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N-acetylglucosamine suppresses osteoclastogenesis in part through the promotion of *O*-GlcNAcylation

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ABSTRACT

Osteoclasts are the only cells in an organism capable of resorbing bone. These cells differentiate from monocyte/macrophage lineage cells upon stimulation by receptor activator of NF-κB ligand (RANKL). On the other hand, osteoclastogenesis is reportedly suppressed by glucose via the downregulation of NF-κB activity through suppression of reactive oxygen species generation. To examine whether other sugars might also affect osteoclast development, we compared the effects of monomeric sugars (glucose, galactose, *N*-acetylglucosamine (GlcNAc), and *N*-acetylgalactosamine (GalNAc)) on the osteoclastogenesis of murine RAW264 cells. Our results demonstrated that, in addition to glucose, both GlcNAc and GalNAc, which each have little effect on the generation of reactive oxygen species, suppress osteoclastogenesis. We hypothesized that GlcNAc might affect osteoclastogenesis through the upregulation of *O*-GlcNAcylation and showed that GlcNAc increases global *O*-GlcNAcylation, thereby suppressing the RANKL-dependent phosphorylation of NF-κB p65. Furthermore, an inhibitor of *N*-acetyl-β-D-glucosaminidase, *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino *N*-phenylcarbamate (PUGNAc), which also increases *O*-GlcNAcylation, suppressed the osteoclastogenesis of RAW264 cells and that of human peripheral blood mononuclear cells. Together, these data suggest that GlcNAc suppresses osteoclast differentiation in part through the promotion of O-GlcNAcylation.

1. Introduction

Bone homeostasis is regulated by the balance between bone formation and resorption (Yamashita et al., 2012). Only a single bone-resorbing cell type is found in the body, known as osteoclasts (Cappariello et al., 2014). Osteoclasts are multinucleated cells that express tartrate-resistant acid phosphatase (TRAP) and differentiate from monocyte/macrophage lineage cells upon stimulation with macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL). M-CSF promotes the expression of RANK, the receptor of RANKL. RANKL stimulation activates downstream signaling molecules including NF-κB and c-Fos, which induce the expression of NFATc1, a master transcriptional regulator of osteoclast differentiation. In turn, NFATc1 induces the upregulation of osteoclast-specific genes including *TRAP*, cathepsin K, and matrix metallopeptidase 9 (Asagiri and Takayanagi, 2007; Kuroda and Matsuo, 2012; Boyce, 2013). Notably, these physiological differentiation processes are well reflected in the RANKL-dependent osteoclastogenic differentiation of RAW264 cells (Hsu et al., 1999).

Osteoclast differentiation is regulated by various molecules, including the monomeric sugar glucose (Glc) at high concentration. Glc suppresses osteoclastogenesis by suppressing the activity of NF-κB through an anti-oxidative mechanism, which entails suppression of the RANKL-induced generation of reactive oxygen species (ROS) (Wittrant et al., 2008), and suppressing the gene expression of several key differentiation molecules including NFATc1 (Xu et al., 2015). It has also been reported that a rare monomeric sugar, allose, inhibits osteoclast differentiation (Yamada et al., 2012). However, the effects of other common monomeric sugars such as galactose (Gal), *N*-acetylglucosamine (GlcNAc), and *N*-acetylgalactosamine (GalNAc) on osteoclastogenesis remain undetermined.

Glc is a well-known major energy source. Monomeric sugars including Glc are metabolized in cells and become activated as nucleotide sugars that are used for, e.g., *N*-glycosylation and *O*-GlcNAcylation (Freeze and Elbein, 2009). *O*-GlcNAcylation is the posttranslational modification of serine or threonine residues in various intracellular proteins by GlcNAc and is reversibly catalyzed by *O*-GlcNAc transferase and β-N-acetylglucosaminidase (*O*-GlcNAcase) (Butkinaree et al., 1800;

Abbreviations: Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcNAc, *N*-acetylglucosamine; M-CSF, macrophage colony-stimulating factor; NF-κB, nuclear factor-κB; PBMC, peripheral blood mononuclear cell; PUGNAc, *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino *N*-phenylcarbamate; RANKL, receptor activator of nuclear factor-κB ligand; ROS, reactive oxygen species; sRANKL, soluble receptor activator of nuclear factor-κB ligand; TRAP, tartrate-resistant acid phosphatase; UDP, uridine diphosphate.

