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Use of Bone Morphogenetic Protein 2 on Ectopic Porous Coated Implants in the Rat

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The ability of recombinant human bone morphogenetic protein 2 to remain osteoinductive and stimulate appositional bone formation on a porous coated implant was tested in a rat quadriceps muscle pouch. Implants with or without hydroxyapatite were used to compare the effects on bone formation of two different doses (23 μg or 46 μg) of recombinant human bone morphogenetic protein 2 against controls as evidenced by contact radiography, histologic examination, and backscatter scanning electron microscopic analysis. Cylindrical plasma sprayed porous titanium implants were placed bilaterally within a muscle pouch surgically created in 48 Lewis rats. Implants treated with recombinant human bone morphogenetic protein 2 formed significantly more bone than did control implants independent of the dose or presence of hydroxyapatite. In all implants with bone formation, osteoinduction via endochondral ossification began within 7 days. By 21 days, cartilage largely was replaced by bone and marrow. The results of this ectopic, nonweightbearing in vivo assay suggest that recombinant human bone morphogenetic protein 2 remains biologically active after application to a titanium implant and may be used to enhance appositional bone formation by direct application to the implant surface.

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Intermediate term results of cementless arthroplasty suggest that subsidence may occur because of the failure of porous ingrowth and bony apposition.[4,19,41] Factors that enhance the amount and maturation of bony ingrowth in cementless arthroplasty may have a definite impact on the ultimate fixation strength and stability at the implant to bone interface. Several investigators have described the fabrication and biology of porous surfaces,[9,18,24] optimal pore size,[2,40] biomechanical requirements promoting bone ingrowth,[25] and the osteoinductive properties of agents designed to enhance bone ingrowth.[13,21,29,38] Experimental models have shown that hydroxyapatite can enhance early bone remodeling,[17] gap heal-

ing,[20] and implant to bone apposition approximately 40% to 50%.[6] Hydroxyapatite treated porous implants have shown enhanced fixation in stable implant models and in intermediate term (less than 5 years) clinical followup.[7,8,11]

Recombinant human bone morphogenetic protein 2, the osteoinductive factor used in this study, has been shown to induce ectopic formation of bone when placed subcutaneously,[36,37,39] and in orthotopic sites in rats, rabbits, dogs, and sheep, resulting in the healing of segmental bone defects and enhanced spinal fusion union rates.[3,12,22,42] However, no studies have used purified recombinant human bone morphogenetic protein 2 alone or in combination with osteoconductive hydroxyapatite to determine its osteoinductive role in enhancing bone formation at a titanium porous coated implant interface.

The current investigation was designed to determine the biologic activity of recombinant human bone morphogenetic protein 2 when applied directly to a titanium plasma sprayed porous implant placed within bilateral intramuscular quadriceps pouches in the rat with or without a hydroxyapatite coating. The experiment tested the hypothesis that recombinant human bone morphogenetic protein 2 applied to a porous coated implant placed in an ectopic environment will produce appositional bone ingrowth from resident progenitor cells without the confounding effects of direct osseous contact normally present in an orthotopic site. It was expected that the presence of hydroxyapatite will induce bone in greater abundance and apposition compared with similar implants without hydroxyapatite.

MATERIALS AND METHODS

Animals

All methodology was approved by the Institutional Animal Care and Use Committee. Forty-eight adult syngeneic Lewis rats weighing 325 to 350 g had 96 implants placed bilaterally within their quadriceps muscle. Rats were caged individually and maintained on rodent chow (Purina, St Louis, MO) and water ad libitum. Unrestricted activity was permitted as tolerated after surgery.

Recombinant Human Bone Morphogenetic Protein

The preparation of recombinant human bone morphogenetic protein 2 has been described previously.[36,37,40] The protein was provided as a carrier stock solution of 2300 μg/ml suspended in 0.5 mol/L arginine and 10 mmol/L histidine (pH = 6.5) buffer. The stock solution was stored at −80° C for less than 1 month before use. Two doses were used (23 μg and 46 μg) in the test groups implanted with recombinant human bone morphogenetic protein 2. Control implants without recombinant human bone morphogenetic protein 2 were coated with an identical carrier solution (20 μl).

