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Decitabine represses osteoclastogenesis through inhibition of RANK and NF-κB☆

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ABSTRACT

DNA methylation is essential for maintenance of stable repression of gene transcription during differentiation and tumorigenesis. Demethylating reagents including decitabine could release the repression, leading to perturbed transcription program. Recently others and we showed that, in B cell lymphomas, decitabine repressed B cell specific gene transcription and activated NF-κB signaling, causing decreased expression of translocated oncogenes including MYC and attenuated tumor cell proliferation. During osteoclastogenesis, changes in DNA methylation occurred in numerous genes, implicating important roles for DNA methylation in osteoclastogenesis. In the present study, we found that decitabine inhibited osteoclastogenesis. The inhibitory effect could be at least partially attributed to reduced expression of multiple osteoclast specific genes including RANK by decitabine. Moreover, decitabine inhibited activity of NF-κB, AP-1 and extracellular signal-regulated kinase (ERK), but not PI3K/Akt pathway. In vivo, using ovariectomized mouse as a model, we observed that decitabine reduced the osteoclast activity and bone loss. In conclusion, our findings demonstrated that decitabine was an inhibitor of osteoclastogenesis by repression of osteoclast specific transcription program including the RANK, NF-κB and AP-1 pathways. DNA methylation might be indispensable for osteoclastogenesis. The use of decitabine could represent a novel strategy in treatment of diseases associated with increased osteoclast activity.

1. Introduction

Osteoclasts are bone-resorptive cells originated from hematopoietic stem cells and develop through the fusion of mononuclear precursors. Together with osteoblasts/stromal cells, osteoclasts are involved in bone development, growth and remodeling [1]. Two essential factors directing osteoclastogenesis are macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor κB (RANK) ligand (RANKL). Mainly produced by osteoblasts and stromal cells, M-CSF binds to c-Fms/CSF1-R on osteoclast precursors and promotes survival and proliferation by activation of multiple pathways including extracellular signal-regulated kinase (ERK) and PI3K/Akt. M-CSF also stimulates RANK expression in osteoclast precursors [2]. RANK is expressed at high levels in osteoclast progenitors and mature osteoclasts [3]. As a cell surface receptor, upon binding of the RANK ligand (RANKL), RANK recruits tumor necrosis factor (TNF) receptor-associated factors (TRAFs), which in turn results in the activation of the multiple signaling pathways including NF-κB, three mitogen-activated protein kinases and AP-1 transcription factor family, initiating osteoclast formation and activity [2,4,5]. Osteoprotegerin (OPG) acts as a decoy receptor for RANKL, blocking the binding of RANKL to its cellular receptor RANK. Both OPG and RANKL are produced mainly by hypertrophic chondrocytes and osteocytes [4,6]. Expression of OPG, RANKL and RANK is tightly regulated and contributing to the coupling between osteoblasts and osteoclasts, such that the balance between bone formation and resorption is maintained.

Epigenetic mechanisms include histone modification and DNA methylation. They are stable and heritable changes that result in alteration of gene expression without any change in their coding sequence. The fact that these epigenetic alterations can be reversed easily by clinically available enzyme inhibitors makes them attractive therapeutic targets. Especially, decitabine (5-aza-2′deoxycytidine) and azacitidine, inhibitors of DNA methyltransferases, have been established as standard treatment for myelodysplastic syndromes (MDS) and were demonstrated to be associated with improved survival [7,8]. DNA methylation is one of the main mechanisms that is responsible for stable repression of lineage-inappropriate genes during differentiation and tumorigenesis [9]. Demethylating reagents including decitabine could release the repression, leading to perturbed transcription program. Recently we and others showed that, in B cell lymphomas, decitabine repressed B cell specific gene transcription at least partly by upregulation of B lineage-inappropriate genes [10,11]. In B cell lymphomas especially Burkitt lymphomas and Follicular lymphomas, decitabine suppressed

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the transcription program supporting the expression of translocated oncogenes MYC and BCL-2, leading to impaired tumor cell fitness. In Burkitt lymphomas characterized by absence of NF-κB signaling [12], decitabine activated NF-κB, which contributes to its tumor suppressing effects [10].

