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Magnesium ion stimulation of bone marrow stromal cells enhances osteogenic activity, simulating the effect of magnesium alloy degradation

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ABSTRACT

Magnesium alloys are being investigated for load-bearing bone fixation devices due to their initial mechanical strength, modulus similar to native bone, biocompatibility and ability to degrade in vivo. Previous studies have found Mg alloys to support bone regeneration in vivo, but the mechanisms have not been investigated in detail. In this study, we analyzed the effects of Mg^{2+} stimulation on intracellular signaling mechanisms of human bone marrow stromal cells (hBMSCs). hBMSCs were cultured in medium containing 0.8, 5, 10, 20 and 100 mM $MgSO_4$, either with or without osteogenic induction factors. After 3 weeks, mineralization of extracellular matrix (ECM) was analyzed by Alizarin red staining, and gene expression was analyzed by quantitative polymerase chain reaction array. Mineralization of ECM was enhanced at 5 and 10 mM $MgSO_4$, and collagen type X mRNA (*COL10A1*, an ECM protein deposited during bone healing) expression was increased at 10 mM $MgSO_4$ both with and without osteogenic factors. We also confirmed the increased production of collagen type X protein by Western blotting. Next, we investigated the mechanisms of intracellular signaling by analyzing the protein production of hypoxia-inducible factor (HIF)-1α and 2α (transcription factors of COL10A1), vascular endothelial growth factor (VEGF) (activated by HIF-2α) and peroxisome proliferator-activated receptor gamma coactivator (PGC)-1α (transcription coactivator of VEGF). We observed that 10 mM $MgSO_4$ stimulation enhanced COL10A1 and VEGF expression, possibly via HIF-2α in undifferentiated hBMSCs and via PGC-1α in osteogenic cells. These data suggest possible ECM proteins and transcription factors affected by Mg^{2+} that are responsible for the enhanced bone regeneration observed around degradable Mg orthopedic/craniofacial devices.

1. Introduction

Every year, more than 6.2 million cases of bone fracture are reported, with 56% of fractures in adults requiring internal fixation with biomedical devices such as plates and screws [1,2]. Bone fixation devices are most commonly made of non-degradable metallic alloys, such as titanium and stainless steel. Drawbacks to these traditional orthopedic alloys include stress shielding due to the mismatch in mechanical properties between the metal and the bone [3], and the need for secondary surgery to remove the fixation devices in some cases. Degradable polymers (e.g. poly (lactic-co-glycolic acid) (PLGA), poly(L-lactic acid) and poly (ethylene glycol)) have been employed in order to avoid the secondary removal surgery; however, their compressive strengths are not ideal for load-bearing fracture repair cases [4], and foreign body reactions to the polymers have been reported [5–7]. In order to address these issues, magnesium alloys have been studied as a candidate material for bone fixation devices due to their bone-like mechanical properties, enhanced osteoconductivity compared to polymers and ability to safely degrade in vivo [3].

Mg alloys were first used for biomedical applications over 200 years ago; however, their development has accelerated in the last 10 years due to advances in alloy manufacturing and processing methods [8]. Numerous research groups have synthesized a wide range of magnesium alloys and characterized their microstructure, corrosion properties, mechanical properties, in vitro cytotoxicity

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and in vivo biocompatibility. In vivo Mg alloy studies have involved implantation of rods into rabbit tibiae [9], ulnae [10] and femora [11,12], rat femora [13] and guinea pig femora [14]. These in vivo studies found through microcomputed tomography, mechanical testing and histology analysis that the magnesium alloys safely degrade and allow osseointegration at the site of implantation. Additionally, comparisons of Mg alloy rods to polymer rods found that mineralization was increased surrounding the Mg samples [14]. Mg^{2+} concentrations were found to be increased in bone tissue immediately surrounding degrading Mg alloys in vivo [15]. This finding suggests that the mechanisms underlying enhanced bone regeneration observed in vivo can be recapitulated using Mg^{2+} salts in vitro.

Most in vitro studies of Mg alloys have focused on cell viability and proliferation to assess cytocompatibility. Previous studies used MTT and WST-1 assays to show that Mg alloys are cytocompatible with primary human mesenchymal stem cells [10], bone-derived cells [16], mouse fibroblasts [11,17], MG-63 human osteosarcoma cells [16], RAW264.7 macrophages [16] and MC3T3-E1 osteoblasts [17,18]. In addition, von Kossa and alkaline phosphatase stains were utilized to examine the effect of magnesium alloys on U2OS human osteosarcoma cell mineralization and osteogenic differentiation [19]. Furthermore, immunohistochemistry and flow cytometry were employed to study the mechanisms of cell adhesion on biomaterials when stimulated by Mg [20]. Overall, these in vivo studies have shown Mg-based devices to be promising for bone fracture fixation, and in vitro studies have shown enhancement of standard osteogenic markers in bone cells. However, to the best of our knowledge, this report is the first identification of specific intracellular signaling pathways through which Mg enhances bone regeneration.

