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Use of Growth Factors to Modify Osteoinductivity of Demineralized Bone Allografts: Lessons for Tissue Engineering of Bone

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Bone grafting materials are used in oral and craniofacial surgery for a variety of applications. Where there is sufficient vascular supply and the amount of bone needed is small, clinicians often can use autologous bone harvested from the surgical site itself. Many different kinds of osteoconductive materials can be used effectively to augment the autologous bone, thereby providing needed structural support at the site. Materials of this kind include calcium phosphate ceramic particles, various forms of bioactive glass, and anorganic bone. Although these materials do not possess inherent osteogenic properties, they are biocompatible and provide surfaces that enable the migration of mesenchymal cells to the site where they differentiate into bone-forming cells and produce new bone.

For some patients, the supply of autologous bone is limited and harvesting bone from extraoral sites has its own morbidity. This is true particularly for older individuals, whose quality of bone stock may be compromised by osteoporosis or other conditions, including diabetes and renal failure. In these cases, it is advantageous to use alternative bone graft materials that are osteoconductive and osteogenic. Osteogenic materials are those that cause bone to form in an orthotopic site to a greater extent than is predicted

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if the material were acting only as a substrate for cell migration and growth. They include bone marrow stromal cells, mesenchymal stem cells, and several growth factors shown to increase mesenchymal cell numbers or enhance osteoblastic differentiation (Box 1).

Osteoinductive materials are those that cause bone to form in sites that otherwise would not support bone formation (eg, muscle or fascia). When implanted in bone, osteoinductive agents recruit undifferentiated mesenchymal cells and induce their differentiation into cells required for bone formation, in particular chondrocytes and osteoblasts. These agents also act directly on osteoprogenitor cells and committed osteoblasts. When implanted in nonorthotopic sites, osteoinductive materials initiate the process of endochondral ossification in a manner similar to embryonic bone formation. Mesenchymal cells attracted to the implant differentiate into

Box 1. Bone graft products used for bone tissue engineering

Osteoinductive
Demineralized freeze-dried bone allograft (DFDBA)
Partially pure proteins (BMP)
BMP-2
BMP-4
BMP-7
BMP-9

Osteoconductive
Freeze-dried bone
Autograft
Ceramics
Bioglasses
Coral-derived
Deproteinized bovine bone
Polylactic acid (PLA)/polyglycolic acid (PGA)

Osteogenic
Mesenchymal stem cells (MSC)
Marrow
Platelet-rich plasma (PRP)
PRP + white blood cells (WBC)
Emdogain
Gene therapy
Fibroblast growth factor (FGF)
Peptide TP508
Peptide P15
Platelet-derived growth factor (PDGF)

chondrocytes, producing and calcifying cartilage matrix. Once this occurs, the calcified cartilage is invaded by blood vessels bringing osteoprogenitor cells. Bone is formed on the calcified cartilage scaffold, which is replaced by bone marrow, resulting in formation of a complete ossicle consisting of hematopoietic marrow surrounded by cortical bone [1].

The canonical osteoinductive material is demineralized bone, first described by Urist in 1965 [2]. At least part of the reason for the osteoinductivity of demineralized bone is the presence and release of osteoinductive proteins collectively known as BMP. Two of these proteins, BMP-2 and BMP-7 (osteogenic protein-1 [OP-1]), have been developed commercially for use as bone inductive materials. Both proteins initiate endochondral bone formation when implanted heterotopically [3,4] and stimulate bone formation clinically [5–7].

Although BMP are the most effective osteoinductive materials known, there are problems associated with their use. BMP are produced by bone cells and stored in the extracellular matrix of bone at low levels and always in the presence of one or more inhibitors [8,9]. This ensures that they are available when needed and that they are active only under specific conditions. When activated, the clearance rate for BMP is rapid. Thus, it is necessary to implant large concentrations of these proteins to have active protein present at the time when an appropriate responding cell population also is present. Moreover, BMPs are most effective when used with a carrier to retain the proteins at the implant site and to provide an osteoconductive matrix.