Implants

Cylindrical titanium implants measuring 5 mm in diameter by 8 mm in length were fabricated at The Hospital for Special Surgery Biomechanics Laboratory (New York, NY) (Fig 1). A porous titanium surface was deposited by a plasma spraying technique (APS-Materials Corporation, Dayton, OH). Because of the relatively small size of the implant, the coat formed was most consistent with a roughened irregular surface, rather than one that was truly porous.

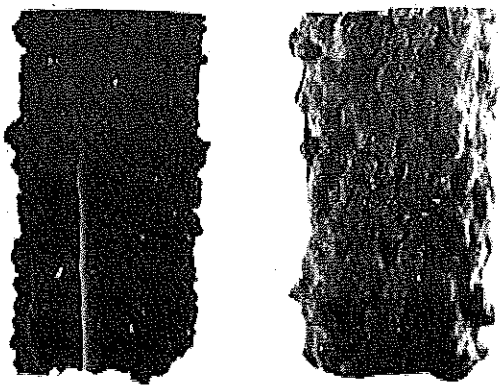

Fig 1. Photograph of porous coated implant. Implants shown are with hydroxyapatite (right) and without hydroxyapatite (left) coating. (See text for exact dimensions, original magnification ×10).

Half of the porous coated implants were plasma sprayed with a hydroxyapatite coating to achieve a 50-μm thick uniform coating (Fig 1). The hydroxyapatite did not obstruct the rough coat morphology and formed a high density, low porosity surface when viewed with a backscatter scanning electron microscope. The hydroxyapatite was prepared in accordance with industry required specifications and quality levels with a purity of more than 95%. Implants were sterilized by gamma irradiation (Isomedix, Whippany, NJ) before treatment with recombinant human bone morphogenetic protein 2.

Implant Preparation

Implants coated with recombinant human bone morphogenetic protein 2 were prepared 24 hours before surgery. Either 23 μg or 46 μg of recombinant human bone morphogenetic protein 2 was pipetted in a 20-μl volume evenly over the implant and allowed to dry overnight beneath a laminar flow sterile hood.

Operative Model

Rats were anesthetized with intramuscular ketamine (100 mg/kg body weight), atropine (0.04 mg/kg body weight), and xylazine (5 mg/kg body weight). Procaine penicillin (600,000 IU/kg body weight) was given intramuscularly for prophylaxis against infection. Using sterile technique, a lateral approach was used to develop an intramuscular pouch within the quadriceps muscle to receive the implant materials. A contralateral quadriceps muscle pouch was similarly prepared to receive a control implant. The enveloping muscle pouch was closed with 3.0 chromic sutures to enclose the composite implant, and the wound was closed in layers using 3.0 chromic and 3.0 nylon sutures.

Experimental Design

Ninety-six implants were placed within the muscle pouches created bilaterally in 48 syngeneic Lewis rats. The rats were divided into four groups, each containing 12 animals, to compare the different implant combinations (Table 1). A control implant without recombinant human bone morphogenetic protein 2 was placed in the left muscle pouch, and a test implant with recombinant human bone morphogenetic protein 2 was placed in the right muscle pouch. Groups I and II contained implants coated with hydroxyapatite. Groups III and IV contained the same implants without hydroxyapatite. Within each group, implants were paired (control versus test) to examine the effects of recombinant human bone morphogenetic protein 2; test implants in Groups I and III had 46 μg of protein applied, and those in Groups II and IV had 23 μg of protein applied.

At two designated times, rats were euthanized by asphyxiation with carbon dioxide. In each group, three animals were sacrificed at 7 days and nine animals at 21 days. Paired implants were excised so as to maintain a soft tissue margin and submitted for radiographic and histomorphometric analysis.

