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Extractable bone morphogenetic protein and correlation with induced new bone formation in an *in vivo* assay in the athymic mouse model★

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Microabstract

A correlation between extractable bone morphogenetic proteins (BMPs) in demineralized bone matrix (DBM) and osteoinduction has been suggested. Extractable BMP-4 and osteoinductivity of DBM from 40 donors were assessed using enzyme-linked immunosorbent assay (ELISA) and *in vivo* athymic mouse assay, respectively. Extractable BMP-4 level averaged 3.7 ± 0.21 ng/g of DBM and correlated with osteoinductivity of the DBM in an *in vivo* assessment of induced newbone formation.

Abstract

Introduction: DBM contains BMPs that are essential factors for endochondral bone formation. Among proteins in the BMP family, BMP-4 is one of the most osteoinductive factors. At present, the most commonly used test method for demonstrating that a material or substance (DBM) is 'osteoinductive', as defined by its ability to cause bone to form *in vivo* at a site that would otherwise not support bone formation, for example heterotopic, is the athymic mouse or rat model. Although this heterotopic implant model is generally deemed to be suitable for specification acceptance, design purposes, service evaluation, regulatory statutes, etc., it should not be presumed to indicate suitability for forming bone in a clinical application. At present, the only reliable assays are *in vivo*, since the property of bone induction or conduction can only be assessed in a heterotopic orthotopic site in a living animal.

In vitro assays, mostly cell culture based, have been described and correlations made to the results obtained in *in vivo* assays. However such *in vitro* assays measure only some biochemical marker presumed to be associated with *in vivo* bone formation and are therefore generally less well accepted as an assay for new bone formation. Thus, only the *in vivo* assay method is currently considered relevant as a standard for assessing new bone formation by biomaterials. The objective of this study was to investigate a possible relationship between extractable BMP-4 in DBM and the ability of that DBM to induce new bone formation in an athymic (nude) mouse assessment model.

Materials and methods: DBM samples prepared from cortical bone derived from 40 donors were extracted by collagenase digestion and implanted heterotopically in an athymic (nude) mouse. BMP-4 in the protein extracts from these DBM samples were measured and quantitated using and ELISA assay method. The

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osteoinductivities of DBM from these donors were assessed using the *in vivo* athymic mouse assay. Correlation between BMP-4 level and osteoinductivity of DBM was analyzed by linear regression analysis (SPSS 10.00).
Results: The extractable BMP-4 levels were variable and ranged between 1.82 and 7.94 ng/g of DBM. We revealed that the less residual calcium left in DBM increased the extractability of BMP-4. The extractability of BMP-4 from DBM varied relative to the particle size such that DBM particles ranging from 550 to 710 μm resulted in the highest level of extractable BMP-4, whereas DBM particles less than 250 μm provided for the lowest level of extractable BMP-4. In the donor age and gender study, the extractable BMP-4 content appears to be age-dependent, with DBM from younger donors being most likely to have greater BMP-4 quantity. In contrast, no difference in the quantity of extractable BMP-4 was observed between male and female donors.
Conclusions: DBM exhibiting high osteoinductivity in the nude mouse bioassay also contained greater BMP-4 levels in the protein extracts from this DBM than did DBM samples possessing low levels of osteoinductivity. There was a positive correlation between quantitative BMP-4 *in vitro* assay and osteoinduction (percentage of new bone formation) determined by the implantation of DBM in the athymic mouse assay with a correlation coefficient of 0.74 ($p < 0.001$), providing that this quantitative BMP-4 *in vitro* assay could be an alternative means of assessing the osteoinductive potential of DBM.

Introduction

Demineralized bone matrix (DBM) has been extensively utilized in orthopaedic, periodontal, and maxillofacial applications and widely investigated as a material to promote new bone formation. Many studies have demonstrated that DBM comprises bone morphogenetic proteins (BMPs) that are substantial regulators for endochondral bone formation. It is postulated that the osteoinductive potential of DBM is predominently attributable to the availability of BMPs present in DBM. The use of DBM in the induction of new bone formation has been quantitatively assessed using *in vitro* cell culture based and *in vivo* animal based bioassays. Several *in vitro* bioassays have been established and utilized for evaluation of osteoinductivity of DBM. The results generally, however, require at least 7 days. The *in vivo* athymic mouse bioassay takes even longer, at least 4 weeks. Therefore, research endeavors have been made to develop a facile and expeditious assay for detection and quantitation of osteoinductive molecules and to correlate that with the ability of bone materials to induce new bone formation in an animal model.