We hypothesized that treating human bone marrow stromal cells (hBMSCs) with $MgSO_4$, resulting in increased exposure of the cells to Mg^{2+}, would enhance osteogenic gene expression, matrix production and mineral deposition. We cultured hBMSCs with various concentrations of $MgSO_4$, either with or without osteogenic factors. These treated cells were then analyzed for their matrix mineralization, gene expression and protein production in order to elucidate the intracellular signaling pathways involved in bone growth around Mg alloys. In this study, we found that increased $MgSO_4$ enhanced protein expression of collagen type X (COL10A1), vascular endothelial growth factor (VEGF), hypoxia-inducible factor (HIF)-1α, HIF-2α and peroxisome proliferator-activated receptor gamma coactivator (PGC)-1α in hBMSCs. COL10A1 is abundant in fractured bone at early stages of healing and VEGF is a major angiogenic signaling protein. This work identified specific osteogenic pathways that are affected by Mg. The identification of these pathways and the optimal Mg concentrations to enhance their activity will lead to improved Mg bone fixation device design and other possible therapeutic uses for Mg.

2. Materials and methods

2.1. Harvest, expansion and experimental culture of hBMSCs

hBMSCs were harvested from surgical waste in accordance with the US NIH regulations governing the use of human subjects under protocol 94-D-0188 or OHRS Assurance No. 4165 and established from colony-forming units as previously reported [21]. The osteogenic differentiation capabilities of these cells were confirmed by bone tissue formation following in vivo transplantation into immunocompromised mice (courtesy of Dr. Pamela Robey at National Institutes of Health). The cells were plated at 40,000 per cm^2 in Minimum Essential Medium Eagle Alpha Modifications (α-MEM; Life Technologies, Grand Island, NY) containing 20% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA), 1% penicillin and streptomycin (Life Technologies) and 1% L-glutamine (Life Technologies). We used this medium formulation as the "expansion medium". Cells were cultured at 37 °C in an atmosphere of 5% CO_2. Non-adherent cells were washed away 24 h later. For subculture, hBMSCs were detached with 0.05% trypsin–EDTA (Life Technologies) and expanded at a 1:3 ratio. Cells were passaged three times, harvested and then plated for experiments.

hBMSCs were cultured in either maintenance or osteogenic medium throughout the experiments. The "maintenance medium" consisted of α-MEM, 5% FBS, 1% penicillin/streptomycin, 1% L-glutamine and a variable amount of $MgSO_4$ (5, 10 and 20 mM for Alizarin red staining assay, 10 and 100 mM for proliferation assay, and 10 mM for gene and protein expression analysis; Sigma Aldrich, St. Louis, MO). α-MEM, as purchased, contains 0.8 mM $MgSO_4$ (this concentration of $MgSO_4$ was considered the control group). Osteogenic differentiation of hBMSCs was induced by culturing in "osteogenic medium", which contained α-MEM, 5% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 50 μM ascorbic acid, 100 nM dexamethasone and 10 mM β-glycerol phosphate (Sigma–Aldrich, St. Louis, MO). Finally, a "SO_4^{2-} control medium" was formulated in the same manner as the maintenance medium, but with the $MgSO_4$ substituted by Na_2SO_4 (Fisher Scientific, Pittsburgh, PA). The contents of all medium used for cell culture are summarized in Table 1.

2.2. Cell proliferation assay

hBMSCs were plated at 1×10^5 per well in six-well plates in expansion medium. After 24 h, the medium was switched to maintenance or osteogenic medium containing 0.8, 10 or 100 mM $MgSO_4$, with three biological replicates per group. Cells were detached with 0.05% trypsin–EDTA at 1, 3, 5 and 7 days, and the number of live cells was counted using a hemocytometer. The dead cells were excluded using the Trypan blue stain.

2.3. Alizarin red staining

hBMSCs were plated in six-well plates at a density of 1×10^5 cells per well in expansion medium. Twenty-four hours after plating, the medium was switched to 0.8, 5, 10 or 20 mM $MgSO_4$ osteogenic medium or Na_2SO_4 (SO_4^{2-} control medium), with three biological replicates per group, and cultured for 3 weeks. The cells were then fixed in 10% formalin for 1 h and washed with phosphate-buffered saline. The calcium nodules in the ECM were stained with a solution of 1% Alizarin red (Sigma Aldrich) in 2% ethanol for 5 min. Following incubation, the stain was removed and washed repeatedly with ddH_2O. Finally, the amount of Alizarin red bound to the calcium nodules was quantified by dissolving the stained ECM into 1% cetylpyridinium chloride (CPC) solution and reading the optical density at 540 nm using a plate reader (Spectramax 190, Molecular Devices, Sunnyvale, CA).

2.4. Assessment of gene expression

2.4.1. RNA extraction and purification

hBMSCs were plated in six-well plates at a density of 1×10^5 cells per well in maintenance or osteogenic medium (0.8 and 10 mM $MgSO_4$), with three biological replicates per group, and cultured for 3 weeks. Total RNA was extracted and purified using RNeasy Mini Kit (Qiagen, Valencia, CA) and treated with RNase-free DNase (Qiagen) to eliminate genomic DNA according to manufacturer's instructions. The quantity and quality of RNA was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Total RNA samples were cleaned using RNA Clean & Concentrator™-5 (Zymo Research Corporation, Irvine, CA) until the ratio of absorbance readings at 230–260 nm was greater than