Vitamin-D Binding Protein Does Not Enhance Healing in Rat Bone Defects

A Pilot Study

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Abstract Vitamin D-binding protein (DBP) has an anabolic effect on the skeleton and reportedly enhances bone ingrowth. We used an in vivo critical bone defect model to determine whether local administration of DBP promotes bone defect healing. We created a 5-mm segmental bone defect in the radial shaft in a rat model. Forty-eight rats were assigned to eight groups: local application of 1 µg, 5 µg, 10 µg, or 50 µg DBP (DBP-1, DBP-5, DBP-10, DBP-50), autogenous bone marrow mononuclear cells with or without 10 µg DBP (BM-DBP-10, BM), 80 µg BMP-2 delivered in gelatin sponge (BMP-2), and the sham operated group. Radiographic evaluation, histological stains, and epifluorescence microscopy were performed. Grossly, all bone gaps of the BMP-2 group were solidly bridged by callus, while all those in the sham operated group remained

unhealed by 9 weeks. Only one specimen of the BM-DBP-10 and DBP-50 groups and three specimens of the BM group were solidly healed; pseudarthroses occurred in all of the other specimens. Histological study and radiographs of the specimens showed similar results. We did not observe the enhanced bone healing reported in a previous study.

Introduction

Congenital or acquired bone defects are a major problem in orthopaedic surgery. Large bone defects resulting from trauma, tumors, osteomyelitis, or implant loosening usually require surgical treatment because spontaneous regeneration is limited to relatively small defects. Such defects in long bones may lead to delayed union or even nonunion despite adequate surgical treatment [3, 5, 6, 9, 20]. To accelerate healing in bone defects, different induction systems have resulted in healing from 30% to 90% or more of the defects [19].

Currently, autografts, allografts, bone substitutes, and callus distraction are the most commonly used techniques for skeletal reconstruction. A fresh graft of autologous

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cancellous bone with hematopoietic bone marrow is generally considered most effective for treating major bone defects [12]. However, harvesting the autograft can prolong surgery and hospital stay, and increase blood loss, the risk of infection, recovery time, and result in pain at the donor site [21]. To avoid the complications associated with autologous bone grafting, numerous investigators have conducted intense research in the field of synthetic bone grafts [2, 4, 7, 8, 10, 15]. The most commonly used bone grafts are calcium phosphate ceramics, such as hydroxyapatite. The porous structure of hydroxyapatite serves as a scaffold and allows bone ingrowth [4]. However, hydroxyapatite is rather inert and has weak osteoinductive activity. Osteoinductive stimulation of bone formation is an alternative: both demineralized bone matrix and growth factors have been used in experimental and clinical defects [2, 7, 8, 15]. However, little is known about suitable combinations, concentrations, and application time points of various growth factors during bone defect healing [10]. The vitamin-D-binding protein (DBP) and a small fragment of the DBP have an anabolic effect on the skeleton of both newborn and young adult rats [16]. Schneider et al. suggested systemic administration might be used to treat osteoporosis and a number of other osteopenia, and local administration might be effective in fractures, bony defect repairs, spinal surgery, and joint replacement [16].

The purposes were to (1) document whether DBP has an osteoinductive effect in a critical sized bone defect; (2) clarify whether the osteoinductive effect of DBP is strong enough to heal the critical-sized bone defect; (3) determine the histomorphologic changes during the osteoinduction or critical-sized defect healing processes.

Materials and Methods

We used a previously reported radial bone segmental defect model [11, 13, 14] to evaluate bone regeneration in critical bone defects. The model has several advantages: it is a simple procedure (no fixation needed), the defects are small and economical, and radiographs are easy to obtain. The DBP was locally applied at the defect area; the power of defect healing in the presence of DBP was examined by radiographic evaluation, histological stains, and epifluorescence microscopy and compared with that of bone morphogenic protein (BMP). We used 48 10-week-old male Sprague-Dawley rats randomized into eight groups (Table 1, Fig. 1). Based on previous literature and our previous experiments on rabbit bone defect models

Fig. 1 Flowcharts illustrate the experimental method.