Other factors contribute to bone formation in a variety of ways. Given the ease of using osteoinductive demineralized bone as a bone graft substitute, it is of interest to find additives that can enhance its osteoinductivity. This article describes factors commonly used in dentistry and shows how these agents affect the osteoinduction ability of demineralized bone.

Methods and materials

Human demineralized freeze-dried bone allograft (DFDBA) was a gift from LifeNet (Virginia Beach, Virginia) and was provided in the form used clinically. DFDBA from 27 different donors was assayed for ability to induce new bone formation when implanted in gastrocnemius muscle of immunocompromised mice [10]. Batches that had high osteoinductivity or low osteoinductivity were selected for the studies (discussed later). The DFDBA was weighed (10 mg/implant), placed in gelatin capsules, and sterilized overnight under UV light. Immediately before implantation, the DFDBA was mixed with the agent of interest.

Recombinant human BMP-2 was supplied in saline (Genetics Institute; now Wyeth, Andover, Massachusetts) and used at a concentration of 100 ng/implant [11]. Emdogain (Biora AG, Malmo, Sweden; now Institut Straumann AB, Basel, Switzerland) was supplied as a powder and used at a concentration of 4 mg/implant [12]. Recombinant platelet-derived growth

factor (PDGF)-BB (BioMimetics, Franklin, Tennessee) was supplied as a powder, dissolved in saline, and used at a concentration of 10 μg/implant [13]. PRP was prepared with the harvest machine [14] using blood from a healthy male subject. Each implant was mixed with 25-μL–activated PRP [15]. Activation of the PRP resulted in a 15-fold increase in total transforming growth factor-beta 1 (TGF-β1). Bio-Oss was extracted with 4 M guanidine hydrochloride and the extract was shown to contain low levels of BMP-2 and TGF-β1, among other proteins [12]. The proteins in the extract were concentrated, dialyzed against saline, and suspended in saline before mixing with DFDBA.

Materials were implanted bilaterally in the hind limb calf muscle of male Nu/Nu mice. These mice have a compromised immune system and, as a result, xenografts do not elicit an immune response. Tissues were examined for the presence of bone at 35 days (BMP-2, Emdogain, PDGF, or PRP) or 56 days (Bio-Oss) post implantation using histology and histomorphometry. The ability of the material to induce new bone formation was determined using a semiquantitative scoring system on paraffin sections stained with hematoxylin and eosin. If no material was found in the implanted tissue, the score was 0. If only the original implant was present, the score was 1. If new bone was present, the score was 2. If two or more ossicles were present, the score was 3; and if the new bone covered more than 70% of the section (magnification ×10), the score was 4. In addition, each section was analyzed by histomorphometric measurement of the area of new bone and the area of residual implant material.

For these experiments, each implant type was assessed using 8 individual implants, 2 identical implants per animal, 1 per leg. Data were analyzed by analysis of variance, and significant differences determined using the Bonferroni modification of the Student *t* test. Because these experiments were conducted at different times, to facilitate comparison of the results, treatment-to-control ratios were compared. In each case, the control was DFDBA alone and the treatment was DFDBA plus the agent. Studies using Bio-Oss are presented using actual values.

Results and discussion

Effects on osteoinduction

The ability of DFDBA to induce new bone formation varied with the additive used (Fig. 1). Addition of BMP-2 increased osteoinduction by almost 50% based on the bone induction score. DFDBA contains BMP-2, but the amount seems to vary among individuals [16]. Moreover, the formatting of BMP-2 within DFDBA is different from that of BMP-2 adsorbed to the surface of the graft particles. Studies suggest that the DFDBA particles must be resorbed for the BMP contained within the matrix to be released. DFDBA becomes, in effect, a time-release carrier for these factors. Surface-adsorbed BMP-2 is released in a burst and, as a result, has its greatest effects on cells

