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Caspase-2 modulates osteoclastogenesis through down-regulating oxidative stress☆

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ABSTRACT

The loss of caspase-2 (Casp-2) in mice results in an osteopenic phenotype associated with increased numbers of osteoclasts in vivo. In this study, we show that Casp-2 is involved in osteoclastogenesis. Protein levels of Casp-2 decrease during the differentiation of macrophages to osteoclasts. Furthermore, siRNA-mediated Casp-2 knockdown in osteoclast precursors or differentiation of bone marrow macrophage (BMM) precursors from *Casp2*$^{-/-}$ mice results in increased osteoclast numbers and tartrate-resistant acid phosphatase (TRAP) activity. *Casp2*$^{-/-}$ osteoclasts are larger in size compared to wild-type osteoclasts and exhibited increased numbers of nuclei, perhaps due to increased precursor fusion. The loss of Casp-2 did not alter earlier stages of differentiation, but had a greater consequence on later stages involving NFATc1 auto-amplification and pre-osteoclast fusion. We have previously shown that the loss of Casp-2 results in increased oxidative stress in the bone. Reactive oxygen species (ROS) is known to play a critical role in late osteoclast differentiation and we show that total ROS and specifically, mitochondrial ROS, significantly increased in *Casp2*$^{-/-}$ BMM precursors after RANKL administration, with a concomitant reduction in FoxO3a and its target antioxidant enzymes, catalase and superoxide 2 (SOD2). Because mitochondrial ROS has been identified as a putative regulator of the later stages of differentiation, the heightened ROS levels in *Casp2*$^{-/-}$ cells likely promote precursor fusion and increased osteoclast numbers. In conclusion, our results indicate a novel role of Casp-2 in the osteoclast as a modulator of total and mitochondrial ROS and osteoclast differentiation.

Introduction

Bone homeostasis is dependent upon a balance between bone resorption by osteoclasts and bone formation by osteoblasts. In conditions where this balance is not maintained, pathological disorders such as osteoporosis are manifested. One of the major underlying factors that have been shown to promote excess bone resorption and lead to the development of bone loss is oxidative stress. For example, oxidative stress was shown to have a significant negative correlation with bone mineral density (BMD) in the lumbar vertebrae and femoral neck in patients with postmenopausal osteoporosis [1]. In addition, several studies have shown that postmenopausal osteoporosis in humans as well as rodent models is associated with decreased antioxidant defenses, which subsequently leads to higher levels of reactive oxygen species (ROS) and bone loss [2–4].

Oxidative stress occurs when there are increased levels of ROS, such as hydrogen peroxide and superoxide, resulting in macromolecular damage within the cell. Interestingly, ROS has been shown to be an instrumental component in promoting osteoclast differentiation and activity [5–8]. In response to receptor activator of nuclear factor kappa-B ligand (RANKL), ROS levels increase and activate signaling pathways such as mitogen-activated protein kinases (MAPKs) including c-Jun N-terminal kinases (JNKs), extracellular signal-related kinases (ERKs), and p38, IKBα, and nuclear factor kappa B (NF-κB), culminating in the activation of the master osteoclast transcriptional regulator, nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) [5,6,9]. Also, the activation of Ca^{2+}-calmodulin and other proteins result in NFATc1 activation and osteoclast gene transcription [10,11]. The initial rise in Ca^{2+} levels also appears to be dependent upon ROS [12]. During this early differentiation stage, the primary focus is on BMM proliferation and maintaining pro-osteoclastogenesis signaling. Later, the cell enters a late-differentiation stage marked by the auto-amplification of NFATc1 driven by long lasting Ca^{2+} oscillations that greatly increases osteoclast gene transcription and drives committed

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osteoclast precursors towards fusion [9,12]. Concurrently, ROS levels also rise, although the identity of the particular species and its source has not been fully elucidated. One possibility is the mitochondria, particularly because increased mitochondrial biogenesis occurs during this point in differentiation [13,14]. In addition, mitochondria are the highest producers of ROS through the electron transport chain, and would likely be primary contributors of ROS during osteoclastogenesis. Depletion of mitochondrial ROS levels leads to a decrease in osteoclast numbers [15,16]. However, downstream mediators of ROS in osteoclast differentiation remain unclear. We posit Casp-2 as a candidate regulatory molecule involved in this process.

Casp-2 is a cysteine aspartate protease that is well known for its role during cellular apoptosis [17–20]. More recent studies have indicated, though, that Casp-2 may be involved in a wider variety of cellular process including the regulation of antioxidant enzymes. Studies by Shalini et al. show that mouse embryonic fibroblasts (MEFs) from *Casp2*$^{-/-}$ mice exhibited higher levels of ROS compared to wild-type (WT) [21]. Furthermore, in cells knocked down for *Casp2*, levels of the antioxidant enzymes superoxide dismutase 2 (SOD2), catalase, and glutathione peroxidase (GSH-Px) were decreased as well as the upstream transcriptional regulator, FoxO3a. Importantly, overexpression of Casp-2 in this model resulted in increased levels of FoxO3a as well as the downstream antioxidant SOD2 and GSH-Px, establishing a link between Casp-2 and FoxO3a expression. The loss of Casp-2 has been shown to lead to a premature aging phenotype [21,22], which includes significantly decreased BMD compared to age-matched WT mice. The decrease in BMD in old mice (>24 mos) was accompanied by decreased bone volume, increased urinary deoxypyridinoline (DPD), and increased numbers of osteoclasts, suggesting that the bone loss phenotype in *Casp2*$^{-/-}$ mice could be osteoclast-based [22]. The observed in vivo increase in osteoclast numbers may be explained by decreased osteoclast apoptosis and/or increased osteoclast differentiation. We have recently addressed the contribution of Casp-2 in osteoclast apoptosis wherein we showed that the loss of Casp-2 attenuated osteoclast apoptosis in response to oxidative stressors [23]. To address the underlying mechanistic role of Casp-2 in osteoclastogenesis and its modulation of and by ROS, we ablated Casp-2 in bone marrow macrophages and osteoclasts using either gene knockdown or used precursors derived from a mouse model with a global *Casp2* deletion. In both cases, we show that deletion of Casp-2 augments osteoclastogenesis that correlates with an increase in oxidative stress. Interestingly, Casp-2 affects the later stage of osteoclast differentiation involving cell fusion.

