-IRES-GFP), respectively [30]. CHO cells were grown in DMEM F12 with 10% FBS. Upon reaching 80% confluence, cells were transferred into DMEM F12 with 10% FBS. Each CM was collected 3-day past confluence and we refer to the concentration of this media as 1×. To inhibit Wnt signaling pathways, cells in 0.25× Wnt-3A CM were co-treated with 0.25× Dkk-1 CM or WIF-1 CM and to suppress mitochondrial biogenesis, co-treated with 10 μM AZT for 72 h. Adipogenic differentiation was initiated 24–48 h after confluence by treatment with 5 μg/ml insulin, 0.1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 50 μM indomethacin for 4 days.

Constructs

Expression vector for human Tfam (pcDNA3-Tfam) was kindly provided by Dr. Youngmi Kim Pak (Kyung Hee University, Korea) and have been described [31,32]. β-catenin adenovirus (Ad-β-catenin) and GFP adenovirus (Ad-GFP) were obtained from Dr. Hyo Soo Kim (Seoul National University, Korea) [33]. TOPflash plasmid was provided by Dr. Roberto Civitellli (Washington University, St. Louis).

Alkaline phosphatase assay

The degree of osteoblastic differentiation was measured by alkaline phosphatase (ALP) activities with ELISA and ALP staining. To assess ALP activities with ELISA, cells in 24-well plates were washed three times with ice-cold PBS, scraped after adding 0.5% Triton X-100 and centrifuged. Enzyme activity assay was performed in assay buffer (10 mM $MgCl_2$ and 0.15 M alkaline buffer, pH 10.3) with 10 mM p-nitrophenyl phosphate as substrate. After adding 3 M NaOH solution to stop the reaction, absorbance was read by an ELISA reader (ThermoMax; Scientific Surplus) at OD_{405}. Relative ALP activity is defined as mmol of *p*-nitrophenol phosphate hydrolyzed per minute per mg of total protein. For ALP staining, cells in 12-well plates were fixed with 4% paraformaldehyde, and then stained with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT).

Measurement of mitochondrial mass

Mitochondrial mass was measured by NAO. Medium was aspirated from 96-well microplates, and 10 mM NAO in 200 μl phenol red-free medium was added and incubated at room temperature for 30 min. Mitochondria uptake NAO independent of mitochondrial transmembrane potential, then it binds specifically to cardiolipin on the mitochondrial inner membrane. Therefore, NAO uptake reflects mitochondrial mass [34–37]. The fluorescence intensity was measured by fluorescent microplate reader (Wallac Victor3V 1420 Multilabel Counter; Perkin-Elmer) at ex/em 485 nm/535 nm.

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was measured by TMRE. Medium was aspirated from 96-well microplates, and 0.5 μM TMRE in 200 μl phenol red-free medium was added and incubated at 37 °C for 30 min. As transmembrane distribution of TMRE is directly related