Table 1. The mean bone gap measurement, mean difference in bone gap measurement, and interobserver coefficients of variation between the two observers. CVinter is the interobserver coefficient of variation

Group	Bone gap measurement							Difference in bone gap measurement		
	Investigator 1		Investigator 2		Two investigators			Mean	S.D.	CVinter (%)
	Mean	S.D.	Mean	S.D.	Mean	S.D.	P value			
Control	2.22	0.97	2.29	1.08	2.26	1.02	—	0.12	0.16	6.98
DBP-1	2.42	0.94	2.47	1.02	2.45	0.98	0.322	0.17	0.12	4.94
DBP-5	1.57	1.05	1.61	1.06	1.59	0.81	0.065	0.10	0.11	6.61
DBP-10	1.43	0.81	1.4	0.76	1.42	0.78	0.024	0.08	0.07	4.95
DBP-50	1.38	0.88	1.33	0.87	1.36	0.88	0.015	0.08	0.09	6.47
BM-DBP-10	1.49	0.53	1.49	0.54	1.34	0.67	0.027	0.03	0.03	2.03
BM	1.169	0.76	1.17	0.77	1.05	0.823	0.004	0.02	0.03	2.70
BMP-2	0	0	0	0	0	0	< 0.001			

Negative control = PBS in gelatin sponge only; DBP-1 = 1 µg vitamin D-binding protein delivered in gelatin sponge; DBP-5 = 5 µg vitamin D-binding protein delivered in gelatin sponge; DBP-10 = 10 µg vitamin D-binding protein delivered in gelatin sponge; DBP-50 = 50 µg vitamin D-binding protein delivered in gelatin sponge; BM-DBP-10 = 1 × 10⁶ bone marrow mononuclear cells in DBP-10; BM = 1 × 10⁶ bone marrow mononuclear cells delivered in gelatin sponge; BMP-2 = Positive control with 80 µg bone morphogenic protein-2 delivered in gelatin sponge.

[11, 18], we chose the original 5-mm bone gap of rats as the negative control samples (sham-operated); while 80 μ g BMP-2 impregnated bone defects of rats as the positive control samples ($n = 6$). The result of the pilot study showed that the critical-sized bone defect was decreased to 2.25 mm (standard deviation, 1.02 mm); while the positive control samples (BMP-2 implanted) were 0 mm (solid union). From this pilot study, the effective sample size was determined based on a difference of bone gaps with a power of 80%, two-tailed, and a confidence interval of 95%. This resulted in a minimum of four samples per group for histological evaluation. The experiments performed in this study were approved by the Animal Ethics Committee, National Taiwan University Hospital, prior to the study and all animal experiments were carried out adhering to Committee guidelines.

After sterile preparation, 5-mm segmental bone defects (with the periosteum left intact) in the radial shaft were created using a double-bladed diamond file on bilateral radii through 1.5-cm longitudinal incisions on the dorsal aspect of the forelimbs. The osteotomy sites were treated following the protocol of each group. The animals received local application of either test substances or PBS delivered in a gelatin sponge measuring 5 mm $\times$ 5 mm $\times$ 10 mm. The four experimental groups received local application of 1 μ g, 5 μ g, 10 μ g, or 50 μ g (DBP-1, DBP-5, DBP-10, DBP-50) vitamin-D-binding protein (DBP) delivered in a gelatin sponge; another two groups received 1×10^6 bone marrow mononuclear cells delivered in gelatin sponge with or without 10 μ g DBP (BM, BM-DBP-10). (The DBP was a gift from Industrial Technology Research Institute, Shin-Chiu, Taiwan, ROC.) In our positive control, we locally inserted 80 μ g BMP-2 (also a gift from Industrial Technology Research Institute, Shin-Chiu, Taiwan, ROC) delivered in a gelatin sponge, while the sham operated negative control group received gelatin sponges soaked with PBS. Bilateral forelimbs received the same treatments. The right-side specimens were assigned to receive histological evaluations, which included decalcification, paraffin-embedded sections, and hematoxylin-eosin stains. The left-side specimens were assigned to receive radiographic examination, and the undecalcified sections were further evaluated by epifluorescence microscopy.

