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Magnesium deficiency results in an increased formation of osteoclasts

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Abstract

Magnesium (Mg^{2+}) deficiency is a frequently occurring disorder that leads to loss of bone mass, abnormal bone growth and skeletal weakness. It is not clear whether Mg^{2+} deficiency affects the formation and/or activity of osteoclasts. We evaluated the effect of Mg^{2+} restriction on these parameters. Bone marrow cells from long bone and jaw of mice were seeded on plastic and on bone in medium containing different concentrations of Mg^{2+} (0.8 mM which is 100% of the normal value, 0.4, 0.08 and 0 mM). The effect of Mg^{2+} deficiency was evaluated on osteoclast precursors for their viability after 3 days and proliferation rate after 3 and 6 days, as was mRNA expression of osteoclastogenesis-related genes and Mg^{2+}-related genes. After 6 days of incubation, the number of tartrate resistant acid phosphatase-positive ($TRACP^{+}$) multinucleated cells was determined, and the TRACP activity of the medium was measured. Osteoclastic activity was assessed at 8 days by resorption pit analysis. Mg^{2+} deficiency resulted in increased numbers of osteoclast-like cells, a phenomenon found for both types of marrow. Mg^{2+} deficiency had no effect on cell viability and proliferation. Increased osteoclastogenesis due to Mg^{2+} deficiency was reflected in higher expression of osteoclast-related genes. However, resorption per osteoclast and TRACP activity were lower in the absence of Mg^{2+}. In conclusion, Mg^{2+} deficiency augmented osteoclastogenesis but appeared to inhibit the activity of these cells. Together, our *in vitro* data suggest that altered osteoclast numbers and activity may contribute to the skeletal phenotype as seen in Mg^{2+} deficient patients.

Keywords: Magnesium deficiency; Osteoclast; Osteoclastogenesis; Osteoclast activity; Bone metabolism; Bone marrow

1. Introduction

The importance of intake of minerals in the maintenance of bone homeostasis has been demonstrated in various studies [1–5]. Of these minerals magnesium (Mg^{2+}) has been shown to play a crucial role in hormone action (e.g., parathyroid hormone), in expression of some neurotransmitters and, more in general, in regulating cellular activity [6,7].

Mg^{2+} is the most abundant divalent cation in cells and serves as an essential structural element in the maintenance of phospholipid bilayers, DNA double helices and protein structure. One of its most important functions is related to the enzymatic activity of cells since all enzymes utilizing ATP require Mg^{2+}. It also acts as an allosteric activator of enzyme action including adenylate cyclase and phospholipase C. This could consequentially facilitate the activation/deactivation of signal transduction pathways [8,9].

It is estimated that 2.5% to 15% of the world population suffers from some level of hypomagnesia [10]. According to Ref. [2], the mean value of daily Mg^{2+} intake in the North American human population is below recommendation. A similar low intake is present in some European countries [10–13].

The normal adult body Mg^{2+} content is about 25 g. Bone can be regarded as the major storage compartment of the body, containing 60%–65% of the total Mg^{2+} content [14]. Part of this magnesium resides on bone surfaces, and it is hypothesized that its main function is to maintain the extracellular Mg^{2+} concentration [14,15].

A study conducted in rats with severe restriction of magnesium intake [0.04% of the Nutrient Requirement (NR)] resulted in increased skeletal fragility; increased bone resorption; decreased bone formation; and increased expression of the neuropeptide substance P, tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β). This finally resulted in an osteoporotic phenotype [16]. Other studies were conducted with a less severe mineral restriction (10%, 25% and 50% of NR) and showed that, even under these conditions, bone loss occurred [1,2,17]. In addition, the Mg^{2+}-deficient diet had a negative influence on the maintenance of osseointegrated implants [18,19].

Mg^{2+} deficiency also potentiates the inflammatory response by increasing the release of cytokines and growth factors that mediate the formation and activity of the body's bone degradation specialist

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cell, the multinucleated osteoclast [20,21]. Osteoclasts are multinucleated cells from the macrophage lineage, specialized in degrading mineralized matrices, such as bone. The formation and activity of these cells are mediated by cytokines like macrophage colony stimulating factor (M-CSF) and the osteoclast differentiation factor RANKL and their receptors, c-fms and RANK, respectively [22]. Deregulation of osteoclast differentiation, function and survival may lead to pathological conditions, such as osteoporosis [23] or osteopetrosis [24,25].

