Content of Bone Morphogenetic Protein-4 in Human Demineralized Bone: Relationship to Donor Age and Ability to Induce New Bone Formation

Sittisak Honsawek*, Dhakoon Dhitiseith*

* *Department of Biochemistry, Faculty of Medicine, Chulalongkorn University*

Background: *Bone morphogenetic proteins (BMPs) are also called growth and differentiation factors (GDFs) and form a subfamily of related proteins within the TGF-β superfamily. BMP-4 is one of multifuntional growth factors with pleiotropic roles in many different cell types and is predominantly present in human bone tissue.*
Objectives: *To analyze the content of extractable BMP-4 in human demineralized bone as a function of age*
Material and Method: *Bone samples were ground and demineralized by exposure to 0.5 N HCl and then extracted by collagenase digestion. Extractable BMP-4 was analyzed using a commercially available enzyme-linked immunosorbent assay (ELISA).*
Results: *63 samples of demineralized bone matrix (DBM) derived from 36 men and 27 women between the ages of 15-65 years. The extractable BMP-4 content appears to be age-dependent, with DBM from younger donors being most likely to have higher BMP-4 quantity. In addition, DBM with high osteoinductivity contained greater amounts of extractable BMP-4 than DBM samples with low osteoinductivity.*
Conclusion: *The BMP-4 in demineralized bone undergoes age-related decrease that may contribute to the reduction of bone volume observed with aging.*

Keywords: *Bone, Demineralized, BMP-4, Donor age, Osteoinductivity*

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Demineralized bone matix (DBM) can induce ectopic endochondral formation that can be utilized to enhance bone healing in a number of clinical applications. DBM can be prepared by acid extraction of allograft bone, leading to loss of some mineralized component but retention of collagen and noncollagenous proteins, including cytokines and growth factors. DBM possesses substantial abilities for regeneration, remodeling, and repair. It has been demonstrated that bone protein extracts, implanted into small animals at heterotopic sites, induce the formation of new bone tissues [1]. The sequential bone development cascade is reminiscent of cartilage and bone morphogenesis, and it is regulated by osteoinductive factors including bone morphogenetic proteins (BMPs) and transforming growth factor-β (TGF-β) [2, 3]. Among proteins in the BMP family, BMP-4 is able to induce de novo chondrogenic and osteogenic formation and appears to be influential for skeletal development during mammalian embryogenesis [4]. BMP-4 also plays a crucial role in bone remodeling and fracture repair, as demonstrated by the increase of the BMP-4 expression during fracture healing [5-7]. Previous studies have shown that the expression of PEB2αA/AML3/CBFA1 gene, a transcription regulator that plays a central part in osteoblast differentiation [8], is regulated by BMP-4/7 heterodimer. The PEB2αA/AML3/CBFA1 gene overexpression inhibits collagen type I and osteocalcin gene expression in osteoblastic cells, therefore BMP-4 is literally associated with the determination of osteoblast phenotype and bone turnover. The objective of the present study was to determine whether the extractable BMP-4 content depends upon the age and gender of the DBM donor. The authers postulate that variability in BMP-4 content extractable from DBM could be attributed to the age and gender of the DBM donor.

Correspondence to : Honsawek S, Department of Biochemistry Faculty of Medicine Chulalongkorn University and Hospital Rama IV Rd, Patumwan, Bangkok 10330, Thailand. Phone: 0-2256-4482, Fax: 0-2256-4482, E-mail: fmedshs@md.chula.ac.th

Material and Method

Preparation of demineralized bone matrix

Bone samples of 63 donors provided by Dr. Lloyd Wofinbarger (36 men between the ages of 15-65 years and 27 women between the ages of 17-65 years) were ground and demineralized by exposure to 0.5 N hydrochloric acid (HCl). Demineralized bone matrices of variable calcium content were obtained by removing bone matrix from the acid at various time intervals. The variably demineralized bone matrices were washed, freeze dried, and stored at –80ºC. The demineralization process was performed aseptically with no additional sterilization of the DBM before use in the bioassays.

Preparation of DBM protein extracts

Ground bone matrix (size from 250 to 850 micron) was demineralized by exposure to 0.5 N HCl, after which the ground demineralized bone matrices were freeze dried and stored at –80ºC. DBM samples were extracted by collagenase digestion as described previously [9]. Briefly, demineralized bone matrices with particle size ranging from 250-850 micron were digested with type I collagenase (Sigma Chemical Co, St Louis, USA) in 200 mM Tris-HCl buffer, pH 7.2 with 3 mM $CaCl_2$, 3 mM $MgSO_4$, 20 mM NaCl, 3 mM N-ethylmaleimide (NEM), 0.1 mM phenylmethanesulfonyl fluoride (PMSF), and 0.1 mM benzamidine-HCl at 37ºC for 24 hours with continuous shaking. The mixture was then centrifuged and the supernatant dialyzed against distilled water at 4ºC overnight. The dialysate was recovered and stored at –20ºC until assayed for BMP quantity.