BMPs belong to the transforming growth factor-β (TGF-β) superfamily. More than 15 BMPs have been identified and characterized. They are unique among growth and differentiation factors that influence osteoblast proliferation and differentiation since they have the ability to institute this process from uncommitted progenitors *in vitro* as well as *in vivo* (Kirker-Head et al. 1995; Rosen et al. 1996; Nifuji et al. 1997). The osteoinductive role of BMPs is multifaceted. BMPs are presumed to function as chemotactic factors, which initiate the recruitment of progenitor cells, as growth factors stimulating proliferation of mesenchymal stem cells, and as differentiation factors promoting maturation of these cells into chondrocytes, osteoblasts, and osteocytes. Among the BMP family of protein factors, BMP-4 is one of the most osteoinductive and has been investigated for clinical applications. In particular, BMP-4 plays a critical role in a variety of processes during embryonic development including bone formation (Leong and Brickell 1996), early mesoderm formation (Schmidt et al. 1995; Watanabe and Le Douarin 1996), and epithelial–mesenchymal interactions (Jones et al. 1991; Bennett et al. 1995). In addition, BMP-4 plays a role in fracture healing, as evidenced by the spatial and transient expression of BMP-4 messenger RNA (mRNA) in the early stages of fracture healing (Nakase et al. 1994), and the potential of BMP-4 to enhance new bone formation and to accelerate the healing process of spinal fusion (Cheng et al. 2002; Guo et al. 2002).

The objectives of the current study were to quantitate BMP-4 in protein extracts of DBM and

investigate the correlation between the levels of extractable BMP-4 from DBM and the osteoinductivity of that DBM in the *in vivo* athymic mouse bioassay. Furthermore, the relationships between residual calcium contents and particle size of DBM and the extractability of BMP-4 from those DBM samples were assessed. The effects of donor age and gender on the extractability of BMP-4 from DBM were also investigated.

Materials and methods

Preparation of demineralized bone matrix

Ground bone matrix (size from 250 to 850 μm) provided by LifeNet was demineralized by exposure to 0.5 N hydrochloric acid (HCl) according to US Patent Nos. 5,275,954 (Wolfinbarger 1994) and 6,189,537 (Wolfinbarger 2001). 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 −80 °C. The demineralization process was performed aseptically with no additional sterilization of the DBM before use in the bioassays.

Quantitation determination of calcium

Residual calcium levels were measured using a modification of the DMA calcium assay kit (DMA, Inc., Arlington, Texas, USA). The variably demineralized bone matrices were dissolved in 4.0 ml of 1 N HCl at 90 °C overnight. Fifty (50) μl aliquots (replicates of three) of each sample were mixed with 4.0 ml of calcium working reagent. Calcium working reagent was prepared by mixing equal amounts of color reagent and base reagent. Absorption was measured at 570 nm. Calcium concentrations were calculated from a standard curve using known concentrations of DMA calcium standard. The calcium content was expressed as weight percent calcium of bone matrix dry weight.

Preparation of protein extracts of DBM

Bone samples of 40 donors provided by LifeNet were ground and demineralized by exposure to 0.5 N HCl as described above, after which the ground demineralized bone matrices were freeze dried and stored at −80 °C. DBM samples were extracted by collagenase digestion as described previously (Jortikka et al. 1993). Briefly, demineralized bone matrices with particle size ranging from 250 to 850 μm 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 37 °C for 24 h with continuous shaking. The mixture was then centrifuged and the supernatant dialyzed against distilled water at 4 °C overnight. The dialysate was recovered and stored at −20 °C until assayed for BMP-4.

