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Review

Biotechnology and Bone Graft Substitutes

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Trauma, disease, developmental deformities, and tumor resection frequently cause bone defects that seriously challenge the skills of orthopedic and maxillofacial surgeons. Currently, repairing osseous deficiencies involves various medical surgical techniques, including autogenous grafts, allografts, internal and external fixation devices, electrical stimulation, and alloplastic implants. The existing technology, though effective in many cases, still is beset with numerous difficulties and disadvantages. A critical need for improved treatment methods exists today. Biotechnology now provides access to new bone repair concepts via administration of protein growth and morphogenic factors. Implantable device and drug delivery system technologies also have advanced. The converging biopharmaceutical, device, and delivery technologies represent an opportunity to improve the quality of health care for individuals with orthopedic and maxillofacial deficiencies. This report reviews current concepts in fracture healing and bone repair and examines existing treatment modalities. It also addresses novel protein drugs that stimulate osseous regeneration and delivery systems for these drugs.

KEY WORDS: biotechnology; bone regeneration; morphogenetic factors; growth factors; devices; orthopedics.

INTRODUCTION

Orthopedic, plastic, and oral surgeons perform more than 250,000 bone grafts annually in the United States (1–3). Surgeons decide to graft when presented with an osseous defect that experience shows will not heal properly. Common causes of bony defects include trauma (motor vehicle collisions, and pedestrian/vehicle accidents, ballistic injuries), birth defects (nasoalveolar cleft, craniofacial deformities), oncologic resections, and disease pathoses (periodontitis, degenerative osteoarthritis, and osteomyelitis). Improper osseous healing has potentially devastating consequences, ranging from disfigurement to loss of function and loss of limb.

The goals in treating such conditions are to restore form and function to patients. Although usually effective in meeting these treatment goals, auto-, allo-, and xenografts are plagued with disadvantages. Autogenous grafting involves harvesting healthy bone from one anatomical site and implanting the graft material in a defect site. Whereas autograft surgery yields the most predictable results, disadvantages of the surgery include

- donor site pain and morbidity,
- potential donor site infection,
- extra blood loss from the donor site, and
- cost.

Furthermore, autografting is ineffective when the defect volume exceeds the volume of healthy graft material. Additionally, a second invasive procedure is contraindicated for geriatric or pediatric patients in a compromised state. The most common alternative to the autograft is human cadaver bone (allograft). Allografts fail more frequently than autografts and allografting has additional disadvantages, including potential host rejection, limited supply in some locations, excessive resorption, unpredictable outcome, and potential disease transmission. Xenogeneic (animal) tissues find rather infrequent application in bone grafting owing to concerns with immunogenicity and disease transmission.

There is a clear and urgent need to provide alternatives to traditional bone grafting. The medical device industry has offered some treatment options, but daunting challenges remain unaddressed by device approaches. Recent recombinant biopharmaceutical advances, however, offer exciting potential for treating osseous defects. Thus, basic bone biology research and genetic engineering have combined to produce highly pure human proteins that *initiate* the bone regeneration process. These *recombinant human bone morphogenetic proteins* (and, to a lesser extent, other polypeptide growth factors) may realistically become the routine treatment alternative to autografts and allografts.

It is also evident that new opportunities exist to apply pharmaceutical science and technology to the problem of incorporating morphogenetic proteins into optimally effective delivery devices engineered to meet the unique physiologic requirements. The primary objective of this Review, therefore, is to describe such opportunities and identify some challenges that lie ahead. The review begins with an introduction to osseous regeneration concepts and then ex-

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amines current and anticipated treatment methods. Bone morphogenetic proteins also are described, to include recent preclinical data obtained using recombinant human bone morphogenetic protein-2 (rhBMP-2).

OSSEOUS DEFECT REPAIR CONCEPTS

Fracture Healing Sequence

The study of fracture healing has provided important insight into the mechanisms of direct bone formation as well as the events that follow orthopedic trauma. These same events also are relevant to either healing of bone grafts or applying morphogenic proteins.

The fracture healing sequence subdivides into five major stages, as shown in Table I (4–6). During the initial phase, a hematoma forms and molecular messengers enter the extracellular compartment. These factors stimulate cellular migration, proliferation, and differentiation and initially control the healing response. Factors known to be present at high concentrations in fractures include platelet-derived growth factor (PGDF), fibroblast growth factor, transforming growth factor β (TGF-β), insulin-like growth factor, and E-type prostaglandins (5,7–11). Bone morphogenetic protein release also may occur during the initial phase, but direct experimental proof is currently lacking.