Autogenous mononuclear cells were isolated from bone marrow aspirates by density gradient centrifugation (Ficoll-Paque PLUS, Amersham Biosciences Inc, Piscataway, NJ). The total cell yield was calculated using a hemocytometer and the trypan blue exclusion assays. The bone marrow mononuclear cells suspension was preserved in a 4°C ice bath for later use. All surgical procedures were performed with the rats under general anesthesia by intraperitoneal injection of 50 mg/kg thiopental. Animals were permitted full weight bearing and unrestricted movement

upon awakening from anesthesia. Normal activity was resumed the second day after surgery.

To visualize the dynamics of bone growth, the rats received sequential fluorochrome labels at 3 weeks (Calceine green, 10 mg/kg IV; Sigma Aldrich, Zwijndrecht, The Netherlands), 5 weeks (oxytetracycline, 32 mg/kg IM; Engemycin, Mycofarm, Amersfoort, The Netherlands), and 7 weeks (Xylenol orange, 80 mg/kg IV; Sigma Aldrich, Zwijndrecht, The Netherlands) [11, 12].

Animals were killed by overdose anesthesia (thiopental 200 mg/kg IP) at 9 weeks. The forelimbs were excised, and the soft tissue was stripped off carefully. The radius and ulna, as a unit, were disarticulated from wrist and elbow joints. The specimens were then immersed in 10% neutral-buffered formalin for 48 hours at room temperature. One animal in the control group, three in the DBP-5 group, and three in the BMP-2 group died during or immediately after the operation. The anesthetic dose was adjusted, and the subsequent animals tolerated the operation well. An additional animal in the DBP-1 group died at the third week. For each of the rats that died we added a similar number of appropriately treated rats to ensure equal numbers ($n = 6$) in each group. Blood biochemistry examinations showed no substantial liver or kidney impairment and no abnormality in electrolytes (data not shown).

The fracture healing status was determined on plain radiographs obtained immediately after sacrifice. The radiographs were taken in both the anteroposterior lateral plane at a distance of 100 cm from the limbs, centered to the middle of the forelimbs. For the radiographic evaluation, cortical bridging was determined on each of the two cortices in both anteroposterior and lateral radiographs (two cortices per plane, a total of four cortices). Radiographic union was defined as solid bridging of three of four cortices in both anteroposterior and lateral plane. Healing of the experimental defects was evaluated by direct measurement of the bone gap on the digital radiographs. To assess the healing process on radiographs, we classified defects into three categories: complete bridging (defect bridged by uniform new bone, cut ends of cortex not or hardly recognizable); incomplete bridging (defect bridged unilaterally or bilaterally but not united, cut ends of cortex recognizable); or no bridging (no change when compared to immediate postoperative appearance or only trace of radiodense/calcified material in defect zone). The radiographs were evaluated by two independent blinded observers (P-YC and J-SS). For each group, the interobserver coefficient of variation (CV_{inter}) was calculated using the mean bone gap measurement (as assessed by the two observers) as well as the standard deviation of this difference in bone gap measurement. CV_{inter} was computed as 100 times the standard deviation of this difference divided by the mean bone gap measurement. The

interobserver coefficients of variation of this study were between 2% and 7% of mean bone gap measurement (Table 1). For the bone defect measurement, the average value of two observers was used for each measurement. The differences in measurement of the distance of bone defect end between each group were determined by using the one-way analysis of variance (ANOVA); we determined differences in distance of critical-sized bone defect between each experimental group and negative control group with the single-tailed student's t-test.

After radiographic evaluation, the right-side specimens underwent decalcification, and then were embedded in paraffin. Paraffin-embedded sections (7 μ m) were stained with hematoxylin and eosin for observational histology. The left-side specimens were immersion-fixed in 5% paraformaldehyde at 48°C, followed by dehydration in ascending concentrations of ethanol, and embedded undecalcified in methylmethacrylate (Merck, Darmstadt, Germany). The blocks were sliced to 200 μ m using a low-speed diamond saw (Isomet, Buehler Ltd, Lake Bluff, Ill.), attached onto glass slides using cyanoacrylic glue, and ground to 30 μ m in thickness. Undecalcified specimens were examined by epifluorescence microscopy. We (P-YC, J-SS) evaluated the degree of bone defect healing using a five-point qualitative scale proposed by Allen et al. [1] with minor modifications. According to this classification system, Grade 4 represents complete bony union, Grade 3 represents an incomplete bony union (presence of a small