Western blot analysis

To determine the presence of BMP-4 in protein extracts of DBM, samples of protein extracts, together with rhBMP-4 (R&D Systems, Minneapolis, MN, USA), in Laemmli sample buffer were applied to 4–15% gradient gels and subjected to SDS–polyacrylamide gel electrophoresis. Western blot analysis was carried out after proteins were electrophoretically transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). Nonspecific hybridization was blocked by incubating the membranes in PBST (phosphate buffered saline with 0.1% Tween 20) containing 3% blocker for 1 h at room temperature. The membranes were then washed three times with PBST for 5 min per wash and placed in PBST containing 1% BSA (Bovine serum albumin) and mouse anti-BMP-4 monoclonal antibody (Chemicon International, Temecula, CA, USA) at a concentration of 1:2000 for 1 h at room temperature. The antibody was removed and the membranes were then washed three times in PBST and incubated in PBST with 1% BSA and goat anti-mouse IgG conjugated with horseradish peroxidase (Bio-Rad, Hercules, CA, USA) at a concentration of 1:5000 for 1 h at room temperature. Subsequently the membranes were washed three times in PBST, and protein bands were visualized by placing the membrane in Opti-4CN (4-chloro-1-naphthol) substrate solution until the desired level of signal was attained. The colorimetric detection was accomplished by rinsing the membrane in deionized H_2O for 15 min.

Quantitative BMP-4 immunoassay (ELISA)

Quantitative sandwich enzyme-linked immunosorbent assay was employed to determine BMP-4 content in protein extracts of DBM using a commercially available kit (R&D Systems, Minneapolis, MN, USA; sensitivity, 1.04 pg/ml; range, 31.2–2000 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 h 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 min 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 4 weeks 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 treatment groups. Tukey-type multiple comparison tests were used for comparing means of more than two groups in one way ANOVA analyses. Correlation between BMP-4 level and osteoinductive capability of DBM was analyzed by linear regression analysis (SPSS 10.00); A p value of less than 0.05 was considered to be statistically significant.

Results

Presence of BMP-4 in protein extracts of DBM

The present study was undertaken to determine whether BMP-4 could be extracted and identified using Western blot analysis. The protein extracts of DBM were separated on SDS–PAGE, transferred to PVDF membranes, and probed with a monoclonal antibody directed against human BMP-4. A serial dilution of recombinant human BMP-4 served as a positive control. Western blot analysis displayed the detection of a single band of 18 kDa that was consistent with that of BMP-4 (Figure 1). The results indicate the presence of BMP-4 in the protein extracts of DBM. However, the levels of extractable BMP-4 were unable to be accurately quantified using this Western blot technique.

The effects of residual calcium on extractability of BMP-4

The effects of residual calcium content on extractability of BMP-4 were assessed using BMP-4

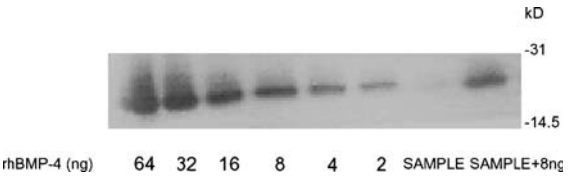

Figure 1. Western blot analysis of protein extracts from human demineralized bone and recombinant human BMP-4. Extracts were prepared as delineated in Materials and methods and then electrophoresed on SDS-gels. After electrophoresis, protein samples were transferred to PVDF membranes and stained with anti-BMP-4 monoclonal antibody. Lanes 1–6: rhBMP-4 64, 32, 16, 8, 4, and 2 ng, respectively; Lane 7: protein extracts of DBM; Lane 8: protein extracts of DBM spiked with 8 ng of rhBMP-4.

ELISA. As shown in Figure 2, the data from samples of bone matrix containing 18–0.05% residual calcium indicated that as the residual calcium level decreased, the extractability of BMP-4 increased. The level of extractable BMP-4 was undetectable in non-demineralized bone matrix (25% residual calcium) with this BMP-4 ELISA (data not shown). In contrast, the DBM demineralized to a residual calcium content of 0.05% produced the highest level of extractable BMP-4 among the variable residual calcium level group tested (Figure 2). In general, the relationship between the residual calcium contents of 25 (non-demineralized bone) to 0.05 weight percent and the extractability of BMP-4 from DBM can be expressed as: $Y = -359.23X + 2198.7$, ($R = 0.9745$), where Y is the extractable BMP-4 level of DBM and X is the residual calcium content of DBM.

ANOVA analysis indicated that there were significant differences among the extractability of BMP-4 from DBM with different degrees of residual calcium ($p < 0.05$). Tukey-type multiple comparison test revealed that the extensively demineralized bone matrix (0.05% calcium content) had significantly higher extractable BMP-4 level than DBM with residual calcium contents of 0.8, 1.8, 9.0, 14.5, and 18.0% and yielded the highest BMP-4 level in the protein extracts derived from DBM.