During the inflammatory phase, neutrophils and macrophages invade the wound site to scavenge debris, remove microorganisms, and express biochemical mediators affecting fibroblastic and angiogenic responses. The inflammatory phase is essential to fracture healing and administering indomethacin or other antiinflammatory drugs is contraindicated in the first few weeks following orthopedic trauma.

After approximately 2 weeks, the so-called soft callus forms. Soft callus is a highly cellular, active environment that spans the fracture gap and features abundant collagenous extracellular matrix. Chondrocytes and osteoprogenitor cells appear. Cartilage formation and revascularization are characteristic of soft callus. Within approximately 2 months, soft callus formation is essentially complete and forms the foundation for hard tissue generation.

The hard callus phase of fracture healing represents the period when cartilage calcifies. In the callus phase, osteoblasts differentiate and elaborate osteoid. Calcified cartilage resorbs, and new bone forms, followed by remodeling. Although cortical bone formation is incomplete during the hard callus phase, the fracture is normally sufficiently strong to restore function and healing is considered complete.

Months and even years after orthopedic trauma, the woven bone evident in hard callus remodels into lamellar and osteonal osseous structure. The remodeling phase returns full strength and normal contour to the fracture area.

Table I. Phases in the Normal Fracture Healing Sequence

Phase	Time	Activities
Induction	0 to 2 days	Hematoma formation Release soluble inductive, growth, and inflammatory factors
Inflammation	2 to 14 days	Polymorphonuclear neutrophils Macrophages
Soft callus	2 to 8 weeks	Highly cellular and collagenous material in fracture gap Chondrogenesis and angiogenesis
Hard callus	2 to 12 months	Woven bone forms Considered healed at this stage
Remodeling	1 or more years	Lamellar bone forms

Cellular Sequence of Bone Morphogenesis

Reddi and Huggins (12–14) used demineralized bone (a source of morphogenic proteins, *vide infra*) to generate ossicles in a rat extraskeletal (subcutaneous) site. The cellular events following induced bone morphogenesis are shown in Table II. Interestingly, the sequence shown in Table II closely resembles epimorphic and embryonic limb formation. Thus, bone morphogenetic proteins apparently recapitulate hard tissue embryogenesis.

Term Definitions

The fracture healing literature describes unique physiologic concepts and specific terminology. Table III summarizes a few key terms that frequently appear in discussions of induced osseous healing (15–17).

It is important to distinguish the terms "osteogenic" and "osteoinductive." Osteogenic materials either (i) contain cells that are committed to osteoblastic phenotypes or (ii) stimulate committed osteoprogenitor cells and mature osteoblasts to proliferate. Bone marrow and autogenous bone graft contain active osteoprogenitor cells and are therefore osteogenic. Bone marrow, bone graft, and "demineralized bone matrix" (DBM; see Table III) contain growth factors that stimulate committed cells. TGF-β and other growth factors stimulate bone cell proliferation and are considered to be osteogenic.

By comparison, osteoinductive materials stimulate uncommitted cells (for example, mesenchymal stem cells) to convert phenotypically to chondroprogenitor and osteoprogenitor cells. The distinction is that cellular proliferation characterizes osteogenesis, whereas cellular differentiation

Table II. Sequence of Induced Bone Morphogenesis in Extraskeletal Sites[a]

Event	Time (days)
Progenitor cell chemotaxis	0 to 2
Mesenchymal cell mitosis	3
Chondrocyte differentiation	5 to 7
Cartilage calcification angiogenesis	7 to 10
Bone formation	10 to 11
Bone remodeling	12 to 18
Hematopoiesis in ossicle marrow compartment	20 to 21

[a] Modified from Reddi and Anderson (1976).