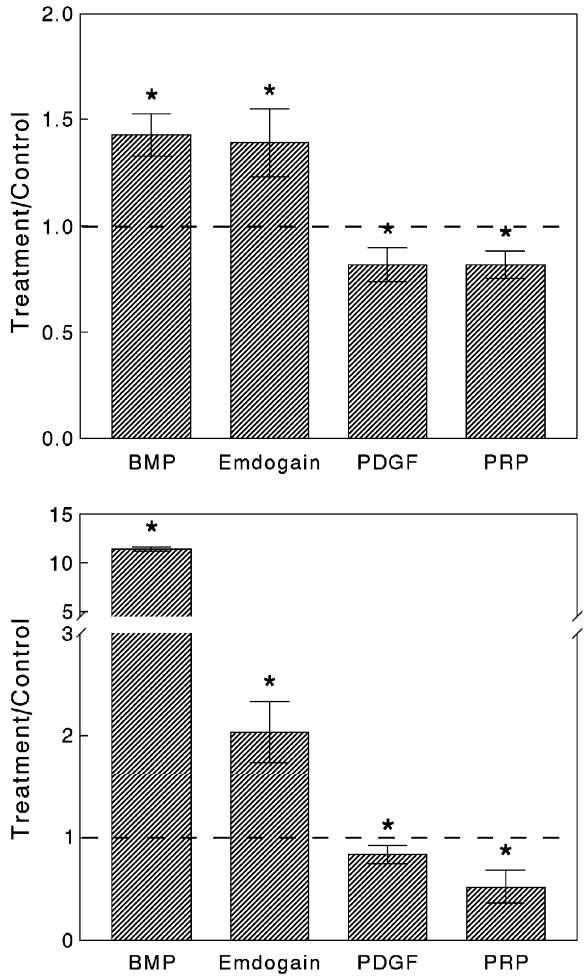

Fig. 1. Effects of bioactive agents on DFDBA-induced bone formation in the gastrocnemius muscle of immunocompromised mice. Nude mice were implanted bilaterally with human DFDBA plus recombinant human BMP-2, Emdogain, recombinant human PDGF-BB, or human PRP. The osteoinduction score was determined using a semiquantitative scale (*top panel*). The area of new bone was determined histomorphometrically (*bottom panel*). Data are treatment-to-control ratios for 8 implants. Values are expressed as means ± SEM. *, $P<0.05$ versus a treatment-to-control ratio of 1.

present at the implant site. Thus, the two forms of the morphogen work on distinctly different cell populations, and the combined effect is additive, if not synergistic. Other factors present in DFDBA also may contribute to the overall tissue response.

Emdogain had the same effect as BMP-2 on the osteoinduction score (see Fig. 1). This was an unexpected result. Emdogain is composed primarily of amelogenin and other proteins present in embryonic porcine tooth germs

[17]. It is reported to contain neither detectable BMP nor TGF-β [18]. Emdogain is believed to function as a matrix, potentially enhancing the recruitment and differentiation of mesenchymal cells [19]. It is possible that a trace component of Emdogain possesses osteoinductive properties. During embryonic development, the interaction of epithelial and mesenchymal tissues is critical for tissue morphogenesis. One or more of the factors that control this process may be present in Emdogain. Amelogenin itself seems important for coupling enamel formation via ectodermal ameloblasts and dentin formation via mesodermal odontoblasts [17]. More recently, amelogenin is shown to act on cells via growth factor–like signaling pathways [20]. Thus, Emdogain may act through similar mechanisms as DFDBA, resulting in an enhancement of DFDBA's osteoinductive properties.

In contrast to the increase in osteoinduction seen when DFDBA is implanted with BMP-2 or Emdogain, PDGF and PRP reduced osteoinductivity by approximately 20% (see Fig. 1). PDGF-BB is produced by platelets and is released by them at sites of injury. Unlike the morphogen, BMP-2, PDGF-BB is a growth factor, and its main effect is to stimulate DNA synthesis and cell proliferation [21]. Thus, in the presence of PDGF-BB, the number of mesenchymal cells, but not necessarily differentiated cells, is increased. If the differentiation stimulus provided by DFDBA is not great enough, then it is possible that the proliferative effect of the growth factor overwhelms the tissue response. The inhibitory effect of PDGF-BB on DFDBA-induced bone formation is concentration dependent and, at high concentrations, causes the chondrogenic phase of endochondral bone formation to persist [22]. At low concentrations, PDGF-BB does not inhibit DFDBA activity and, in an orthotopic site where other osteogenic signals are present, its effect on mesenchymal cell proliferation may result in increased bone formation. Clinical studies suggest that this is the case [23].