Although Mg^{2+} deficiency leads to loss of bone mass, it is not known whether Mg^{2+} influences osteoclast formation and activity. Therefore, the aim of this study is to evaluate the effect of magnesium restriction on osteoclast formation and activity. Precursors were obtained from marrow of long bone and jaw and cultured in the presence of M-CSF and RANKL. Precursor cells from two different sites were analyzed because several studies have suggested phenotypic differences among osteoclast subpopulations, such as functional differences in formation and resorption activity [26–29].

2. Materials and methods

2.1. Mice

Prior to their sacrifice, 6-week-old male C57BL/6 mice (Harlan, Horst, the Netherlands) were maintained for 1 week at the animal facility of the VU University. Permission for the experiments was obtained from the animal ethical committee of the VU University, Amsterdam.

2.2. Bone marrow isolation

Bone marrow cells from long bones (femur and tibia) and jaws were isolated as described before [30]. The cells were resuspended through a 21-gauge needle, filtered over a 100-μm pore size cell strainer filter (Falcon/Becton Dickinson, Franklin Lakes, NJ, USA) and kept on ice in culture medium without magnesium (MEM alpha without nucleotides, with L-glutamine; Promocell, Heidelberg, Germany) supplemented with 5% FCI (HyClone, Logan, UT, USA), 100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B (antibiotic antimycotic solution; Sigma-Aldrich, St. Louis, MO, USA) until further use.

2.3. Osteoclastogenesis

Bone marrow cells were plated in 96-well flat-bottom tissue culture-treated plates (Costar, Cambridge, MA, USA) at a density of 1.0×10^5 cells/well in 150 μl of culture medium containing 30 ng/ml recombinant murine M-CSF (R&D Systems, Minneapolis, MN, USA) and 20 ng/ml recombinant murine RANKL (RANKL-TEC, R&D Systems, Minneapolis, MN, USA). Cells were cultured in four different concentrations of magnesium: 100% Mg^{2+} or 0.8 mM Mg^{2+}, as supplied with standard alpha-MEM. The other concentrations, 50% (0.4 mM) and 10% (0.08 mM), were made by mixing Mg^{2+}-free alpha-MEM (0%) with 100% Mg^{2+} containing alpha-MEM. Cells were cultured on plastic or on 650-μm-thick bovine cortical bone slices. Culture media were replaced every 3 days. The cells were assessed once a day every day. Hardly any fibroblast-like cells were seen; hence, it is likely that expression data can be most of all be contributed to osteoclasts and their precursors. After 3 days of culturing, cells were washed with phosphate-buffered saline (PBS) and harvested for polymerase chain reaction (PCR) analysis. The cells were also washed with PBS and harvested for DNA analysis after either 3 or 6 days. Supernatants were collected for viability assay. Typical for osteoclastogenesis cultures was that they were subconfluent (less than 25% of the well surface covered) at all stages. On day 6, wells were washed with PBS and fixed in 4% PBS-buffered formaldehyde, stored at 4°C and stained for tartrate-resistant acid phosphatase (TRACP). On day 8, water was added to osteoclast cultures on bone slices for bone resorption analysis. This experiment was repeated two times, each experiment with $n=3$ mice for a total of $n=6$ mice.

2.4. TRACP staining

Cells from femur/tibia and jaw were cultured for 6 days on plastic or on bone and stained for TRACP activity using the leukocyte acid phosphatase kit (Sigma-Aldrich, Saint Louis, MO, USA). Nuclei were stained with diamidino-2-phenylindole dihydrochloride. To evaluate osteoclast formation, the number of TRACP-positive multinucleated cells ($TRACP^+$-MNCs) was counted. These multinucleated cells were categorized in cells containing 3–5, 6–10 or more than 10 nuclei.

2.5. Magnesium and calcium measurements

To determine the concentration of magnesium and calcium in the culture medium, the supernatants of the four different magnesium-containing media were collected after 6 days of culturing on plastic and on bone ($n=6$). The media were also incubated at the same conditions but without cells ($n=3$). Magnesium and calcium concentrations were analyzed by atomic absorbance spectrometry (Analyst100; Perkin-Elmer Corporation, Norwalk, CT, USA).