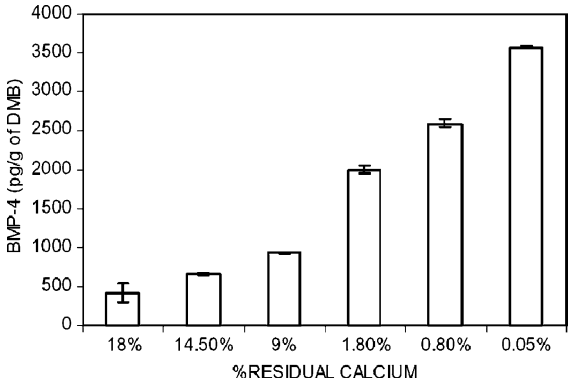

Figure 2. BMP-4 levels in protein extracts of demineralized bone particles containing variable levels of residual calcium. DBM with different levels of residual calcium were extracted and extractable BMP-4 levels were determined using BMP-4 ELISA. The residual calcium levels were measured using the DMA Calcium assay. Particle sizes of DBM used here were 250–850 μm. Data are shown as means with error bars representing standard error of the mean (SEM).

The effects of particle size on extractability of BMP-4

The effects of particle size on extractability of BMP-4 were assessed using BMP-4 ELISA. The effects of particle size differences on extractability of BMP-4 are illustrated in Figure 3. DBM in the 550–710 μm size range exhibited the highest levels of extractable BMP-4 when compared to similarly demineralized DBM of different particle size ranges. From the <250 μm particle size range of DBM to the 550–710 μm particle size range of DBM, as the particle size increased the extractability of BMP-4 from DBM increased. Smaller particle sizes and larger particle sizes resulted in lower levels of extractable BMP-4, such that DBM bone particles in the 355–550 μm and 710–850 μm particle size groups provided for equivalent levels of extractable BMP-4. DBM in the <250 μm bone particle size range showed extractable BMP-4 levels which approximated 64% of the highest extractable BMP-4 levels observed, and DBM in the 250–355 μm bone particle size range yielded extractable BMP-4 levels approximating 72% of the highest extractable BMP-4 levels observed. ANOVA analysis revealed that there were significant differences among the extractable BMP-4 levels of DBM in the different bone particle size groups ($p < 0.05$). Tukey-type multiple comparison test indicated that the 550–710 μm bone particle size group had significantly greater extractable BMP-4 than DBM in all other

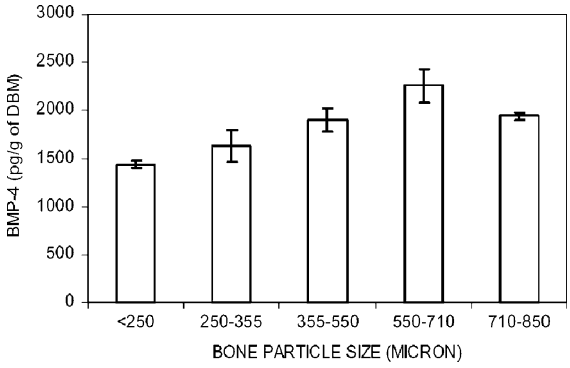

Figure 3. BMP-4 levels in protein extracts of demineralized bone particles of variable particle size ranges. DBM with different particle size ranges were extracted and the extractable BMP-4 levels were determined using BMP-4 ELISA. Data are shown as means with error bars representing standard error of the mean (SEM).

bone particle size groups tested. There were no significant differences in extractable BMP-4 levels of DBM between <250 and 250–355 μm, 250–355 and 355–550 μm, and 355–550 and 710–850 μm bone particle size groups.