PDGF is a major component of PRP but not the only component. TGF-β also is enriched in PRP and, like PDGF, stimulates mesenchymal cell proliferation [24]. At high concentrations, TGF-β1 blocks terminal differentiation of growth plate chondrocytes and osteoblasts [25,26]. Whether or not this is a factor in the inhibition of bone formation via DFDBA is not known. PRP is used in dental surgery based on the hypothesis that it is an enrichment of autologous growth factors involved in wound healing. Many publications indicate that it improves bone healing in oral surgical applications. Part of this effect may be that PRP improves the handling properties of DFDBA. There is an increasing body of literature, however, that supports the authors' observation that PRP is inhibitory [27–30].

Effects on bone formation are specific to the factor used

The osteoinduction score indicates whether or not bone formation has occurred and gives some idea of the robustness of the effect, but it does not provide information about the quality of the osteoinduction. As shown

in Fig. 1, BMP-2 had a much greater stimulatory effect on bone formation than Emdogain, even though both additives resulted in a comparable osteoinduction score. This can be attributed to the mechanisms involved. BMP-2 upregulates expression of transcription factors, such as RUNX-2, that regulate osteoblast phenotypic expression [31]. Thus, there are more bone cells and more bone. The lack of BMP in Emdogain suggests that its ability to enhance the osteoinductivity of DFDBA is the result of its properties as a bioactive matrix.

PDGF reduces new bone formation by DFDBA to a much lesser extent than PRP (see Fig. 1), suggesting that other components of PRP also are involved. TGF-β1 is one of these factors. PDGF and TGF-β1, however, can act directly on committed osteoblasts as autocrine and paracrine regulators of osteoblastic activity [24]. Although TGF-β1 is shown to block terminal differentiation [26], it does stimulate early states of osteoblastic maturation, including increased alkaline phosphatase activity. This supports the hypothesis that the environment is critical and that growth factors and cytokines present in an orthotopic site may be as important as those that are implanted.

Role of resorption rate in bone tissue engineering

Factors used to augment DFDBA also affect the rate of resorption of the implant material (Fig. 2). BMP-2 results in greater resorption of DFDBA than is seen when the allograft is implanted alone. This effect of BMP-2 is noted with other carriers, including polylactic acid/polyglycolic acid copolymer scaffolds implanted in nude mouse muscle [32] and when used clinically with bone graft [33]. One reason for this may be that BMP-2 activates resorbing cells in general; another may be that it stimulates the entire remodeling cycle and the graft or carrier material is resorbed as a byproduct of the osteoclastic resorption of bone [34]. For the most part, the net increase in bone formation overcomes any initial loss of carrier. In sites where retention of a carrier or bone graft substitute for longer periods of time is desirable, BMP-2 may not be the best approach overall.

Other additives, such as PDGF, are neutral with respect to DFDBA resorption (see Fig. 2). Even though PDGF was inhibitory with respect to bone formation, it was not because it stimulated bone resorption, supporting the interpretation of results (discussed previously). Emdogain and PRP delayed the rate at which DFDBA was resorbed, suggesting that factors present in these two complex agents might modulate bone remodeling in addition to bone formation. These are two different and complex mixtures that had distinctly different effects on DFDBA-induced osteogenesis, but it is likely that some factors are in common.