Table III. Fracture Healing and Bone Morphogenesis Terminology[a]

Term	Definition	Example(s)
Osteogenic	Contains osteoprogenitor cells	Bone marrow
	Stimulates (committed) osteoprogenitor cell proliferation	Bone graft
Osteoconductive	Scaffold on which committed osteoprogenitor cells produce bone	Demineralized bone matrix
		Bone graft
	Bony ingrowth from fracture ends	Hydroxyapatite ceramics
Osteoinductive	Bone morphogenesis	Demineralized bone matrix
	Stem cell phenotypic conversion to osteoblasts	Bone graft
		Bone morphogenetic proteins (BMPs)
Demineralized bone matrix (DBM)	Bone extracted with organic solvent and hydrochloric acid to remove fats and inorganics	Essentially collagenous matrix containing active protein factors
Inactive collagenous bone matrix (ICBM)	DBM extracted with guanidine or urea to remove noncollagenous proteins	Collagenous matrix devoid of active factors

[a] Reference 3 provides an excellent description of this terminology.

characterizes osteoinduction. Bone morphogenetic proteins (but not TGF-β and other growth factors) are osteoinductive. Bone marrow, bone graft, and DBM all contain bone morphogenetic proteins and are therefore osteoinductive as well as osteogenic.

Osteoconduction, a third important concept, has two different meanings. Some references use the term osteoconduction to describe the process by which bony ingrowth progresses from fracture ends into a fracture gap. More commonly, osteoconduction refers to substrates that provide a favorable scaffolding for vascular ingress, cellular infiltration and attachment, cartilage formation, and calcified tissue deposition. Osteoconductive materials may support osseous regeneration via the scaffolding effect.

It is now generally believed that osseous regeneration can be promoted by combining osteoconductive materials with osteogenic and/or osteoinductive materials. Most current efforts to improve traditional grafting treatments for severe osseous defects seek practical approaches that combine osteoconductive and osteogenic/osteoinductive materials.

OSSEOUS DEFECT REPAIR PRACTICES

Currently Approved Practices

Table IV summarizes osteogenic, osteoconductive, and osteoinductive materials currently approved for use in the United States. Autograft is the most widely used and accepted material, followed by allograft. Both auto- and allograft contain osteogenic and osteoinductive factors as well as osteoconductive collagenous and noncollagenous substrate.

Table IV. Approved Materials for Bone Grafting

Category	Material	Source[a]
Osteogenic, osteoinductive, and osteoconductive	Autogenous bone graft	Patient
	Allogeneic demineralized bone matrix	*Osteotech*
Osteogenic and osteoinductive	Autogenous bone marrow	Patient
Osteoconductive	Bovine bone mineral	*W. Lorenz*
	Coralline hydroxyapatite	*Interpore*

[a] Italics indicate corporate source.

Osteotech markets a product under the trade name, Grafton. Grafton is human DBM processed aseptically from cadaver bone provided by the American Red Cross (3). Grafton is a viscous semisolid and is available in prefilled syringes. As with other DBMs (19–21), Grafton is considered to be osteogenic, osteoconductive, and osteoinductive.

Autogenous bone marrow also finds significant application in clinical practice as an osteogenic and osteoinductive material (1,18).

Finally, two inorganic osteoconductive materials (Pro Osteon and Bio-Oss) are available. Pro Osteon is a coralline hydroxyapatite implant for orthopedic and maxillofacial indications. The literature documents the osteoconductive properties of hydroxyapatite (HA) and tricalcium phosphate (β-TCP) porous ceramics (22–26). Pro Osteon material is HA derived from coral and processed to provide pore and channel structure consistent with osteoconduction. The coralline HA implants provide mechanical strength during healing and permit osseous integration via the internal pore structure (27,28). Hydroxyapatite may slowly resorb, however, and Pro Osteon implants remain for months (and possibly years) after surgery.

The Bio-Oss implant is an anorganic derivative of bovine bone. The manufacturing process removes essentially all organic material, leaving a porous HA matrix. Marketed primarily for maxillofacial and dental applications, the Bio-Oss implants, like the Pro Osteon material, allow osseous integration throughout internal pore structures (32).

Investigational Procedures

In addition to the bone grafting techniques and materials currently approved for use, several materials are under study in preclinical and clinical settings. At least some investigational materials will receive approval for medical practice and therefore warrant discussion. Table V lists relevant ex-

Table V. Investigational Materials for Bone Grafting

Category	Material	Phase	Source[a]
Osteoconductive	Collagen and HA/TCP composite	Phase III	*Collagen Corp.* *Zimmer*
	Poly(lactic acid) foam	Preclinical	*Danek*
	Fibrin sealant	Preclinical	*Immuno* *American Red Cross*
	Calcium sulfate	Physician	
		IND	N/A
Osteogenic, osteoinductive, and osteoconductive	Bovine demineralized bone matrix	Physician	
		IND	N/A
Osteoinductive	human BMP	Physician	
		IND	Urist
	rhBMP-2	Phase I	*Genetics Institute*

[a] Italics indicate corporate source.

amples and the following paragraphs address selected characteristics of the investigational materials.