amount of cartilage in the callus), Grade 2 represents a complete cartilaginous union (well-formed plate of hyaline cartilage uniting the fragments), Grade 1 represents an incomplete cartilaginous union (retention of fibrous elements in the cartilaginous plate), and Grade 0 indicates the formation of a pseudarthrosis (most severe form of arrest in fracture repair). In this part of study, we obtained no interobserver variability for this qualitative radiographic interpretation (Appendix 1).

Results

Bone morphogenic protein has potent osteoinductive effects and high doses of DBP had some osteoinductive effect on this critical-sized bone defect model. Bone morphogenic protein, a higher dose of DBP, and supplementary bone marrow mononuclear cells decreased the distance of bone gap; while the lower dose of DBP had no osteoinductive effect (Fig. 2A–H). We observed decreases in the bone gap of the DBP-10, BM, BM-DBP-10, DBP-50, and BMP-2 groups when compared with that of negative control (Fig. 3, Table 1).

Although higher dose of DBP and supplementary bone marrow mononuclear cells had some beneficial effect on bone-defect healing, they did not attain as solid a union as that which occurred in the BMP-2 group. Grossly, the bone gaps were solidly bridged by callus in all 12 specimens of

Fig. 2A–H The bone defects in each group were evaluated by radiograph. The groups pictured are (A) negative control group: PBS in gelatin sponge only; (B) DBP-1 group: 1 μ g vitamin-D binding protein (DBP) delivered in gelatin sponge; (C) DBP-5 group: 5 μ g DBP delivered in gelatin sponge; (D) DBP-10 group: 10 μ g DBP delivered in

gelatin sponge; (E) DBP-50 group: 50 μ g DBP delivered in gelatin sponge; (F) BM-DBP-10 group: 1×10^6 bone marrow mononuclear cells in DBP-10; (G) BM group: 1×10^6 bone marrow mononuclear cells delivered in gelatin sponge; and (H) BMP-2 group (positive control group): 80 μ g bone morphogenic protein-2 delivered in gelatin sponge.

Fig. 3 We observed decreases in the bone gap of the DBP-10, DBP-50, BM, BM-DBP-10 and BMP-2 groups. Pictured are the negative control group; DBP-1 group; DBP-5 group; DBP-10 group; DBP-50 group; BM-DBP-10 group; BM group; and BMP-2 group (positive control group).

the BMP-2 group (union rate, 100%), while all samples in the negative control group remained unhealed by 9 weeks. Only one specimen in the BM-DBP-10 and DBP-50 groups (union rate, 8.3%) and three specimens in the BM group (union rate, 25%) were solidly healed; bone gap or pseudarthrosis was noted in all other specimens. As the degree of bone defect healing measurement, all samples of BMP-2 group attained solid union status (Grade 4), the healing in samples with bone marrow mononuclear cells implantations (BM and BM-DBP-10 groups) distributed relatively evenly in different degrees; while the other groups attained only incomplete cartilaginous union (Grade 1) and even nonunion (Grade 0) (Table 2). In the histological study of the BMP-2-treated group, there was hypertrophic callus bridging both ends of the bone defects; while in the other non-BMP-2 and un-united specimens, the histological study showed either soft tissue filled the bone defect or the bone ends healed to adjacent ulnar bone. In the BMP-2-treated group, all the specimens united with a small amount of cartilage in the callus; while in the other non-BMP-2 treated groups, most of the other specimens developed pseudarthroses. Microscopically, there was hypertrophic callus bridging the two ends of the bone defects in the BMP-2-treated group with areas of hyaline cartilage (C) and areas of mature trabecular bone (B) with marrow tissues (M) (Fig. 4A); however, at the end of experiment, the callus was still not completely mature by 9 weeks. In the un-united specimens, there were two patterns of histological findings. In the first pattern, atrophic bone ends (AB) extended from the proximal and distal