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Hanover et al., 1800). *O*-GlcNAcylation is thought to exhibit crosstalk with phosphorylation and has been shown to affect the activities of a variety of signaling molecules including those known to have important roles in osteoclastogenesis such as p38, ERK, NF-κB, c-Fos, and Akt (Butkinaree et al., 1800; Hanover et al., 1800).

In the present study, we compared the effects of simple sugars (Glc, Gal, GlcNAc, and GalNAc) on the RANKL-dependent osteoclastogenic differentiation of murine RAW264 cells. We also investigated the role of *O*-GlcNAcylation in this process by examining the effect of sugars thereon and the effects of an inhibitor of *N*-acetyl-β-$_D$-glucosaminidase (PUGNAc), which increases *O*-GlcNAcylation, on RAW264 and human peripheral blood mononuclear cell (PBMC) osteoclast differentiation.

2. Materials and methods

2.1. Cell culture

The mouse macrophage-like RAW264 cell line was obtained from the RIKEN Cell Bank (Tsukuba, Japan) and maintained in modified Eagle's medium alpha (MEMα medium (Wako, Osaka, Japan) containing 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) and 1× penicillin/streptomycin (Wako) under a humidified atmosphere containing 5% CO_2 at 37 °C. Uncharacterized cryopreserved human PBMCs were obtained from Cellular Technology, Ltd. (Shaker Heights, OH, USA) and cultured in MEMα medium containing 10% heat-inactivated fetal bovine serum and 1× penicillin/streptomycin.

2.2. Osteoclast differentiation and TRAP staining

RAW264 cells were seeded on a 96-well plate (1000 cells/well) and cultured for 1 day. Thereafter, the cells were treated with 250 or 500 ng/mL soluble RANKL (sRANKL) (Oriental Yeast, Tokyo, Japan) in the presence of 20 mM Glc, Gal, GlcNAc, GalNAc, or *O*-(2-acetamido-2-deoxy-$_D$-glucopyranosylidene) amino *N*-phenylcarbamate (PUGNAc; an inhibitor of *N*-acetyl-β-$_D$-glucosaminidase) (all from Wako) and allowed to differentiate for 4 days. The sugars were dissolved in phosphate-buffered saline (PBS); PUGNAc was dissolved in DMSO. Human PBMCs were seeded on a 96-well plate (1×10^5 cells/well) and cultured for 1 day. Then the cells were treated with 50 ng/mL sRANKL and 25 ng/mL human M-CSF (PeproTech, Rocky Hill, NJ, USA) in the presence of 20 mM GlcNAc or 10 μM PUGNAc and allowed to differentiate for 8 days. Media were replenished every 2 days.

Differentiated cells were washed with PBS and then treated with 4% paraformaldehyde solution for 10 min at room temperature. After being washed again with PBS, the cells were treated with PBS and then stained with a TRAP staining solution containing 50 mM sodium tartrate, 45 mM sodium acetate, pH 5.2, 0.1 mg/mL naphthol AS-MX phosphate (Sigma-Aldrich, St. Louis, MO, USA), and 0.6 mg/mL fast red violet LB (Sigma-Aldrich), pH 5.2, for 1 h or longer at room temperature. The cells were viewed under a TC5400 microscope (Meiji Techno, Saitama, Japan) equipped with a Moticom 2000 digital camera (Shimadzu, Kyoto, Japan), and TRAP-positive cells that stained red and contained three or more nuclei were counted. Photographs were taken with a 10× objective.