The case of Bio-Oss

Many of the approaches used to improve the predictability of DFDB-induced bone formation, such as those described previously, also are used

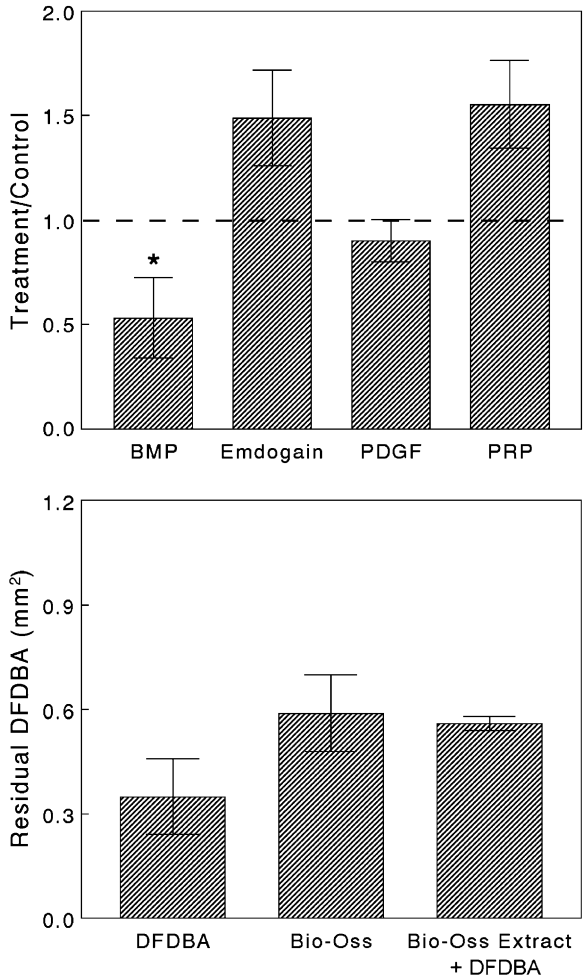

Fig. 2. Effects of bioactive agents on residual implant after DFDBA-induced bone formation in the gastrocnemius muscle of immunocompromised mice. At harvest, implanted tissues were examined for the presence of residual implant materials and the area measured. The top panel compares the effects of BMP-2, Emdogain, PDGF-BB, and PRP as treatment-to-control ratios. The bottom panel compares the amount of residual Bio-Oss to low-activity DFDBA with and without concentrated Bio-Oss extracts containing BMP-2 and TGF-β1. Data are means ± SEM for 8 implants of each kind.

with osteoconductive bone graft substitutes. The design of many of these materials is based on the chemical and physical structure of bone. The mineral phase of bone is a carbonate-substituted apatite and the crystallites are organized in a complex organic matrix. Various manufacturers have focused on the calcium phosphate chemistry and many of the new bone graft substitutes are fabricated to be resorbable within a reasonable time frame. The

goal is for a material to be removed as new bone is formed, but this cannot always be achieved with ceramic materials of this kind.

An alternative approach is to produce materials that have the physical structure of bone mineral but with the organic matrix removed. The most common method used for this is to essentially incinerate the organic matrix, resulting in a sintered mineral phase that has the physical properties of calcified bone. Early studies indicate that although lower temperatures could remove most of the organic components of bone and retain bone mineral–like crystal structure, protein is protected by the mineral phase and could persist [35]. This is the case with Bio-Oss, which essentially is anorganic bovine bone, albeit with low levels of bioactive protein present, including BMP-2 [12]. As shown in Fig. 3, Bio-Oss by itself is not osteoinductive. After 56 days of implantation in nude mouse muscle, only the implant remained. When concentrated extracts of Bio-Oss proteins were added to low-activity DFDBA, however, osteoinduction ability of the composite implant was comparable to that seen typically in high-activity DFDBA. This is because of a twofold increase in new bone formation (see Fig. 3), similar to that seen with Emdogain. The Bio-Oss extracts were added to low-activity DFDBA [12], whereas Emdogain was added to high-activity DFDBA [36]. Thus, the effect of the Bio-Oss extract likely was the result of the added BMP-2. Despite this, the amount of DFDBA remaining at the implant site was unchanged (see Fig. 2). In an orthotopic site, a material, such as Bio-Oss, may be advantageous not only for its structural properties but also for the potential stimulus of inherent factors that stimulate bone formation.