Bovine liquid collagen plus HA/TCP ceramic granules comprise a composite (Collagraft) that has completed Phase III clinical trials and awaits approval for use (29–31). It is recognized that both insoluble collagen (33–36) and HA/TCP ceramics (*vide supra*) are osteoconductive. Adding autogenous bone marrow or autograft to the Collagraft composite yields a bone graft "extender" for orthopedic applications. Although clinical trial results apparently demonstrate that Collagraft works effectively, concerns remain about the immunogenicity of xenogeneic collagen and the long-term effects of nonresorbable HA ceramic on bone strength.

Clinical studies have examined bovine demineralized bone matrix (without added ceramic material) as an osteoconductive and osteogenic substrate (37–39).

Several groups (40–45) report using lactide and glycolide polymers and copolymers as absorbable matrices for osseous regeneration. These polymers lack immunogenic potential and have a long history of safe use in suture materials. Despite their promise as osteoconductive materials, however, synthetic polymers have not progressed beyond the research investigation stage.

Human fibrin sealant has been studied preclinically as an osteoconductive substrate for osteoinductive materials (46–48). Fibrin sealant is currently available in Europe and the American Red Cross has fibrin under development in the United States. As with other allogeneic materials, however, the fibrins may pose some threat of disease transmission.

Calcium sulfate (plaster of Paris) has received attention as a filler material for osseous repair (49–51), but the reported studies are rather dated and do not pertain to combined use of calcium sulfate with osteogenic or osteoinductive materials. Moreover, plaster of Paris bioabsorption is rather unpredictable.

Finally, several bone morphogenetic proteins (BMPs) are in early clinical trials. Urist has studied human BMP purified from fresh cadaver bone. The hBMP studies used several osteoconductive substrates including allogeneic bone (52,53), polylactide onlays (54–56), and TCP ceramic (57). Two recombinant human BMPs, rhBMP-2, and rhBMP-7, have also entered initial clinical testing. Unlike purified human BMP, the recombinant proteins present no disease transmission risk and are available in high purity and potentially unlimited amounts. The promise afforded by the rhBMPs warrants special attention and the following section examines these newest osteoinductive materials in detail.

BONE MORPHOGENETIC PROTEINS

Overview

Osteoinduction studies date to 1889 when Senn (58) implanted a decalcified ox tibia containing iodoform into a canine calvarial defect. The objective was to treat the dog's osteomyelitis. A positive, unexpected result was osseous regeneration suggesting the presence of an "inductive" factor in the graft material. In 1937, Huggins (59) observed bone formation following implantation of renal epithelial tissues in an extraskeletal site. In 1965, Urist (60) generated osseous tissue in an extraskeletal (subcutaneous) site with rat DBM implants, thereby demonstrating the existence of an autoinductive factor. Urist *et al.* further showed (61,62) that treating DBM with aqueous guanidinium hydrochloride removes the inductive factors and that reconstituting inactivated DBM with the aqueous extract restores osteoinductive activity. Urist thus coined the term "bone morphogenetic protein(s)" to describe the extractable inductive factor(s).

BMPs are present in bone matrix at extremely low concentrations (on the order of 1 μg BMP/kg DBM). Wang, Wozney, and co-workers (63,64) isolated, identified, and cloned individual BMPs. Seven individual BMPs are now known (65,66), and the published amino acid sequence information demonstrates that some osteoinductive proteins

Q A K H K Q R K R L K S S **C** K R H P L Y	20
V D F S D V G W N D W I V A P P G Y H A	40
F Y **C** H G E **C** P F P L A D H L N S T N H	60
A I V Q T L V N S V N S K I P K A **C C** V	80
P T E L S A I S M L Y L D E N E K V V L	100
K N Y Q D M V V E G **C** G **C** R	114

Fig. 1. rhBMP-2 amino acid sequence showing cysteines (boldface) and glycosylated asparagine (underlined).