Donor-related effects on extractability of BMP-4

It is postulated that donor age and gender may influence the amounts of BMP-4 extractable from DBM of similar particle size ranges and degree of demineralization. Accordingly, demineralized bone samples from variable age groups of both male and female donors were extracted and quantified using BMP-4 ELISA. All DBM used in this donor age and gender study contained approximately 2% residual calcium and contained bone particles in the size range from 250 to 850 μm. DBM from different donors were classified into three age groups: 0–25, 26–44, and greater than 45 years. As shown in Figure 4, there was an age-dependent decrease in the level of extractable BMP-4, as measured by ELISA when the DBM was analyzed within these specific age ranges. When the results were analyzed according to both donor age and gender, as illustrated in Figure 5, there were both donor age and gender-related effects on the quantity of extractable BMP-4, as measured in the BMP-4 ELISA, obtained from DBM. Interestingly, the results of this study revealed that DBM derived from female donors contained the highest extractable BMP-4 when derived from donors in the age group 0–25 years of age than from donors in all other age groups tested, whereas there was no statistically significant difference in the extractable BMP-4 among DBM samples derived from male donors (Figure 5). When compared within each age group, no significant difference in the levels of extractable BMP-4 was observed between male and female donors in the age group 0–25 years of age. In contrast, DBM from male donors in the age group 26–44 years of age and 45 years of age and older possessed significantly more extractable BMP-4 ($p < 0.05$) than those from female donors in the same age group. In general, however, there was no statistically significant difference in the extractable BMP-4 levels of DBM between male and female donors, regardless of donor age (data not shown).

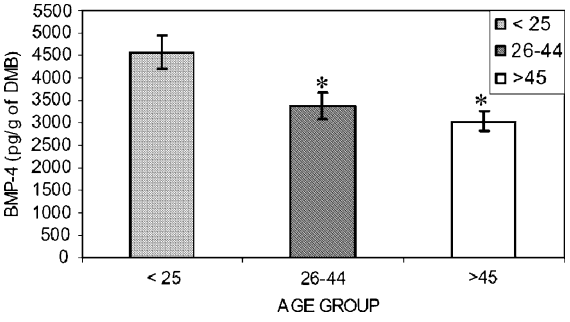

Figure 4. The effect of the age of the donor on the extractability of BMP-4 from DBM ($n = 40$). DBM from different donors were divided into three age groups as described and extracted by collagenase digestion. Extractable BMP-4 level of DBM was measured using BMP-4 ELISA and expressed as pg of BMP-4 per g of DBM. Data are shown as means with error bars representing standard error of the mean (SEM). $*p < 0.05$ for comparison with <25 years old age group.

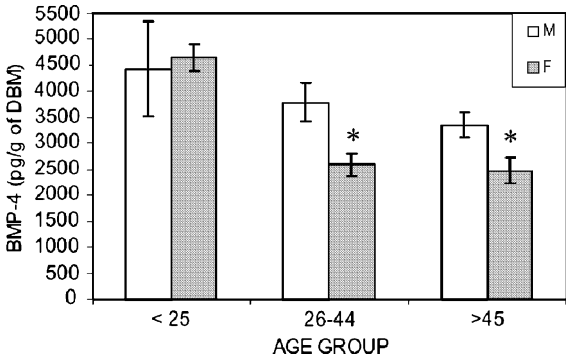

Figure 5. The effect of donor age and gender on the extractable BMP-4 levels present in DBM ($n = 40$). DBM from different donors was categorized into the age and gender groups as described and extracted by collagenase digestion. Extractable BMP-4 level of DBM was measured using BMP-4 ELISA and expressed as pg of BMP-4 per g of DBM. Data are shown as means with error bars representing standard error of the mean (SEM). M, male; F, female. $*p < 0.05$ for comparison between male and female within the same age group.

Correlation between extractable BMP-4 levels in DBM and osteoinductivity

It is presumed that the osteoinductive potential of DBM is predominantly attributed to the availability of BMP present in DBM. Among the BMP family, BMP-4 is one of the most osteoinductive

factors and has been investigated for clinical applications. Therefore, it is essential to investigate the relationship between the levels of extractable BMP-4 from DBM and the osteoinductivity of that DBM in the *in vivo* athymic mouse bioassay. Bone samples of 40 donors demineralized under production conditions at LifeNet were extracted and analyzed by BMP-4 ELISA. As demonstrated in Figure 6, the amounts of the extractable BMP-4 in the protein extracts of DBM were variable among DBM samples. The extractable BMP-4 levels ranged between 1.82 and 7.94 ng/g of DBM (mean = 3.7 ± 0.21 ng BMP-4/g of DBM). In addition, the osteoinductivity of DBM from the same donors quantitated by BMP-4 ELISA were assessed using the *in vivo* athymic mouse bioassay. After 4 weeks (28 days), the implants were recovered and processed for histology. Histomorphometric analysis was used to measure the area of new bone formed and the area of residual implant material. The results revealed that inactive DBM (negative control) showed no new bone formation and the implants were surrounded by connective tissue (Figure 7a). Active DBM, in contrast, demonstrated new bone formation after 4 weeks in the athymic mouse assay model (Figure 7b). New bone formation was visible along the edge of residual DBM particles. Most formed cartilage was replaced by the newly formed bone. Figure 8 illustrates that DBM samples possessing high osteoinductivity in the nude mouse bioassay contained greater BMP-4 levels in the protein extracts from these DBM than those DBM samples exhibiting low levels of osteoinductivity. There was a good positive correlation between the quantitative BMP-4 *in vitro* assay and osteoinduction (percentage of new bone formation) determined by the implantation of DBM in the *in vivo* nude mouse bioassay with a correlation coefficient of 0.74 ($p < 0.001$).