Quantitative BMP-4 immunoassay (ELISA)

Quantitative sandwich enzyme-linked immunosorbent assay was utilized to analyze BMP-4 content in protein extracts of DBM by a commercially available kit (R&D Systems, Minneapolis, MN, USA; sensitivity, 1.04 pg/ml; range, 31.2 to 2,000 pg/ml). Briefly, 200 µl of sample or appropriate standard were added to each well of a 96 well-microtiter plate precoated with anti-BMP-4 monoclonal antibody and incubated for 2 hours at room temperature. Following a wash to remove any unbound antibody-enzyme reagent, 200 µl of a substrate solution was added to the well and incubated for 30 minutes at room temperature. The color development was stopped with 50 µl of 2 N sulfuric acid and the intensity of the color was measured using a microplate reader (Multiskan Ascent, Labsystems, Franklin, MA, USA) at 450 nm. The concentration of BMP-4 in sample extracts was determined by comparing the optical density of the sample to a standard curve.

In vivo athymic mouse bioassay

Eight to ten-week old male athymic mice were anesthetized. Implants of rehydrated DBM were packed into muscle pouches created bilaterally within the longissimus dorsi muscle. After 28 days of implantation, the implants were isolated from the muscle pouches and cleaned of excess tissue. The explants were then fixed in 10% neutral phosphate buffered formalin, decalcified in 10% formic acid solution, embedded in paraffin, sectioned, and subsequently stained with hematoxylin and eosin. The areas of new bone and total bone (new bone and implant bone) were measured using histomorphometric analysis. The percentage of new bone formation was expressed relative to the total cross-sectional area measured.

Statistical analysis

The data are represented as means with error bars representing standard error of the mean (SEM). Analysis of variance (ANOVA) was used to determine the significant differences among the age . Tukey-type multiple comparison tests were used for post comparing means of more than two groups in one way ANOVA analyses. A p value of less than 0.05 was considered to be statistically significant.

Results

It is hypothesized that donor age and gender may affect the amounts of BMP-4 extractable from DBM of similar particle size ranges and degree of demineralization. Accordingly, demineralization bone samples derived from different age groups of both male and female donors were extracted and analyzed using BMP-4 ELISA. All DBM utilized in this donor age and gender study comprised approximately 2% residual calcium and contained bone particles in size ranging from 250 to 850 microns. DBM from variable donors were categorized into five age groups: 0-19, 20-29, 30-39, 40-49, and greater than 50 years. As illustrated in Fig. 1, the content of BMP-4 in DBM declined by 41% in male donors (Fig. 1a) and by 43% in female donors (Fig. 1b) between the age group 0-19 years of age and 50 years of age and older. Analysis of the BMP-4 contents of the DBM samples demonstrated a significant reduction of the BMP-4 content in male donors between the 30-39 year-old age group and the 40-49, $\geq$ 50 year-old age group from 3998.2 to $\geq$ 2746.0 pg/g of DBM (P=0.04, Fig. 1a). A similar decrease was observed in female donors between the 0-19 year-old age group and the 30-39, 40

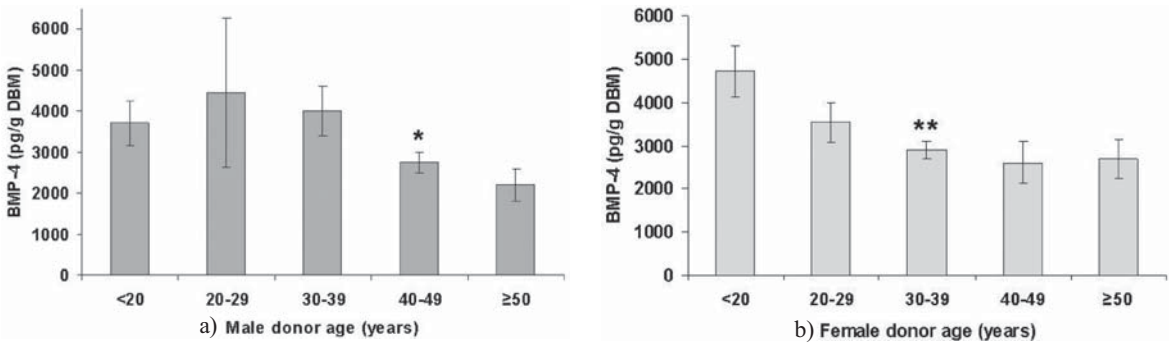

Fig. 1 Donor age-related alterations in BMP-4 content from DBM samples in (a) Male population (n= 36) (b) Female population (n= 27). Data are expressed as means with error bars representing SEM