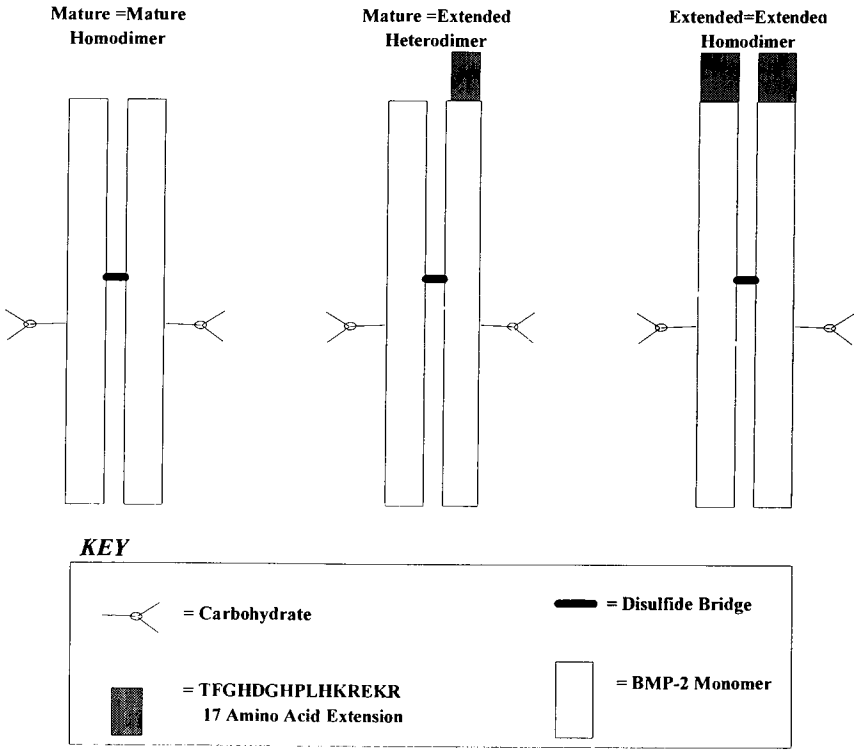

Fig. 2. Schematic representation of rhBMP-2 primary sequence heterogeneity. Three isoforms (two homodimers and one heterodimer) are possible.

reported earlier are, in fact, BMPs. Thus "osteogenin" has the rhBMP-3 amino acid sequence (67) and "osteogenic protein-1" is actually BMP-7 (68,69).

The various rhBMPs are all present in bone matrix and may function in a complex synergy. It is known, however, that rhBMP-2 (in the absence of other BMPs) implanted in an extraskeletal site induces bone formation through endochondral ossification (70) and therefore it has received extensive characterization.

rhBMP-2

rhBMP-2 is a 32-kDa, highly basic (p*I* > 8.5), homodimeric protein (66). Each "mature" monomer contains 114 amino acids, including 7 cysteines, and 1 consensus region for N-linked glycosylation. Figure 1 shows the amino acid sequence for the "mature" monomer.

Recent work indicates that Chinese hamster ovary (CHO) cell expression systems produce three rhBMP-2 isoforms (71). A 17-amino acid N-terminal extension to the mature monomeric polypeptide, and the mature monomer give two homodimeric combinations and one heterodimeric combination to produce the three isoforms as shown schematically in Fig. 2. Glycosylation contributes to additional protein microheterogeneity. Mass spectrometric analysis (71) of reduced and alkylated rhBMP-2 reveals five rhBMP-2 glycoforms (*N*-acetylglucosamine with 5, 6, 7, 8, or 9 mannose residues). Since rhBMP-2 is a dimer, there are theoretically 15 different glycoforms.

Interestingly, capillary electrophoresis provides the analytical resolution to separate successfully rhBMP-2 into its individual glycoforms. Figure 3 shows overlays of capillary electropherograms for intact rhBMP-2 and deglycosylated (*endo-H*-treated) protein. The deglycosylated sample shows the three rhBMP-2 primary structural isoforms and the intact sample shows 14 of the 15 available glycoforms.

PRECLINICAL STUDIES WITH rhBMP-2

Initial studies in rats (70) demonstrated that rhBMP-2 is osteoinductive in extraskeletal (subcutaneous) implant sites using allogeneic ICMB as an osteoconductive substrate. This work left three important questions unanswered. First, can rhBMP-2 promote bone regeneration in an orthotopic (bony) site? Second, can rhBMP-2 promote bone regeneration in higher animals? Third, can rhBMP-2 promote bone regeneration when combined with an osteoconductive substrate other than collagenous bone matrix?

