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Basic nutritional investigation

Milk basic protein supplementation enhances fracture healing in mice

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ABSTRACT

Objectives: There is an unmet need for agents that can stimulate bone healing. The goal of this study was to evaluate the effects of basic proteins from milk whey (milk basic protein [MBP]) on fracture healing in mice.
Methods: Closed tibial transverse fractures were generated in 6-wk-old male C3 H/HeJ mice given either tap water or MBP-supplemented water for 3, 7, 14, 28, and 56 d after fracture generation. The tibial tissues were analyzed by radiography, μCT, and a three-point bending test. The expression levels of genes associated with bone metabolism were analyzed by real-time reverse transcription-polymerase chain reaction.
Results: Quantitative μCT analysis showed that MBP-treated fractured tibiae had a larger hard callus in the sectional area and a larger volume compared with fractured tibiae without MBP treatment. The expression levels of genes associated with chondrogenesis and osteogenesis showed greater increases in fractured tibiae with MBP treatment. Significant increases in the callus mechanical properties were found in MBP-treated tibiae.
Conclusions: MBP supplementation has the potential to improve fracture healing and bone strength in mouse tibiae. MBP could be a potential safe, low-cost, and easily administered nutritional element to prevent secondary fractures in patients with bone fractures.

Introduction

Bone fractures are among the most common traumatic injuries. Recently, osteoporosis-related fractures have been the fastest-growing healthcare problem for our aging society with a significant negative economic effect [1]. The increasing life expectancy of the general population creates an ever-increasing risk of fractures. Long bone fractures, which normally require a few months for recovery, confer a large burden to the patient associated with inconvenience, pain, and frequent disability. Therefore, there is significant interest in treatments that can enhance the rate of fracture repair and provide more rapid recovery.

Recently, there have been several new anti-bone resorption agents available for osteoporosis patients, and some of them have been reported to affect fracture union and bone repair [2–4]. There have been potential pharmacologic therapies such as systemic treatment with the anabolic agents teriparatide, 1–34-amino-acid fragment of recombinant human parathyroid hormone, and bone morphogenetic proteins (BMPs), and local or systemic bisphosphonate treatment. However, there are inconclusive reports for human clinical treatments. Furthermore, because these agents require specialized medical care and are expensive, there is still a need for safe nutritional supplementation for management of fractures to regain normal biomechanical properties.

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Bovine milk protein contains positive modulatory effectors of bone health [5–7]. The active components of dairy products that directly affect bone metabolism have previously been reported [5]. Takada et al. [8] demonstrated that milk soluble protein whey enhanced bone strength in ovariectomized rats. Furthermore, Toba et al. [9] reported that basic proteins fractioned from milk whey (milk basic protein [MBP]) suppressed bone resorption in vitro and that the breaking energy of excised femurs from ovariectomized rats with MBP supplementation in their diet showed higher values than those from rats with a normal diet. In humans, MBP supplementation led to increased bone mineral density (BMD) in the lumbar spine in both young [10] and older [11] women. Possible active components of MBP that inhibit bone resorption are cystatin C [12] and lactoperoxidase [13]. There are few studies demonstrating the effects of milk whey on bone remodeling processes.

Fracture healing is a unique and complex physiological process that results in unscarred restoration of bone tissue [14]. Endochondral fracture healing requires primary cartilage formation and subsequent replacement of the cartilaginous soft callus by a hard bony callus with ossification. Over the time course of fracture healing, multiple cellular lineages give rise to cartilage, bone, vascular, and hematopoietic tissues that constitute skeletal tissues.

To understand whether systemic MBP supplementation affects fracture healing, we created a mouse model of mid-diaphyseal fracture stabilized with an intramedullary pin, and added MBP to the drinking water. We analyzed the tissue responses to MBP administration after bone fracture by radiography, μCT, and a three-point bending test. Furthermore, to elucidate the possible mechanisms for how MBP affects bone remodeling, the mRNA expression levels of modulators of bone metabolism were evaluated by real-time reverse transcription-polymerase chain reaction (RT-PCR).

Materials and methods

Animals

The protocols for animal experimentation in this study were reviewed and approved by the Animal Care and Use Committee, Kyushu University. Six-wk-old male C3 H/HeJ mice were obtained from Japan SLC Inc. (Shizuoka, Japan) and kept on a 12-h/12-h light/dark cycle at 20–25°C under specific pathogen-free conditions. The mice were fed a standard rodent diet (CE-2; CLEA Japan, Tokyo, Japan) and had free access to water. Body weights and amounts of food and water consumption were measured after the designated experimental time periods.

Tibial fracture and MBP treatment

Unilateral closed transverse fractures were produced in the tibiae of isoflurane-anesthetized 6-wk-old male mice according to the standard experimental fracture procedure described in the Bonnarens and Einhorn three-point bending method in 1984 [15]. Briefly, a short incision was made through the medial skin of the proximal tibia and a sterilized 0.3-mm cobalt chrome wire was inserted into the intramedullary canal through the tibial condyle. The pin was cut as close as possible to the knee articular cartilage. Fractures were then created using a customized three-point fracture apparatus. All bones were assessed by radiography at surgery and at harvesting. Fractures that did not meet the standard criteria were omitted from the subsequent analyses.