Radiographic Evaluation

The intact specimens with surrounding tissue were contact radiographed at 40 kV potential for 12 seconds (Faxitron, Hewlett Packard, Santa Clara, CA)

TABLE 1. Distribution of Implants to Four Paired Treatment Groups

Group	Side	Number of Rats	Treatment
I	L	12	Hydroxyapatite
	R		Hydroxyapatite/rhBMP-2 (46 μg)
II	L	12	Hydroxyapatite
	R		Hydroxyapatite/rhBMP-2(23 μg)
III	L	12	Implant
	R		Implant/rhBMP-2(46 μg)
IV	L	12	Implant
	R		Implant/rhBMP-2 (23 μg)

L = left; R = right; rhBMP-2 = recombinant human bone morphogenetic protein 2.

using X-Omat film (Kodak, Rochester, NY). Each specimen was scored as being positive or negative for bone formation.

Histomorphometric Evaluation

Histologic Analysis

The specimens were processed for undecalcified histologic analysis by dehydrating in a graded series of alcohols, clearing in xylene, and embedding in methylmethacrylate. Serial sections were cut perpendicular to the long axis of the implant, attached to slides, ground to a nominal thickness of 100 μm, and stained with toluidine blue and basic fuscia. Soft tissue adjacent to the implant was qualitatively and quantitatively evaluated.

The central most section from each implant was used for quantitative analyses. These sections were viewed under a light microscope with a standard eyepiece fit with an 11 by 11 grid creating 121 sampling areas covering the entire field. At the magnification used (×40), the distance between test lines was 0.238 mm, meaning that the grid sampled an area of 6.85 mm^2 (121 test points × 0.056644 mm^2 per test point). The grid was superimposed randomly over one quadrant of the implant and the adjacent soft tissue. The type of material underlying each grid intersection was recorded as (1) implant, (2) bone, (3) marrow, (4) fibrocartilage, (5) fibrous tissue, or (6) muscle. The grid was repositioned further from the implant as needed to cover all of the adjacent tissue. The remaining three quadrants were scored similarly. The average number of intersections sampled per specimen was 495.

A nodule was defined as soft tissue adjacent to the implant containing bone, marrow, or fibrocartilage. The area of the forming nodule was estimated by summing the number of intersections overlying these three tissue types. The volume fraction of each tissue type within the nodule was determined by dividing the number of intersections for that particular tissue by the sum of the intersections for bone, marrow, and fibrocartilage and multiplying by 100%.

To measure bone apposition, the grid was placed perpendicular to the implant at four 90° increments. Each intersection with the implant edge was scored as positive or negative for bone so that the amount of direct bone to implant contact could be quantified. The total number of intersections was 44 per implant.

Backscatter Electron Microscopic Study

Selected unstained sections were examined by backscatter scanning electron microscopic analysis (model 35c; JEOL, Tokyo, Japan). Specimens were prepared, and images were made at a magnification factor of 20 as described elsewhere.[31]

Statistical Analysis

Radiographic evaluation was performed by two observers blinded to the treatment type. McNemar's test for paired data was used to compare the number of implants forming bone as shown by contact radiography at 21 days. Intragroup comparisons were made between the control and test limbs.

Histologic type was evaluated by one trained observer blinded to the treatment groups. Contingency table (chi squared) analysis was performed on the data comparing the two time points for nodule formation and bone apposition. Comparisons between nodule size and relative tissue areas of each tissue type at 7 and 21 days were performed using a Student's t test. Data from test implants harvested at 21 days were subjected to analysis of variance.

RESULTS

There were no complications related to the anesthesia or the surgery. Postoperatively, no animals showed signs or symptoms of infection or rejection of the implant. All wounds healed uneventfully.