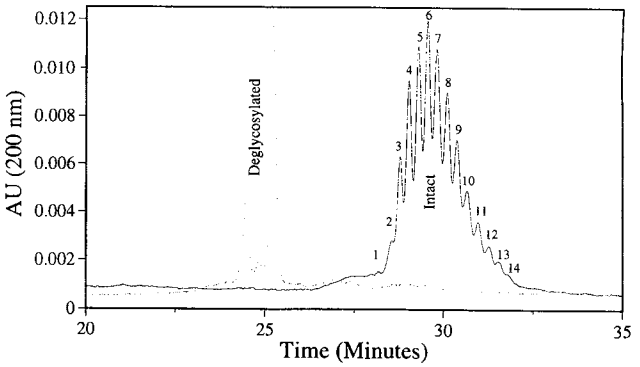

Fig. 3. Overlayed capillary electropherograms for intact rhBMP-2 and deglycosylated (by *endo-H* treatment) rhBMP-2. Fourteen (of 15 possible) glycoforms are seen for intact rhBMP-2.

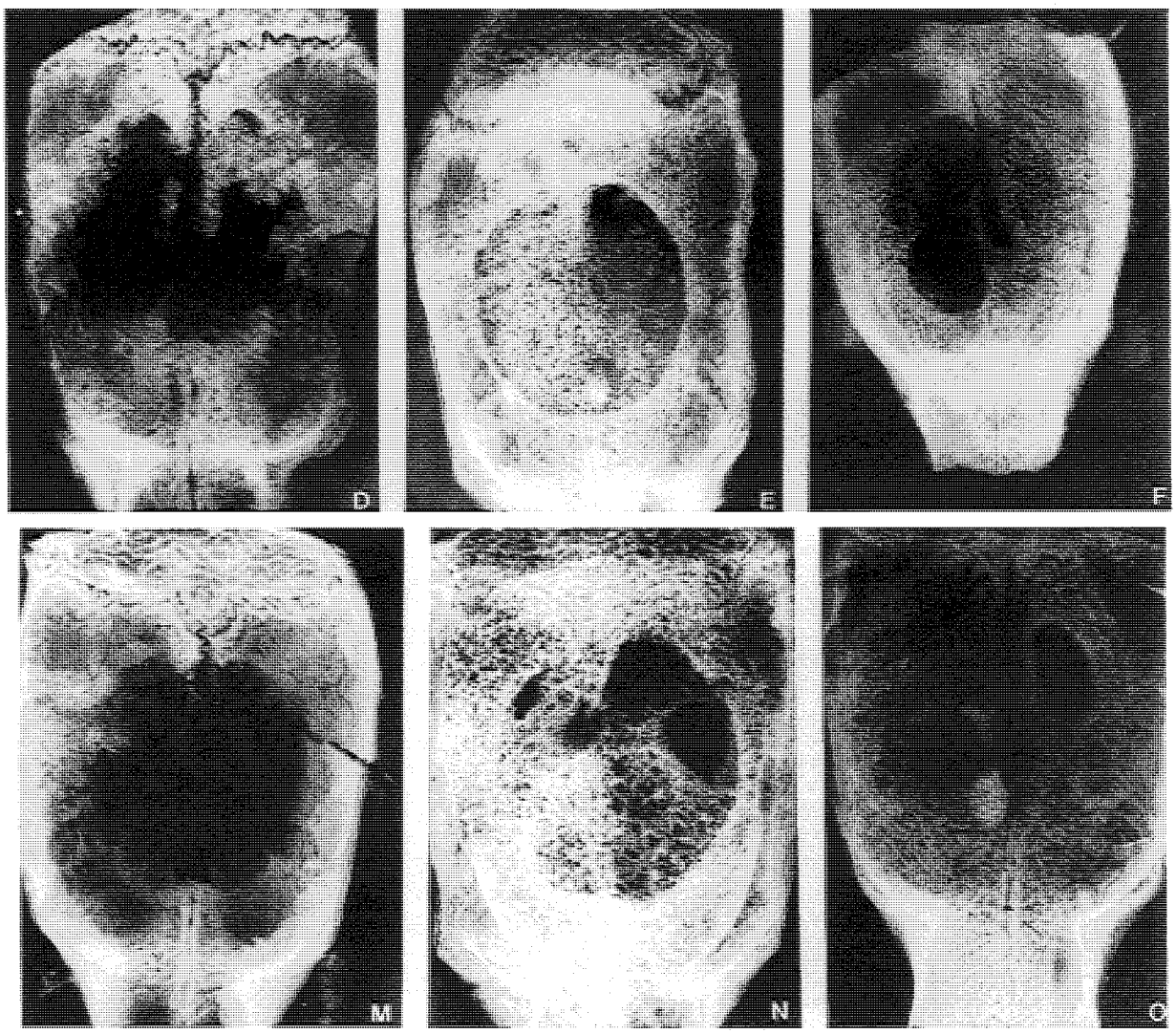

Fig. 4. Radiographs of rat calvarial defects at 3 weeks. (D–F) ICBM implants at 0, 10, and 30 μg rhBMP-2, respectively. (M–O) PLGA/blood implants at the same doses.

To address the first issue, Yasko *et al.* (72) implanted rhBMP-2 with allogeneic ICBM in a rat segmental femoral defect model. In this model, a 5-mm transcortical segment is resected and internal fixation applied. The 5-mm gap is a critical-size defect (73) insofar as untreated defects do not proceed to osseous union. Treatment groups received either ICBM alone, ICBM plus 1.4 μg rhBMP-2, or ICMB plus 11 μg rhBMP-2. Using radiographic, histologic, and biomechanical test methods, animals were evaluated at timed intervals through 9 weeks postimplantation. This study verified that untreated and ICBM-treated animals did not achieve osseous union. Both rhBMP-2 treatment groups, however, progressed to union and demonstrated essentially normal histology and biomechanical strength. Thus, rhBMP-2 effectively regenerates osseous tissue in a bony site.

Toriumi and co-workers (74) extended the study of rhBMP-2 effectiveness to dogs. These authors used rhBMP-2 with allogeneic ICBM in a canine mandibular segmental defect model. Untreated control groups demonstrated that a 3-cm mandibular segment is a critical-size defect. Treatment groups with 250 μg rhBMP-2 per implant, however, demonstrated excellent radiographic union and woven bone formation (by histology) at 3 and 6 months postimplantation.