2.3. TRAP enzyme activity assay

RAW264 cells were allowed to differentiate as described in Section 2.2. After 4 days, the cells were washed with PBS and lysed with 100 μL TRAP buffer (50 mM sodium tartrate, 50 mM sodium acetate, 150 mM KCl, 0.1% TritonX-100, 1 mM sodium ascorbate, and 0.1 mM $FeCl_3$, pH 5.2) for 10 min at 4 °C. The prepared cell extract (10 μL) was then added to 100 μL TRAP buffer containing 2.5 mM *p*-nitrophenyl phosphate (Thermo Fisher Scientific) as a TRAP substrate, and the reaction mixture was incubated for 1 h at 37 °C. After the addition of 50 μL 0.9 M NaOH to the mixture to stop the reaction, the absorbance at 405 nm was measured using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.4. Real-time PCR

RAW264 cells were allowed to differentiate for 4 days as described in Section 2.2. Total RNA extraction and cDNA synthesis were performed using the Power SYBR® Green Cells-to-CT™ Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instruction. In brief, cells differentiated in a 96-well plate were washed with PBS and lysed with 50 μL lysis solution containing DNase I. A portion of the lysate (10 μL) was used for reverse transcription with both random primers and oligo dT. Real-time PCR was performed with the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the Power SYBR® Green PCR Master Mix (Life Technologies). All PCR products were amplified with 40 cycles of denaturation (95 °C, 15 s) and annealing and extension (65 °C, 15 s). Hypoxanthine guanine phosphoribosyl transferase (*Hprt*) was used as an internal control, and data were analyzed using the $2^{-\Delta\Delta Ct}$ method. The primers used for PCR were as follows: *Hprt*, 5′-GCT CGA GAT GTC ATG AAG GAG-3′ and 5′-CAG CAG GTC AGC AAA GAA CTT-3′; cathepsin K, 5′-GGC TGT GGA GGC GGC TAT-3′ and 5′-AGA GTC AAT GCC TCC GTT CTG-3′; and matrix metallopeptidase 9, 5′-AAA GAC CTG AAA ACC TCC AAC CT-3′ and 5′-GCC CGG GTG TAA CCA TAG C-3′. The primers for *Hprt* were designed according to the method described by Nairn et al. (2008), and the others were according to that by Kim et al. (2014)).

2.5. Staining and measurement of ROS

For ROS staining, RAW264 cells were seeded on a 12-well plate (6000 cells/well) and cultured for 1 day. Then the cells were treated with 500 ng/mL sRANKL in the presence of 20 mM sugars and allowed to differentiate for 4 days. Differentiated cells were washed twice with PBS and then stained with PBS containing 5 μM fluorescent ROS detection reagent (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA); Life Technologies) for 30 min at room temperature. Thereafter, the cells were washed twice with PBS, and the resultant green fluorescence was viewed using a FLoid™ Cell Imaging Station (Life Technologies) with a 20× objective.

For ROS measurement, RAW264 cells were seeded on a 96-well white plate (1000 cells/well) and cultured for 1 day. Then the cells were treated with 500 ng/mL sRANKL in the presence of 20 mM sugars and allowed to differentiate for 4 days. Differentiated cells were stained with PBS containing 10 μM CM-H_2DCFDA for 1 h at room temperature. The cells were washed twice with PBS, and the fluorescent dye was extracted by incubation with 100 μL PBS containing 0.2% TritonX-100 for 30 min at 4 °C. Thereafter, the fluorescence (excitation 485 nm, emission 538 nm) was measured using a SpectraMax M5 microplate reader.

2.6. Western blotting

For immunoblotting analysis, cells were washed with PBS and lysed in 200 μL sample buffer (50 mM Tris–HCl, pH 6.8, 1% sodium dodecyl sulfate, 10% glycerol, 0.01% bromophenol blue, and 2% 2-mercaptoethanol) with sonication. After boiling and centrifugation, the resulting supernatants were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the separated proteins were transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Berkeley, CA, USA) using a wet electroblotting system for the detection of *O*-GlcNAcylated proteins or using the iBlot Gel Transfer Device (Life Technologies) for the other proteins of interest. Immunoblotting was performed on an iBind™ Western Device (Life Technologies) according to the manufacturer's instructions using horseradish peroxidase-conjugated anti-*O*-GlcNAc (CTD110.6) mouse monoclonal antibody, anti-IκBα mouse monoclonal antibody,