Table 2. Histological findings scored on the Allen scale [1]

Group	Grade 4	Grade 3	Grade 2	Grade 1	Grade 0
Control	0	0	0	2	10
DBP-1	0	0	0	4	8
DBP-5	0	0	0	2	10
DBP-10	0	0	0	2	10
DBP-50	0	0	0	4	8
BM-DBP-10	0	1	1	3	7
BM	1	2	2	3	4
BMP-2	12	0	0	0	0

Negative control = PBS in gelatin sponge only; DBP-1 = 1 μ g vitamin D-binding protein delivered in gelatin sponge; DBP-5 = 5 μ g vitamin D-binding protein delivered in gelatin sponge; DBP-10 = 10 μ g vitamin D-binding protein delivered in gelatin sponge; DBP-50 = 50 μ g vitamin D-binding protein delivered in gelatin sponge; BM-DBP-10 = 1×10^6 bone marrow mononuclear cells in DBP-10; BM = 1×10^6 bone marrow mononuclear cells delivered in gelatin sponge; BMP-2 = Positive control with 80 μ g bone morphogenic protein-2 delivered in gelatin sponge.

sides. The defects were filled with muscle or fibrous tissue (F) and scanty cartilage tissue (C) on the bone ends (Fig. 4B). Most of the specimens fell in this category. In the second pattern, there is a piece of newly formed bone in the defect, based on the adjacent ulnar bone (U) (Fig. 4C). It may heal to one of the bone ends; it is not clear whether it is derived from the ulnar bone or solely formed within the defect.

Sequential fluorochrome labeling demonstrated the bony tissue metabolized actively; while the BMP-2 treatment and bone marrow mononuclear cells supplement promoted active bony regeneration and mineralization. The cortical bone showed highly ordered layering of bone mineralization to expand the diameter of the regenerated bone in the BMP-2 group (Fig. 5A). Specimens sectioned from the osteotomy sites of the negative control, DBP-1, DBP-5, DBP-10, and DBP-50 groups showed only scanty calcein-stained bone trabeculae at the ends of well-organized cortical bone (Fig. 5B). In contrast, the specimens in BM, BM-DBP-10, and BMP-2 groups, whether united or not, showed prominent active mineralization at 3 weeks (calcein, green fluorescence). These early woven bone structures were partially remodeled by laminar bone-forming Haversian systems (unstained layers; yellow layer: oxytetracycline, 5 weeks; red layer: xylenol orange, 7 weeks) (Fig. 5C). In either group, there was always a patch of new woven bone at the defect site with or without direct contact with the cortical bone of the ulna in un-united specimens; while in united specimens, these ulna-based woven bone patches merged into the bridging calluses. This finding might imply the periosteum or bone marrow mononuclear cells as the cell's source (Fig. 5D).

Fig. 4A–C Histology was evaluated in all groups with two types of staining (left column, Stain: hematoxylin and eosin, original magnification, $\times 40$; right column, Stain: Alcian blue, original magnification, $\times 40$). There are hypertrophic callus bridging the two ends of the bone defects in the (A) united group (the BMP-2 treated group). At the end of experiment, there are areas of hyaline cartilage (C), as well as more mature areas of trabecular bone (B) with marrow tissue (M). In the un-united group (B and C): There were two patterns of histological findings. In the first pattern, atrophic bone ends (AB) extended from proximal and distal sides. The defects were filled with muscle or fibrous tissue (F), and some scanty cartilage tissues (C) were also visible on the bone ends (B). In the second pattern, there was a piece of newly formed bone in the defect, based on the adjacent ulnar bone (U) (C).

Discussion

Vitamin-D-binding protein (DBP) and a small fragment of the DBP are reported to have an anabolic effect on the skeleton of both newborn and young adult rats [16]. The purposes were to (1) document whether DBP has an osteoinductive effect in a critical sized bone defect; (2) clarify whether the osteoinductive effect of DBP is strong enough to heal the critical-sized bone defect; (3) determine the histomorphologic changes during the osteoinduction or critical-sized defect healing processes.