During osteoclastogenesis, hypermethylation and hypomethylation changes occurred in several thousand genes, most of which are harboring PU.1, NF-κB, and AP-1 (Jun/Fos) binding motifs [13]. These observations implicate a pivotal role for DNA methylation in osteoclastogenesis. However, whether inhibition of DNA methylation has any influence on osteoclastogenesis has yet to be determined. Moreover, inhibitors of DNA methyltransferases are already being used for treatment of MDS, and are undergoing clinical trials for other malignancies especially acute myeloid leukemia (AML). The potential impacts of these drugs on bone health have not been evaluated. In this study, we investigated whether decitabine could inhibit osteoclast formation and function using *in vitro* and *in vivo* models; and if so, what the involved molecular mechanisms are.

2. Materials and methods

2.1. Cell culture and treatment

Bone marrow mononuclear cells (BMMCs) were obtained from the femoral and tibial bone marrow of C57BL/6 mice at 6–8 weeks of age. Briefly, the marrow cavity of the bones was exposed; cells were flushed out and RBCs were lysed. The obtained cells were cultured overnight in medium supplemented with 25 ng/mL M-CSF (R&D Systems China) to remove adherent bone marrow stromal cells, then non-adherent cells (BMMCs) were collected. BMMCs and RAW264.7, a murine monocytic cell line, were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, M-CSF (25 ng/mL, for BMMCs), penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37 °C and 5% CO_2. Decitabine (5-aza-dC, Dec) was purchased from Calbiochem (Beijing, China) and dissolved in dimethyl sulfoxide (DMSO). The final DMSO concentration in the culture medium was less than 0.05% and did not show any cytotoxic effect. For decitabine treatment, cells were seeded in complete medium one day before treatment, after incubation with decitabine (0.5 μM unless otherwise specified) or vehicle for 24 h, medium was changed completely and cells were allowed to grow further for one day. Then cells were treated with RANKL (50 ng/mL, R&D) to induce osteoclast formation. Four days after RANKL treatment, cells were used for TRAP staining and TRAP enzyme activity assay, or collected for mRNA and protein preparation.

2.2. Cytotoxicity assay

For cytotoxicity assay, cells were seeded in 96-well plates at 1×10^4 cells/well in complete medium. After 24 h, cells were treated with different concentrations of decitabine (0, 0.1, 0.25, 0.5, 1, 2 μM). Medium was changed completely 24 h later. A Cell Counting Kit-8 kit (CCK8, Beyotime, Jiangsu, China) was used to measure cytotoxicity at one day, three days and five days after decitabine treatment.

2.3. TRAP staining and TRAP enzyme activity assay

TRAP staining was done in cultured RAW264.7 cells, and paraffin sections with a TRAP staining kit (Sigma-Aldrich) according to the manufacturer's protocol. TRAP-positive cells with three or more nuclei were identified as osteoclasts [14]. Cell images were taken using a digital camera attached to a Nikon ECLIPSE TE2000-S microscope (Nikon, Japan). TRAP enzyme activity was measured with an assay kit (Sigma-Aldrich) following the manufacturer's instructions. Briefly, cultured medium was collected from osteoclasts formed by BMMCs and RAW264.7 cells, respectively. TRAP enzyme activity was measured with a Synergy fluorescence plate reader at 405 nm on a colorimetric plate reader.