Discussion

Bone morphogenetic proteins were originally identified as protein regulators of cartilage and bone formation. They have been implicated in embryogenesis and morphogenesis of various tissues and organs. They can regulate growth, differentiation, chemotaxis and apoptosis of a variety in cell types, including mesenchymal, epithelial, hematopoietic and neuronal cells. BMP-4 plays an

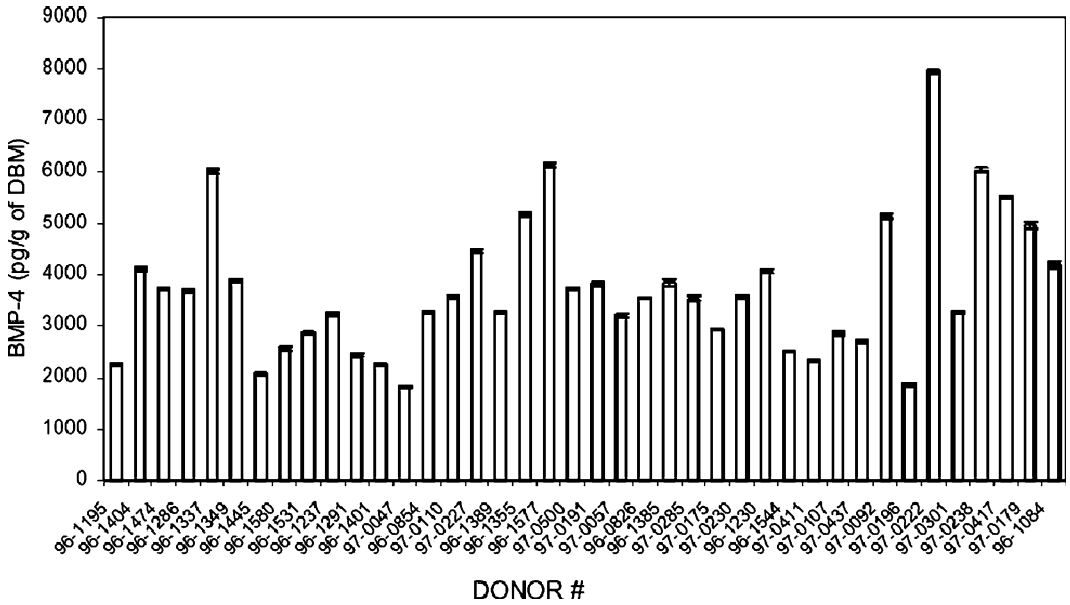

Figure 6. Levels of extractable BMP-4 in protein extracts of ground demineralized bone matrix. BMP-4 contents in protein extracts of 40 different DBM samples were analyzed using BMP-4 ELISA. All results are shown as means with error bars representing standard error of the mean (SEM).