* P=0.04 between the age group 30-39 and 40-49, ≥50 years

** P<0.05 between the age group <20 and 30-39, 40 – 49, ≥ 50 years

– 49, ≥ 50 year-old age group, from 4712.6 to ≤ 2894.8 pg/g of DBM (p<0.05, Fig. 1b). When DBM were analyzed within these specific age ranges, the results showed an age-related decline in the bone matrix level of BMP-4 (Fig. 2). The average content of extractable BMP-4 from DBM was 3248.5 pg/g of DBM. Interestingly, the BMP-4 contents in these bone samples from donors in the age group 0-19 years of age were almost two fold higher compared with the BMP-4 contents of 50 years of age and older, indicating that BMP-4 contents markedly decline during the fourth and fifth decade of life (Fig. 2). When compared according to donor gender, BMP-4 did not show any significant variation between male and female donors (data not shown). Not all of the DBM samples yielded the same capability to induce new bone formation. The osteoinductivity of DBM materials is presumably attributable to the availability of bone growth factors and/or BMP present in DBM. Hence, it is critical to determine the association between the osteoinductivity in the athymic mouse assay and the contents of BMP-4 extractable from DBM. The abilities to induce new bone formation of DBM from the same donors quantified by BMP-4 ELISA were analyzed using the in vivo athymic mouse assay. Following 28 days, the implants were recovered and processed for histomorphometric analysis measuring the area of new bone formed and the area of residual implant. The results showed that samples of DBM with low osteoinductivity produced a small amount of new bone formation and numerous DBM particles were not surrounded by cellular differentiation and activity. For good and excellent osteoinductivity, the DBM particles were surrounded by mesenchymal cells, calcified cartilage, or newly formed bone with osteoblasts and osteocytes. As displayed in Fig. 3, the data showed that

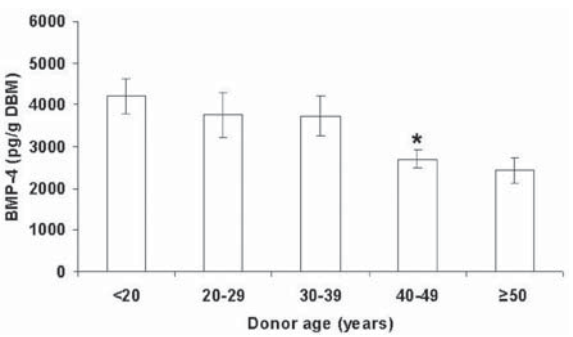

Fig. 2 Donor age-dependent decrease in BMP-4 content from DBM samples in the total population (n= 63). Data are expressed as means with error bars representing SEM. * P<0.05 between the age group 30-39 and 40-49, ≥ 50 years

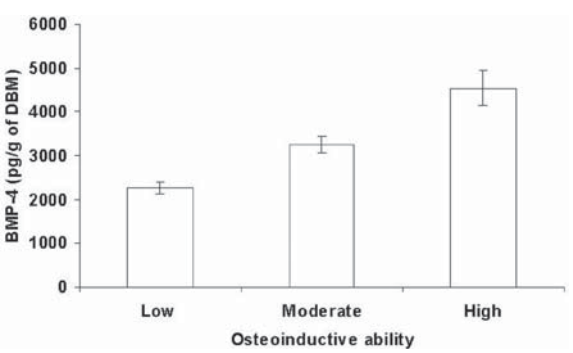

Fig. 3 Relatioship between extractable BMP-4 contents determined by ELISA and osteoinductive ability of 63 different donors of DBM determined by histomor-phometric analysis. Data are expressed as means with error bars representing SEM

DBM samples possessing high osteoinductivity in the athymic mouse assay contained greater extractable BMP-4 levels from these DBM than those DBM samples exhibiting low activity of osteoinduction.