Radiographic Evaluation

Contact radiographs of the 24 implants harvested at 7 days revealed no bone formation, regardless of the presence of recombinant human bone morphogenetic protein 2 or hydroxyapatite. The number of implants in each group showing bone formation at 21 days is shown in Table 2.

In Group I (hydroxyapatite coated testing 46 μg of recombinant human bone morphogenetic protein 2), Group III (uncoated implant testing 46 μg of recombinant human bone morphogenetic protein 2), and Group IV (uncoated implant testing 23 μg of recombinant human bone morphogenetic protein 2), test implants with recombinant human bone morphogenetic protein 2 showed a

TABLE 2. Number of Implants With Bone Formation Revealed by Contact Radiograph at 21 Days

Group	Side	Bone	Treatment	McNemar's*
I	L	0/9	Hydroxyapatite	p = 0.004
	R	9/9	Hydroxyapatite/rhBMP-2 (46 μg)	
II	L	3/9	Hydroxyapatite	NS
	R	6/9	Hydroxyapatite/rhBMP-2 (23 μg)	
III	L	0/9	Implant	p = 0.004
	R	9/9	Implant/rhBMP-2 (46 μg)	
IV	L	0/9	Implant	p = 0.008
	R	8/9	Implant/rhBMP-2 (23 μg)	

L= left; R = right; rhBMP-2 = recombinant human bone morphogenetic protein 2; NS = not significant.
*McNemar's test for paired data (L versus R).

statistically greater incidence of nodule formation than did controls independent of the dose used. A representative contact radiograph of a hydroxyapatite coated porous implant with 46 μg of recombinant human bone morphogenetic protein 2 is shown in Figure 2.

In Group II (hydroxyapatite coated testing 23 μg of recombinant human bone morphogenetic protein 2), hydroxyapatite coated control implants formed bone in three of nine implants and hydroxyapatite coated test implants with recombinant human bone morphogenetic protein 2 formed bone in six of nine. However, these differences were not statistically significant.

Histomorphometric Evaluation

Histologic Analysis

Histologically, control implants with or without hydroxyapatite were completely surrounded by fibrous tissue in all implants harvested at 7 days and in 92% (33 of the 36 implants) at 21 days. The three implants with a bone forming nodule in the hydroxyapatite coated control group at 21 days (left, Group II implants) were similar in appearance to the implants coated with recombinant human bone morphogenetic protein 2 at 21 days.

In the recombinant human bone morphogenetic protein 2 treated implants, there were no differences between groups in terms of the incidence of nodule formation, but a greater fraction of the implants had nodules at 21 days (31 of 36) than at 7 days (6 of 12) (chi squared = 6.64, $p < 0.01$). At 7 days, the nodule was characterized by a preponderance of undifferentiated fibrocartilage (Fig 3), although occasional areas of membranous bone or mineralized fibrocartilage were seen. At 21 days, bone formed a cylindrical

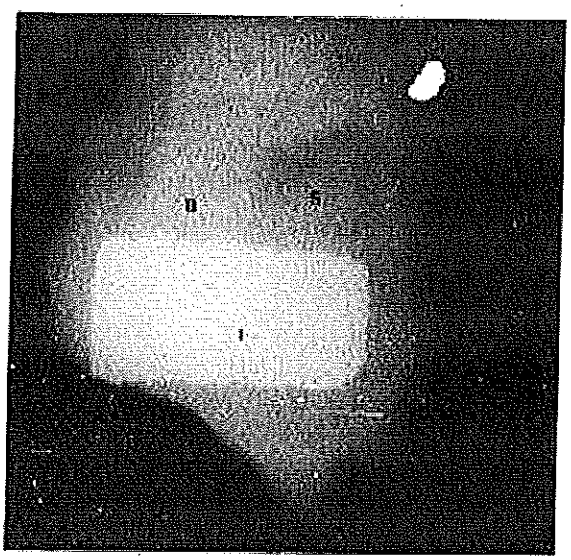

Fig 2. Sample contact radiograph of hydroxyapatite coated implant with 46 μg of recombinant human bone morphogenetic protein 2 after removal at 21 days. Note bony (B) apposition to implant (I) surface. Soft tissue (S) remains around the implant.