Materials and methods

Mice

Casp2$^{-/-}$ mice were originally generated by Dr. Junying Yuan of Harvard University and kindly provided by Dr. Carol Troy of Columbia University with Dr. Yuan's consent. The mice were backcrossed with C57Bl/6 for ten generations. All mice were housed in micro-isolator-topped cages and maintained in a pathogen-free environment at the AAALAC-accredited UTHSCSA animal facility following the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals. We used male WT and *Casp2*$^{-/-}$ mice aged 3–6 weeks for these studies.

Cell culture

All cells were grown in Gibco αMEM (Life Technologies, Grand Island, NY) with 10% fetal bovine serum (Biosera, Kansas City, MO) and antibiotics. Primary bone marrow was isolated according to standard protocols. In brief, mice were sedated with isofluorane (Baxter, Deerfield, IL) and sacrificed by cervical dislocation. Bone marrow cells were collected by washing the marrow cavity αMEM delivered via a 21 G needle. Cells were cultured overnight and the non-adherent fraction was separated and allowed to expand for 3 days in 30 ng/mL CSF-1 (R&D Systems, Minneapolis, MN). BMMs were harvested and plated with 30 ng/mL CSF-1 and 10 ng/mL RANKL (R&D Systems) for differentiation into osteoclasts.

Cell transfections with siRNA

RAW 264.7 cells were plated and allowed to grow for 24 h. Cells were then transfected in OPTIMEM containing Lipofectamine® RNAiMAX (Life Technologies) and 33 nM siRNA duplexes, following the manufacturer's protocols. Casp-2 siRNA (Qiagen, Valencia, CA; MM_Casp2_3 SI00941717 5′-CAGGGTCACTTGGAAGACTTA-3′) or AllStars Negative Control (Qiagen) was used. After 1 h, RANKL was added to each well. Cells were allowed to differentiate for 2 days and were either scraped in RIPA buffer (Santa Cruz Biotechnology, Dallas, TX) or were given fresh media containing siRNA duplexes and RANKL. At day 4 cells were scraped again or were used for TRAP staining or TRAP activity assays.

TRAP (tartrate-resistant acid phosphatase) staining and activity assays

TRAP staining was accomplished with a Leukocyte Acid Phosphatase Staining Kit (Sigma. St. Louis, MO) according to the manufacturer's protocols. Total numbers of TRAP^{+}, multi-nucleated (>3) cells (MNCs) per well of a 48-well plate were counted. In addition, images of these wells were used to assess up to 10 osteoclasts for nuclear number and cell size with ImageJ software and numbers of nuclei/unit area were calculated. A total of 100 osteoclasts were assessed per group. Osteoclasts in these images were also categorized according to number of nuclei and compared among groups. To measure TRAP activity, live cells were incubated for 30 min at 37 °C in a pre-warmed solution containing 4% acetate, 4% tartrate, 0.1% sodium dodecyl sulfate, and 6 tablets of SIGMA FAST™ p-nitrophenyl phosphate (Sigma). The resulting supernatant was transferred to a fresh 96 well plate and read at 405 nm on a BioRAD iMark Microplate Reader (Bio-Rad Laboratories, Hercules, CA). Experiments were performed in triplicate with at least three replicates per experiment.

Western blotting

Cells at different stages of differentiation were lysed with RIPA buffer (Santa Cruz Biotechnology, Dallas, TX). Protein concentration was assessed with a Micro BCA protein assay kit (Thermo Scientific, Waltham, MA) and 30 μg protein was loaded per lane on 10% SDS-PAGE gels. Following transfer, nitrocellulose membranes were blocked with either 5% nonfat dry milk or 5% BSA and incubated with primary antibody overnight at 4 °C. Primary antibodies were used to detect Casp-2 (Millipore, Billerica, MA), GAPDH (Life Technologies), cathepsin K (CtsK; Abcam, Cambridge, MA), NFATc1 (Santa Cruz Biotechnology), catalase (Santa Cruz Biotechnology), SOD2 (Santa Cruz Biotechnology), FoxO3a (Cell Signaling Technology, Danvers, MA), and MAPK (total and phosphorylated Akt, IκBα, ERK, p38, and JNK; Cell Signaling Technology). Secondary antibodies compatible with the LI-COR infrared imaging system were used that included anti-rat, mouse, or rabbit IRDye® 800CW or IRDye® 680RD (LI-COR, Lincoln, NE). Blots were imaged on a LI-COR Odyssey system and assessed by densitometry using in Image Studio Lite V3.1 software. Results were normalized to the GAPDH and in some cases also to the day 0 (macrophage/M0 or BMM) samples. Experiments were performed at least in triplicate.

Immunocytochemistry

Primary BMMs and osteoclasts were cultured in 24-well plates on glass coverslips pretreated with 4.7% HCl. Cells were first incubated with mouse IgG to block non-specific binding, rinsed with phosphate-buffered saline (PBS) and fixed with 2% paraformaldehyde for 15 min at room temperature. After treating with blocking solution (2% goat

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