Discussion

Bone morphogentic protein-4 (BMP-4) is one of fifteen structurally related BMPs belonging to the transforming growth factor-β (TGF-β) superfamily of secreted proteins. Mature BMP-4 is a dimer that binds to a multimeric transmembrane receptor with serine/threonine kinase activity. Although discovered because it stimulates bone formation in adult mammals, BMP-4 plays a central role in a variety of processes during embryonic development including bone formation [10], early mesoderm formation [11, 12], and epithelial-mesenchymal interaction [13]. BMP-4 also is involved in prenatal and postnatal endochondral ossification [10]. Some have suggested that deregulation of BMP-4 expression is associated with fibrodysplasia ossificans progressiva [14]. In addition to the heterotopic bone formation, it is suggested that BMP-4 plays a role in bone fracture repair. BMP-4 is an osteogenic growth factor that has unambiguously been shown to promote the osteogenic phenotype in vitro [15] and is expressed during endochondral bone formation in normal fracture healing [6].

Aging is described by a decrease in bone volume, a reduction that may be related with insufficient coupling of bone formation to bone resorption (i.e. net loss of bone exceeds net gain of bone). If age-associated bone loss is attributed in part to insufficient or impaired bone coupling, and if bone coupling is determined in part by bone growth factor content, then a decline in the content of bone growth factors could contribute to age-associated bone loss. Recent evidence has demonstrated that bone matrix contents of insulin-like growth factor-I (IGF-I), -II, and TGF-β decrease with aging in humans [16-19]. Accordingly, the authors postulated that donor age and gender may influence the BMP-4 contents extractable from DBM. Based on the quantitative determination of BMP-4 in the DBM, the present study reveals that there is an inverse relationship between the BMP-4 extractable from DBM and donor age, similar to the decline of IGF-I, -II, and TGF-β in bone matrix with age described in previous studies [16-19]. This decline in BMP-4 content with donor age is presumably ascribed to the direct age-related decreases in BMP-4 production by bone cells. In addition, the extractable BMP-4 content in DBM was significantly positively associated with the osteoinductive potential of DBM in the in vivo athymic mouse bioassay.

Three possible explanations could account for age-associated bone loss in terms of growth factor activities. First, the bone growth factor content could decrease as a function of aging, since the rate of bone growth factor synthesis by bone cells decreases with age. This interpretation would be consistent with the present results. Secondly, the rate of production of essential binding proteins that may be responsible for the deposition of bone growth factor could decrease as a function of age. Finally, the responses of bone cells to bone-derived growth factors decrease as a function of aging.

In conclusion, the present study has demonstrated an age-dependent decrease in the BMP-4 contents of human DBM. The present results indicate that the decrease in bone matrix BMP-4 with age may play a role in the reduction of ability to induce bone formation observed with aging.

Acknowledgement

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ความสัมพันธ์ระหว่างปริมาณของโปรตีนแยกสกัดชนิด bone morphogenetic protein-4 จากเนื้อเยื่อกระดูก demineralized bone matrix อายุของผู้บริจาค และความสามารถในการชักนำสร้างกระดูก

สิทธิศักดิ์ หรรษาเวก, ฐากูร ฐิติเศรษฐ์

คณะผู้ศึกษาได้ศึกษาปริมาณของโปรตีนแยกสกัดซึ่งเกี่ยวข้องกับการชักนำสร้างกระดูกชนิด bone morphogenetic protein-4 (BMP-4) จากเนื้อเยื่อกระดูก demineralized bone matrix (DBM) ของผู้บริจาคซึ่งมีอายุระหว่าง 15-65 ปี จำนวน 63 ราย และศึกษาความสัมพันธ์กับกลุ่มอายุของผู้บริจาค และความสามารถในการชักนำสร้างกระดูก พบว่ากลุ่มผู้บริจาคในวัยสูงอายุมีปริมาณของโปรตีน BMP-4 ที่แยกสกัดได้จากเนื้อเยื่อกระดูก DBM ต่ำกว่ากลุ่มผู้บริจาคที่มีอายุน้อยอย่างมีนัยสำคัญ นอกจากนี้ เนื้อเยื่อกระดูก DBM ของกลุ่มซึ่งมีความสามารถในการชักนำสร้างกระดูกได้ดี มีระดับของโปรตีน BMP-4 สูงกว่ากลุ่มที่มีความสามารถในการชักนำสร้างกระดูกได้ไม่ดี ปริมาณของโปรตีนแยกสกัดชนิด BMP-4 ที่ต่ำลงในผู้บริจาคที่มีอายุมากขึ้นนี้ อาจมีส่วนเกี่ยวข้องกับปริมาณเนื้อกระดูกที่ลดลง ซึ่งพบได้ในภาวะกระดูกโปร่งบางหรือกระดูกพรุนของผู้บริจาคที่มีอายุมากขึ้น