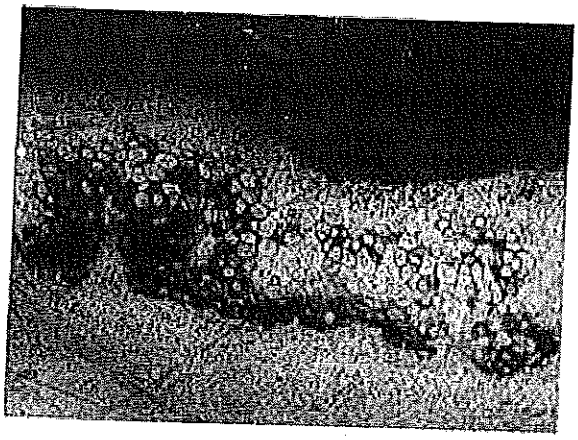

Fig 3. Implant coated with hydroxyapatite and recombinant human bone morphogenetic protein 2 (23 µg) showing cartilage (C) formation at 7 days. Note the presence of a nodule of bone (B) formation removed from the implant surface by a distance of approximately 30 µm. (Plastic embedded specimen surface. Stain, basic fuscia and toluidine blue; original magnification, ×125.)

ring surrounding the implant, with marrow occupying most of the space between the ring of bone and the implant.

Quantitatively, there were no differences in tissue area of the forming nodule or in the relative proportions of bone, marrow, and fibrocartilage among the four recombinant human bone morphogenetic protein 2 treated implants harvested at 7 or 21 days. However, there were significant differences between the recombinant human bone morphogenetic protein 2 treated implants harvested at 7 days and those harvested at 21 days (Fig 4). For this comparison, only the treated implants positive for nodule formation were included in the analysis. From Day 7 to Day 21, there was a fourfold increase in the size of the nodule ($p = 0.016$). This increase was attributable to a 10-fold increase in the amount of marrow ($p = 0.014$) and a threefold increase in the amount of bone ($p = 0.007$). The proportion of the nodule occupied by bone did not change from 7 to 21 days (Fig 5), but there was a sevenfold increase in the relative amount of marrow ($p < 0.001$) and a 55-fold decrease in the relative amount of fibrocartilage ($p = 0.027$).

The number of implants with direct bone to implant contact varied from six to nine per group at 21 days, although one implant had direct contact at 7 days. The incidence of direct contact did not vary significantly among treatment groups at either time but was greater at 21 days (29 of 36) than at 7 days (one of 12) (chi squared = 20.03; $p < 0.001$). For the 29 implants with direct bone to implant contact at 21 days, the mean number of intersects in the groups with hydroxyapatite coating (5.3 ± 2.4 for 23 µg recombinant human bone morphogenetic protein 2 and 4.4 ± 2.3 for 46 µg recombinant human bone morphogenetic protein 2) were not statistically different. The corresponding implants with recombinant human bone morphogenetic protein 2 without hydroxyapatite had a mean number of intersects (3.7 ± 2.7 and 3.3 ± 3.8, respectively), which also did not differ statistically.