Summary

Bone is a highly vascularized tissue and, under normal circumstances, even large defects heal with bone because of the local supply of mesenchymal stem cells within the bone marrow environment and the presence of osteoinductive agents within the bone matrix. In addition, factors released at the wound site or present within the hematoma enhance osteogenesis by recruiting progenitor cells and stimulating their proliferation and differentiation. When this process becomes dysregulated, either because of the size of the defect or because of other host-dependent factors, some form of bone tissue engineering may be necessary. Addition of multipotent autologous cells, such as those in bone marrow, and the addition of autologous bone as a structural element provide the optimal therapeutic approach. Often one or both of these are in limited supply and alternative approaches are needed. The lessons learned from the authors' study of DFDBA-induced bone formation illustrate those features of osteogenesis that may confound the use of tissue engineering strategies.

- The behavior of material may be different orthotopically and heterotopically. Factors that promote osteoinduction in muscle likely are effective

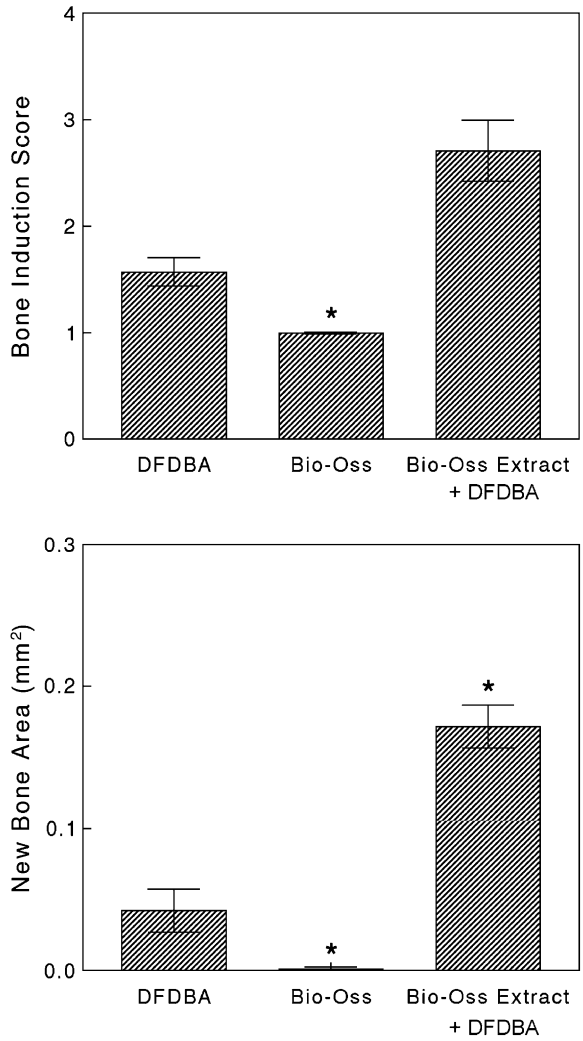

Fig. 3. Effect of Bio-Oss extracts containing BMP-2 on the ability of low-activity human DFDBA to induce bone formation in the gastrocnemius muscle of immunocompromised mice. The osteoinduction score was determined using a semiquantitative scale (*top panel*). The area of new bone was determined histomorphometrically (*bottom panel*). Values are expressed as means $\pm$ SEM for 8 implants. *, $P<0.05$ versus DFDBA alone.

in a bone site, but those factors that inhibit osteoinduction may have usefulness orthotopically as long as the site is not overly compromised.

- Simply adding a growth factor or cell attachment ligand may be sufficient in fresh fractures in healthy rats but may not be effective in patients, especially when confounding variables are present, in particular those involving age and pharmacology.

- The rate of graft resorption may be a critical variable depending on the intended use of a material and should be considered when designing any kind of bone graft substitute.
- Each bioactive factor acts through a distinct mechanism that influences its relative function when used clinically. Understanding those mechanisms is important when selecting a material for a specific application.

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