Notwithstanding these promising initial preclinical results with rhBMP-2 in bony defects, severe practical difficulties (such as potential rejection, immunogenicity, supply limitations, and disease transmission) complicate the use of allogeneic or xenogeneic bone matrix (ICBM) in medical practice. The ideal complement to recombinant human bone morphogenetic protein should be noncollagenous, bioabsorbable, and osteoconductive. The literature, however, does not directly establish precedents for successfully combining rhBMPs with noncollagenous matrices. Indeed, Ma *et al.* (33) claim that collagenous substrate is an essential requirement for osseous regeneration using osteogenin (BMP-3) as osteoinductive factor.

To explore this issue, we combined rhBMP-2 with noncollagenous substrates (75) and treated 8-mm (critical-

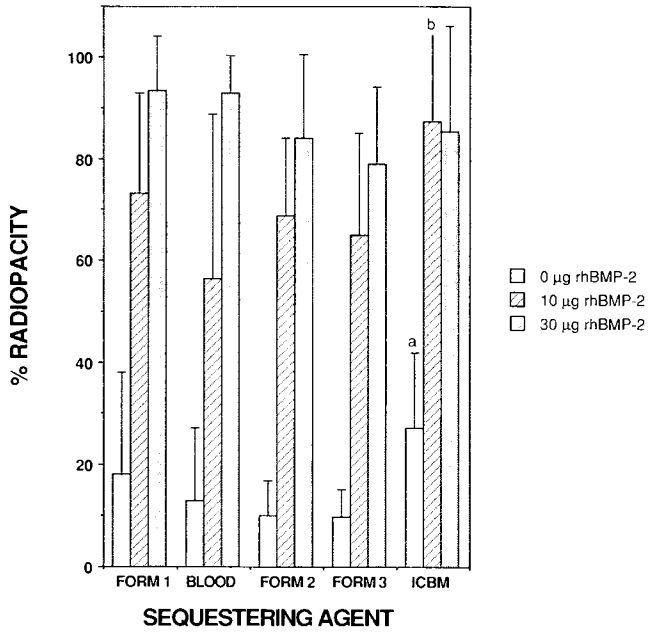

Fig. 5. Percentage radiopacity (by radiographic image analysis) in rat calvarial defects at 3 weeks postimplantation. The percentage radiopacity is shown for five treatment groups and three rhBMP-2 doses. Error bars represent the standard deviation of 9 to 13 replicates. (a) Significantly different from Formulations 2 and 3 and blood at same dose of rhBMP-2 ($P < 0.05$). (b) Significantly different from Formulation 3 and blood at same dose of rhBMP-2 ($P < 0.05$).