2.4. Bone pit formation by osteoclasts

BMMCs and RAW264.7 cells were seeded onto a Corning Osteo Assay Surface (Corning Incorporated Life Science, USA) in a multiple well plate in complete medium one day before treatment. Cells were treated with 0.5 μM decitabine or vehicle for 24 h, then medium was changed. One day later, RANKL was added to the medium to induce osteoclast differentiation. Cells were cultured for another six days. Then the disc was washed with 5% sodium hypochlorite for 5 min, and images were taken and the resorption area was quantified by image analysis (Bioquant Image Analysis, Nashville, TN).

2.5. RNA extraction, cDNA synthesis and real-time PCR (RT-PCR)

Quantitative real-time polymerase chain reaction (qRT-PCR) was done as described [10,15]. Briefly, total RNA was prepared from RAW264.7 cells using TRIzol reagents (Invitrogen), first-strand cDNA was synthesized with MMLV reverse transcriptase (Promega, Madison, WI). Templates were amplified with the SYBR GreenMaster Mix (Invitrogen China Limited).

The primers used were as follows (sequences 5′ to 3′, sense and antisense): RANK: CAGGAGAGGCATTATGAGCA and GGTACTTTCCTG GTTCGCAT; TRAP: GATGCCAGCGACAAGAGGTT and CATACCAGGGGA TGTTGCGAA; cathepsin K (CK): GAAGAAGACTCACCAGAAGCAG and TCCAGGTTATGGGCAGAGATT; matrix metalloproteinase-9 (MMP-9): CTGGACAGCCAGACACTAAAG and CTCGCGGCAAGTCTTCAGAG and β-actin: ATTTCTGAATGGCCCAGGT and CTGCCTCAACACCTCAACC.

2.6. Western blot analysis and electrophoretic mobility shift assay (EMSA)

Immunoblot was done in RAW264.7 cells as described [10,15]. Primary antibodies which included mouse anti-β-Actin (1:500 dilution) were from BOSTER (Wuhan, China). Antibodies (1:1000 dilution) specific for RANK, phospho-Akt (Ser-473), Akt, phospho-ERK1/2 (Thr202/Tyr204), and ERK were from Cell Signaling (Boston, MA, USA). Antibodies (1:500 dilution) specific for TRAP (ab96372), CK (ab19027) and MMP-9 (ab38898) were from Abcam (Shanghai, China). Secondary antibodies included goat anti-rabbit IgG-horseradish peroxidase (HRP; sc-2004, from Santa Cruz Biotechnology) and donkey anti-goat IgG-HRP (sc-2020; Santa Cruz Biotechnology).

EMSA was performed as described before [10]. The DNA-binding activity of NF-κB and AP-1 was detected using a LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific, China). RAW264.7 cells (5×10^5 cells/well) were treated similarly as described in the "Cell culture and treatment" section. Briefly, 24 h after seeding the cells, 0.5 μM decitabine or vehicle was added and incubated for 24 h. Then medium was changed, one day later, cells were stimulated with RANKL (50 ng/mL) for 30 min, and nuclear protein was isolated using a Nuclear and Cytoplasmic Protein Extraction Kit according to the manufacturer's instructions (Beyotime Institute of Biotechnology, Jiangsu, China) and quantified. Nuclear extracts were mixed with poly (dI–dC), biotin-labeled probe, binding buffer, 5 mM $MgCl_2$, 2.5% glycerol and NP-40 and incubated at room temperature for 10 min. Equal amounts of protein–DNA complexes were electrophoresed on a 6% polyacrylamide native gel. The biotin end labeled DNA was detected using a Streptavidin–HRP conjugate and a chemiluminescent substrate. The AP-1 and NF-κB probes (Beyotime Institute of Biotechnology, Jiangsu, China) used for EMSA, containing the consensus recognition sites for AP-1 and NF-κB, were as follows: AP-1, 5′-CGCTTGATGACTCAGCCGGAA-3 and NF-κB, 5′-AGTTGAGGGGACTTTCCCAGGC-3′.

Band densities were quantified using Image Lab 5.1 software (Bio-Rad, Hercules, California, U.S.A.) and normalized to that of controls.