We note several limitations to our study. First, biomechanical tests were not feasible due to insufficient healing of the bone gaps in both the sham-operated negative control group and most of the experimental groups. However, all bones in the BMP-2 positive control group in this study attained solid union; this fact still can validate that there is a difference in the mechanical strength between the

positive control samples and the un-united samples. Second, although the dosages of locally applied DBP ranged from 1 μg to 50 μg , the dosage of 50 μg DMP probably did not attain the actual effective dosage of DBP. However, the dosage applied is much higher than reported by Schneider et al., which is 1 μg applied locally [16]. Third, the study population was small and the study duration was probably not long enough, consisting of only a 9-week end point on the fracture healing. Again, the efficacy of this model was confirmed by the 100% solid union in the BMP-2 positive control group. Fourth, we had no detailed information about the amount of newly produced bone. However, there were qualitative histological and epifluorescence analyses performed and we believe that the validity of this experiment was evidenced by the 100% union rate of the BMP-2 group and the 0% union rate of the negative control group.

With radiographic examination and quantitative radiographic evaluation we found bone gaps treated with bone

Fig. 5A–D Epifluorescence study for the dynamics of bone growth is shown. **(A)** Cross-sectional study of control specimen showed the dynamics of bone remodeling. The cortical bone showed highly ordered layering of bone mineralization to expand the diameter of the regenerated bone in the BMP-2 group. Autofluorescence in the yellow light range was also noted in the marrow space. **(B)** Specimens sectioned from the osteotomy sites of the negative control, DBP-1, DBP-5, DBP-10, DBP-50 groups showed only scanty calcein stained bone trabeculae at the ends of well-organized cortical bone. **(C)** The specimens in BM, BM-DBP-10 and BMP-2 groups showed prominent active mineralization. These early woven bone structures were partially remodeled by laminar bone forming Haversian systems. **(D)** In either group, patch of new woven bone at the defect site in direct contact with the cortical bone of the ulna in un-united specimens was usually observed. In united specimens, these ulna-based woven bone patches merged into the bridging calluses. (A1, B1, C1, D1: Epifluorescence microscopic image. A2, B2, C2, D2: Bright field image.).

morphogenic protein, higher dose of DBP, and supplementary bone marrow mononuclear cells decreased the distance of the bone gap while the lower dose of DBP did not have any effect on the bone defect healing (Figs. 2A–H and 3). An anabolic effect of DBP has been reported in one previous investigation [16]. In systemic administration, it can increase the total bone density, while local injection in

the femoral metaphysis can also enhance the osteogenesis at the local bone marrow [16]. In this in vivo study, we demonstrated that the local application of bone morphogenic protein, higher dose of DBP, and supplementary bone marrow mononuclear cells had an osteoinductive effect.

In all samples of the BMP-2 group, there were hypertrophic calluses bridging both ends of the bone defects with

areas of hyaline cartilage, mature trabecular bone, and marrow tissues; the union rate was 100% in this group. All negative control samples remained unhealed by 9 weeks. In this study, we demonstrated that the bone morphogenic protein possesses osteoinductive effect and can promote the healing of bone defect; higher doses of DBP and supplementary bone marrow mononuclear cells had some beneficial effect on bone defect healing; however, this beneficial effect was still not good enough to assure bone defect healing even at the highest dosage of 50 μ g DBP applied locally.

BMP-2 provides optimal healing environments by stimulating new bone formation at the site of implantation [10]; DBP does not attain the same potential as that of BMPs. In most samples in the un-united specimens, atrophic bone ends extended from the proximal and distal sides with the defects filled with muscle or fibrous tissue. The addition of bone marrow mononuclear cells improved the bone gap healing with some samples in the BM and BM-DBP-10 groups attaining solid union. Schneider et al. reported “new bone formation” around the injection site in the bone marrow. Unlike BMPs, subcutaneous or intraperitoneal injection of DBP did not result in ectopic bone formation [16]. The effect of the BMPs goes through endochondral osteogenesis, that is, bone formation in newly formed hyaline cartilage. There was no cartilage noted around the local injection site of DBP in Schneider’s report. The authors postulated that the DBP or DBP peptide need mesenchymal cells to express bone anabolic effect, perhaps via the intramembranous pathway [16]. In our study, mesenchymal cells could exist in the hematoma or migrate from the open ends of the bone marrow. In most of the specimens in the DBP groups and control group, the repair of the defect seemed to progress from both sides of the opened medullar canal, with gradual tapering of the bone width, resulting in an atrophic picture of the bone ends. We observed interposition of newly formed bone in some samples. However, these bone segments were triangular in shape, with a wide base integrated on the underlying ulnar bone, raising the possibility that the new bone formation may be derived from the injured periosteum of ulnar bone instead of within the defect itself. On the other hand, the BMP-2 group showed a completely different pattern, having robust callus formation within the defect itself (ie, within the BMP-2-treated gelatin carrier).