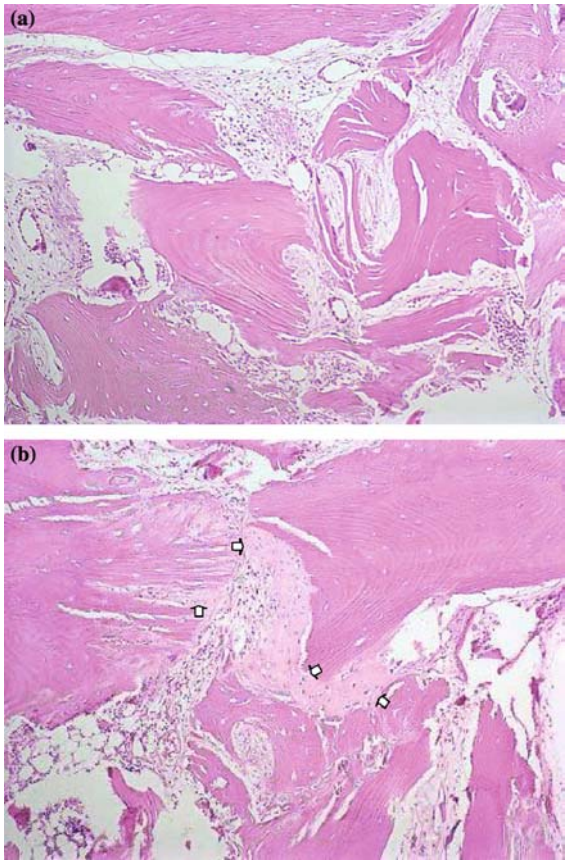

Figure 7. Hematoxylin and eosin stains of demineralized bone implanted in muscle pouch sites for 4 weeks in the athymic mouse assay model (20×). (a) Histological evaluation of inactive DBM reveals no new bone formation and inactive DBM were surrounded by connective tissue. (b) Histological evaluation of active DBM shows new bone formation along the edge of residual DBM particles. The newly formed bone is indicated by the arrows.

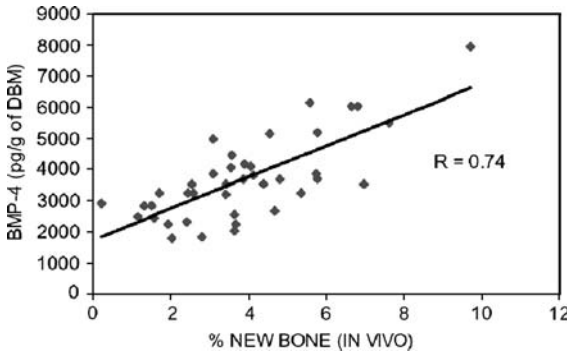

Figure 8. Correlation between extractable BMP-4 levels of DBM determined by ELISA and percentage of new bone formation (*in vivo*) determined by histomorphometric analysis. A total of 40 DBM samples from 40 different donors were divided into two portions, one portion was used in the quantitative BMP-4 *in vitro* assay with the second portion used in the *in vivo* athymic mouse bioassay. Correlation was analyzed by the linear regression analysis (SPSS 10.00). Regression coefficient (R) was 0.74 ($p < 0.001$).

essential role in the onset of bone formation and dorsal/ventral patterning (Graff 1997) and has been implicated in the commitment of embryonic mesodermal cells to a hematopoietic fate in a number of systems (Wozney 1992; Leong and Brickell 1996; Reddi 1998). BMP-4 is also involved in prenatal and postnatal endochondral ossification. Deregulation of BMP-4 expression is associated with fibrodysplasia ossificans progressiva (Xu and Shore 1998; Shore et al. 1998). In addition to this heterotopic bone formation, it also is suggested that BMP-4 plays a role in bone fracture repair. It has become evident that early in the process of fracture healing, the concentration of BMP-4 increases (Bostrom et al. 1995). Recombinant human bone morphogenetic protein-4 (rhBMP-4) effectively enhances new bone formation and accelerates fusion of the rabbit posterolateral posterior spinal fusion model in a dose-dependent fusion (Cheng et al. 2002; Guo et al. 2002). Additionally, recent studies showed that rhBMP-2-containing demineralized bone allograft resulted in a dose-dependent increase in new bone formation (Niederwanger and Urist 1996; Schwartz et al. 1998). It is generally accepted that the BMPs present in demineralized bone materials reflect to their ability to induce new bone formation.

In the current study, we investigated whether BMP-4 could be identified and quantitated in the protein extracts of DBM. The data demonstrated that BMP-4 was present and could be detected in the protein extracts of DBM using Western blotting and ELISA. A recent study has shown that BMP-4 was observed in the extracts of demineralized freeze-dried bone allograft (DFDBA) by slot blot analysis (Shigeyama et al. 1995). It is plausible that BMPs other than BMP-4 are also present in the extracts of DFDBA since BMP-2 and BMP-7 have been demonstrated to be present in the extracts of DFDBA, although we did not specifically investigate for these BMPs. Both BMP-2 and BMP-7 are also effective osteoinductive

proteins that are able to induce heterotopic bone formation (Wozney and Rosen 1998). Future investigations with BMP-2 and BMP-7 will need to determine the presence and quantities of BMP-2 and BMP-7 in the protein extracts of DBM as they are relevant to the potential for induction of new bone formation.