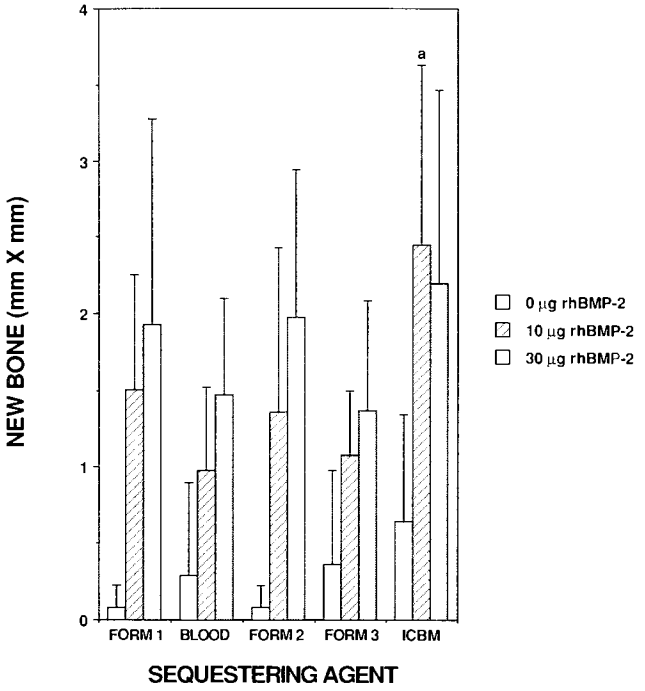

Fig. 6. Area occupied by new bone from histomorphometric analysis of rat calvarial defects at 3 weeks postimplantation. Data for five treatment groups and three rhBMP-2 doses. Error bars represent standard deviation of 8 to 13 replicates. (a) Significantly different from all other formulations at same dose of rhBMP-2 ($P < 0.05$).

size) calvarial defects in rats. This study used 0, 10, and 30 µg rhBMP-2 per 0.1-mL implant and compared powdered ICBM controls with microparticulate poly(lactide-*co*-glycolide) (PLGA) implants. ICBM powder was mixed with a rhBMP-2 solution to yield a moldable paste. PLGA/rhBMP-2 mixtures were formulated as pastes with allogeneic blood clot ("blood") or with three different thickening agents ("Form 1," "Form 2," and "Form 3") to provide implantable semisolids. Radiographic, radiomorphometric, histomorphometric, and histologic evaluations were performed at 3 weeks postimplantation. Figure 4 shows radiographs for three rhBMP-2 doses in ICBM versus PLGA treatment groups. Significant radiopacity is clearly evident at the 10- and 30-µg doses (but not the 0-µg dose) for both treatment types.

Figures 5 and 6, respectively, show that quantitative radiomorphometry and histomorphometry verify the radiographic results. In general, the responses are dose dependent. At the 10-µg dose, ICBM treatment groups showed statistically significantly more bone regeneration than all other treatments. At the 30-µg dose, however, there were no statistically significant differences between treatment groups with respect to radio- and histomorphometric response. Thus, it appears certain that *noncollagenous substrates combined with rhBMP-2 do support osseous regeneration.* Understanding the clinical significance of these preliminary observations awaits the outcome of ongoing trials.

CONCLUSIONS

Extensive, massive osseous defects are a major reconstructive challenge. Available treatments have significant disadvantages. Recombinant human bone morphogenetic proteins represent a watershed opportunity for developing safe and effective bone graft substitutes. A century after Senn's original report, and three decades after Urist proved the principle of osteoinduction, biotechnology now promises to provide highly pure, active rhBMPs for clinical use. Although considerable daylight exists between today's research and tomorrow's approved therapy, it is truly exciting to contemplate the potential for successful outcome. In this context, the authors consider that pharmaceutical disciplines are now particularly well positioned to impact positively the developing alternatives to bone grafting. Drug delivery technology is advanced in conventional (parenteral and nonparenteral) pharmaceutical applications, and this technology may transfer to implantable carrier systems for osteoinductive proteins. Thus, pharmaceutical sciences are now poised (both literally and figuratively) to bridge the gap between biotechnology and bone repair.

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