Although the histological and epifluorescence observations confirmed the addition of bone marrow mononuclear cells did not heal the defect, we did observed enhanced or accelerated ingrowth of bone as reported in previous experimental study [16]. We observed scanty calcein-stained bone trabeculae at the bone ends in the negative control, DBP-1, DBP-5, DBP-10, and DBP-50 groups; while in the BM, BM-DBP-10, and BMP-2 groups, there

was active mineralization, and a patch of new woven bone at the defect with partially remodeled Haversian systems. The possible mechanisms of failure of DBP in the present model cannot be exactly explained by our data. A possible mechanism of failure could be the large size of the cortical defect. In the present study, the implantation of BMP-2 yielded the best new bone formation and a radiological consolidation in all samples was observed.

Enzymatic processing of the DBP O-linked carbohydrate in the third domain results in the transformation of the DBP into a potent macrophage-activating factor called DBP-MAF. DBP-MAF can rapidly activate macrophages during the host defense response, and can induce macrophage cell death by upregulating caspase activity. DBP-MAF can also stimulate osteoclast activity and bone resorption [16]. Recently, the biologically active form of vitamin D₃ (1- α ,25-OH₂-vitamin D₃) has been described not only to influence bone metabolism but also to exert immunomodulating activities, which may have an impact on bone formation/resorption as well [17]. However, it is not known whether DBP-MAF has direct effect on the proliferation, differentiation, or anabolic function of osteoblasts. The observed increase in bone mineral density and “new bone formation” in Schneider’s study may also be explained by the inhibition of the osteoclast function, and the DBP-MAF may not possess a true anabolic effect on bone. To further delineate whether DBP has the potential to promote bone regeneration, an *in vitro* study to evaluate their effects on the proliferation, differentiation, and function of osteoblasts or mesenchymal cells is warranted.

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Appendix 1. Observer classification of radiographic healing (Allen et al. [1])

Specimen#												
OBSERVER-1	1L	1R	2L	2R	3L	3R	4L	4R	5L	5R	6L	6R
Group												
Control	1	0	1	0	0	0	0	0	0	0	0	0
DBP_1	1	0	0	0	1	0	0	0	1	1	0	0
DBP_5	0	1	0	0	0	0	0	0	0	0	0	1
DBP_10	0	1	1	0	0	0	0	0	0	0	0	0
DBP_50	1	1	0	0	0	0	1	0	1	0	0	0
BM-DBP-10	3	1	1	2	1	0	0	0	0	0	0	0
BM	1	4	1	0	3	0	2	3	1	0	2	0
BMP-2	4	4	4	4	4	4	4	4	4	4	4	4

Appendix 1. continued

Specimen#												
OBSERVER-2	1L	1R	2L	2R	3L	3R	4L	4R	5L	5R	6L	6R
Group												
Control	1	0	1	0	0	0	0	0	0	0	0	0
DBP_1	1	0	0	0	1	0	0	0	1	1	0	0
DBP_5	0	1	0	0	0	0	0	0	0	0	0	1
DBP_10	0	1	1	0	0	0	0	0	0	0	0	0
DBP_50	1	1	0	0	0	0	1	0	1	0	0	0
BM-DBP-10	3	1	1	2	1	0	0	0	0	0	0	0
BM	1	4	1	0	3	0	2	3	1	0	2	0
BMP-2	4	4	4	4	4	4	4	4	4	4	4	4

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