The mice were randomly divided into three groups: Fracture group; fracture plus 0.165% MBP; and fracture plus 1% MBP group. In the MBP-supplemented groups, MBP was dissolved in drinking water at 0.165% or 1% for administration. The MBP concentration was determined by reference to a report by Toba et al. [9], who demonstrated that 0.1% MBP-supplemented ovariectomized rats showed greater bone strength and higher BMD than ovariectomized rats without MBP supplementation. We used 0.165%, based on our estimated concentration of bovine cystatin C as a possible active component of MBP. We also added 1% MBP to our trial.

MBP was kindly provided by the Milk Science Research Institute (Megmilk Snow Brand Co. Ltd., Kawagoe, Japan) as described by Toba et al. [9]. Briefly, MBP was prepared from fresh skimmed milk. The protein concentration of MBP was 98%, and the MBP fraction contained several minor components including cystatin C [12], angiogenin [16], and lactoperoxidase [13].

Quantitative μCT evaluation

For μCT analysis, mice were perfused transcardially with phosphate-buffered saline, followed by a fixative comprising 4% paraformaldehyde in phosphate buffer (pH 7.4) at 14, 28, and 56 d after fracture or treatment. Intramedullary pins were removed from the tibiae before imaging. Images were acquired using a SkyScan 1076 (SkyScan, Kontich, Belgium) at 9-μm resolution, 48 kV, and 200 μA with a 0.5-mm-thickness aluminum filter and 1.1° rotation between frames. The CT images were reconstructed in 4000 × 2672 pixel matrices using a standard convolution-back projection procedure. The resulting gray-scale images were processed using InstaRecon software (InstaRecon Inc., Champaign, IL, USA). The volume of interest in each fractured tibia was defined by the entry points of 200 slices both proximal and distal to the anterior fracture line in the axial direction and by the outer diameter of the callus in the radial direction. This resulted in a 3609-μm-thick square cuboid comprising any mineralized tissue surrounding the fracture (Appendix Fig. 1). Analyses of bone parameters were performed using CTan software (ver. 1.8.1.5; SkyScan). The bone parameters of bone volume relative to total tissue volume (BV/TV), trabecular thickness (Tb.Th.), trabecular number (Tb.N), and trabecular separation (Tb.Sp) were measured. BMD was calibrated with known BMD values as the volumetric density of calcium hydroxyapatite at 0.25 and 0.75 g/cm^3 with the CTan software.

Quantitative real-time RT-PCR

The mice were euthanized by an overdose of pentobarbital at 3, 7, 14, or 28 d after the onset of the experiments. We compared the fracture group and fracture plus 0.165% MBP group, because there were significant relative differences between these groups. Total RNA was extracted from the tibiae using the TRIzol reagent (Life Technologies, Tokyo, Japan). cDNA was synthesized from the total RNA using a ReverTra Ace qPCR RT Kit (Toyobo Co. Ltd., Osaka, Japan). Aliquots (1 μg) of the RNA extracts were treated in parallel in the presence or absence of reverse transcriptase. PCR was performed with gene-specific primer sets based on published sequences using Mac Vector (Ceres Bioscience, Saitama, Japan) to obtain PCR products of 50–250 bases (Table 1). All primer sets were designed to span exon–exon junctions to minimize the possibility of amplifying genomic DNA. Quantitative PCR was performed using SYBR premix EX Taq (Takara Bio, Otsu, Japan) and a Rotor Gene 300 (Qiagen K.K., Tokyo, Japan) according to the manufacturer's protocols. We evaluated actin, ATP synthase, H^+ transporter, mitochondrial Fo complex, subunit B1 (ATP5 f1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase 1 (HPRT1),

Table 1
Primer sequences used for quantitative RT-PCR

Gene	Amplicon size	Forward (5′–3′)	Reverse (5′–3′)	GenBank Acc. No.
Runx2	97	GACGAGGCAAGAGTTTCACC	GGACCGTCCACTGTCACTTT	NM_001146038.1
Osterix	166	CCACTGGCTCCTCGGTTCT	GTCCCGCAGAGGGCTAGAG	NM_130458.3
ALP	68	CTGATCATTCCCACGTTTTC	GAGCCAGACCAAAGATGGAG	NM 007431.2
Sox 9	144	AGTACCCGCATCTGCACAAC	TACTTGTAATCGGGGTGGTCT	NM_011448.4
RANKL	197	TGTACTTTCGAGCGCAGATG	CCACAATGTGTTGCAGTTCC	NM 011613
OPG	225	CTGCCTGGGAAGAAGATCAG	TTGTGAAGCTGTGCAGGAAC	NM_008764.3
TRAP	131	ACCTTGGCAACGTCTCTGCAC	GTCCAGCATAAAGATGGCCACA	NM_001102404.1
β-catenin	159	ACCTTTCAGATGCAGCGACT	TGGCACACCATCATCTTGTT	BC048153
Collagen III	173	CAGGCCAGTGGCAATGTAAAGA	CTCATTGCCTTGCGTGTTTGATA	NM_009930.2
ATP5 f1	109	AACATGATGCGTCGCAAGGA	CAATGCACTTGGCAATGGTCTC	NM_009725.3