In apparent contrast to our results, BMP-2 and BMP-4 were reported to be undetectable in the extracts of DFDBA by immunoprecipitation coupled with Western blot analysis (Li et al. 2000). This discrepancy could be attributable to different extraction methods, inability of the antibody used in that study to recognize its target epitope, and/or sensitiviy of assays utilized. The results of the present study suggest that BMPs are covered by the minerals and tightly associated with bone matrix molecules. Furthermore, the amount of BMP is small. Therefore, BMPs would not be detectable without extensive demineralization and appropriate extraction. However, the findings in the present study are in accordance with Wozney (1992), who reported that BMPs are found in microgram quantities per kilogram of cortical bone. The present study demonstrated that the level of extractable BMP-4 in DBM was in the range of nanograms per gram of DBM.

The data from this study revealed that non-demineralized bone matrix did not yield extractable BMP-4 whereas slightly demineralized bone matrix provided a degree of extractability of BMP-4 as the mineral content decreased the extractability of BMP-4 increased. Expectedly, the observation that the non-demineralized bone matrix showed no detectable BMP-4 level supports the widely held concept that BMPs are trapped by the minerals, and demineralization will expose BMPs relatively proportional to the degree of demineralization. Obviously, the more bone matrix is demineralized, the more minerals are removed, and the more BMPs become liberated and extractable from the DBM. That the osteoinductivity of DBM demineralized to less than about 2% residual calcium has been reported (Zhang et al. 1997) to decrease could relate to the acid lability of BMPs yet not render their degradation detectable by an immunoassay targeting an antigenic component of the molecule. Additionally, in the particle size study, it was also illustrated that bone particle size had a striking effect on the extractability of BMP-4 from DBM. Bone particles between 550 and 710 μm provided for the highest levels of extractable BMP-4 and appear to represent the optimal size range in that bone particles of larger or smaller sizes provided lower extractable BMP-4 levels. Interestingly, this finding is in agreement with the previous observation in our laboratory that DBM in the 550–710 μm bone particle size range provided for maximum osteoinductive potential in *in vitro* and *in vivo* bioassays (Zhang et al. 1997).

Additional data presented in this study revealed that there were donor age and gender-related effects on the extractability of BMP-4 from DBM. DBM derived from female donors appears to contain maximally and significantly more extractable BMP-4 level when derived from donors in the age group 0–25 years of age than from donors in all other age groups tested, whereas there was no statistically significant difference in the extractable BMP-4 level among DBM samples derived from male donors. When compared within each of age group, DBM from male donors in the age group 26–44 years of age and 45 years of age and older possessed more extractable BMP-4 than those from female donors in the same age group.

Collectively, we have investigated the levels of extractable BMP-4 and the osteoinductive potential of DBM derived from 40 donors. The data designated a positively direct correlation between the extractable BMP-4 levels in DBM using the quantitative *in vitro* assay and the osteoinductivity of DBM from the *in vivo* bioassay. A linear regression analysis between these two assessment methods exhibited a correlation coefficient (R) of 0.74 ($p < 0.001$). Therefore, the quantitative BMP-4 *in vitro* assay could be an alternative means of assessing the capability of DBM to induce new bone formation. Additional data suggest that availability (i.e., extractability) of BMP-4 depends upon the demineralization process. Hence, it is essential to the production of a reproducibly osteoinductive DBM product that the demineralization process be controlled and reproducible irrespective of the nature of the bone materials being demineralized.

The principal benefit of the quantitative BMP-4 *in vitro* assay for quantitating the osteoinductivity of demineralized bone products is time saving; results are available within 2 days. In contrast, the

in vitro cell culture based and *in vivo* animal based bioassays required a minimum of 1 and 4 weeks respectively to obtain information which may be used as a quality assessment criteria for release of produced DBM. Moreover, these two bioassays are labor consuming and involve a considerable amount of work, including complicated biological systems. In addition, surgical problems with implanted animals can be eliminated. There is no need to be concerned about immunological and inflammatory responses of tested animals to demineralized bone products. The quantitative BMP-4 *in vitro* assay described in the current study is sensitive, reproducible, inexpensive, and requires less than 2 days to perform.

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