2.6. DNA quantification

Cell proliferation was measured using DNA quantification. The bone marrow cells of six mice were collected on day 0 at a density of 1.0×10^5 cells and after 3 and 6 days of incubation on plastic in the four different magnesium concentration media with M-CSF and RANKL. The amount of DNA was determined using the CyQuant cell proliferation assay kit (Invitrogen Corporation, Carlsbad, CA, USA) and measured in the Synergy HT plate reader (BioTek Instruments, Winooski, VT, USA).

2.7. Cell viability

To determine the cell viability, the supernatant of cells cultured on plastic were collected after 3 days. For this assay, Triton was added to samples at a concentration of 0.5% as a positive control. The cytotoxicity assay was performed according to the manufacturer's instructions (ToxiLight BioAssay kit; Lonza Rockland Inc., Rockland, ME, USA). This assay quantitatively measures the leakage of adenylate kinase (AK) from damaged cells. The measurement of the release of AK from the cells allows the determination of cytotoxicity. This kit provides the sensitivity with a detection limit of 10 cells per microwell with a dynamic range of over five orders of magnitude.

2.8. Real-time quantitative PCR

For real-time quantitative PCR analysis, bone marrow samples of six mice were collected after 3 days of culture with M-CSF and RANKL. RNA isolation and real-time quantitative PCR were performed as described [31]. Real-time PCR primers were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA) (Table 1). To avoid amplification of genomic DNA, each amplicon spanned at least one intron. To test for PCR efficiencies, one of the osteoclast samples was used to generate a standard curve for all of the genes studied. The PCRs of the different amplicons had equal efficiencies. Expression of porphobilinogen deaminase (PBGD) was not affected by the different Mg^{2+} concentrations and was used as housekeeping gene. Samples were tested for transient receptor potential-melastin-like 7 (TRPM-7), MRS2, c-fos, RANK, c-fms, dendritic cell-specific transmembrane protein (DC STAMP), m-calpain, μ-calpain, IL1-β and TNF-α. Subsequently, expression was normalized for the expression of PBGD by calculating the ΔCt ($Ct_{\text{gene of interest}}-Ct_{PBGD}$), and expression of the different genes was expressed as $2^{-(\Delta Ct)}$.

2.9. TRACP activity assay

The supernatant of cells obtained from six mice seeded on plastic cultured in four different magnesium concentrations in the presence of M-CSF and RANKL was collected after 6 days of incubation. TRACP activity was assayed in 96-well plates with *p*NPP as substrate in 5 μl of supernatant with final concentrations of 10 mM *p*NPP, 1

Table 1
Primer sequences used in the study

Primer	Sequence	Amplicon length (bp)	Accession no.
c-fms	GAAACGCGACCTTCAAAAACA GGCCGGATCTTTGACATACAA	100	ENSMUSG00000024621
c-fos	TCACCCTGCCCCTTCTCA CTGATGCTCTTGACTGGC TCC	64	ENSMUSG00000021250
DC STAMP	TGTATCGGCTCATCTCCTCCAT GACTCCTTGGGTTCCTTGCTT	100	ENSMUSG00000022303
IL-1β	GGACCCATATGAGCTGAAAGCT TGTCGTTGCTTGGTTCTCCTT	100	ENSMUSG00000027398
MRS2	GGGTCCTGATTGACGATTCG TCACATTGCGATGGCTGTCT	62	ENSMUSG00000021339
TNF-α	GCCACCACGCTCTTCTGTCT GTCTGGGCCATAGAACTGATGAG	100	ENSMUSG00000024401
TRPM-7	CAACCGGAGCTTGGATTTTAAC TGAGGGCATCACCAACATGT	68	ENSMUSG00000027365
RANK	TGGGCTTCTTCTCAGATGTCTTT TGCAGTTGGTCCAAGGTTTG	59	ENSMUSG00000026321
PBGD	AGTGATGAAAGATGGGCAACT TCTGGACCATCTTCTTGCTGA	122	ENSMUSG00000032126