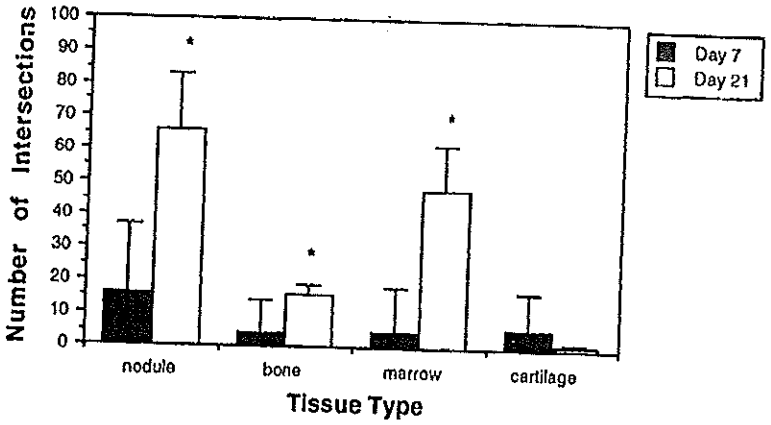

Fig 4. Mean tissue areas (recombinant human bone morphogenetic protein 2 treated implants) with 95% confidence intervals. Results of histomorphometry at 7 days (n = 6) and 21 days (n = 31).

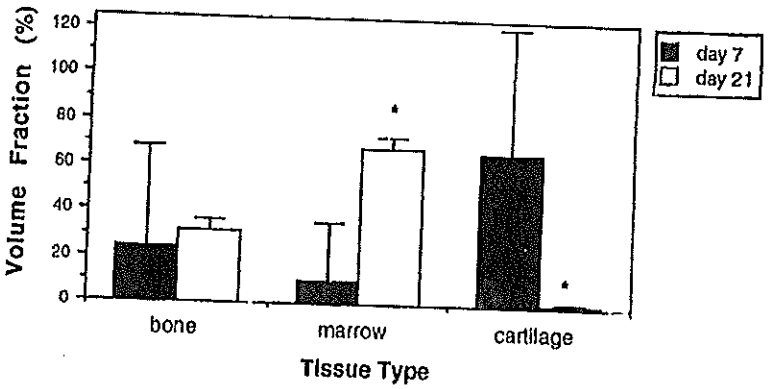

Fig 5. Relative tissue areas (recombinant human bone morphogenetic protein 2 treated implants) with 95% confidence intervals. Results of histomorphometry at 7 days (n = 6) and 21 days (n = 31).

Backscatter Electron Microscopic Analysis

Of the test (recombinant human bone morphogenetic protein 2 treated) implants forming bone, the implants without hydroxyapatite (right limb, Groups III and IV) formed bone characterized as a thin rim encapsulating the implant (Fig 6). Implants with hydroxyapatite and recombinant human bone morphogenetic protein 2 (right limb of Groups I and II) tended to contain bone formed in direct apposition to the hydroxyapatite in addition to the encircling rim of bone (Fig 7).

DISCUSSION

The investigation of methods to enhance bony ingrowth and implant fixation using osteoinductive and osteoconductive materials is critical to the success of cementless arthroplasty. The osteoinductive activity of recombinant human bone morphogenetic protein 2 in an ectopic site without the presence of an implant has been reported.[36,37,39] However, until now there have been no reports of recombinant human bone morphogenetic protein 2 showing enhanced bone formation when applied directly to a porous hydroxyapatite coated implant.

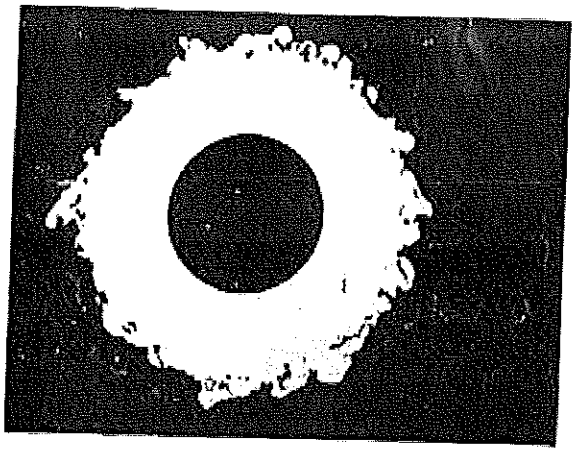

Fig 6. Backscatter scanning electron micrograph of recombinant human bone morphogenetic protein 2 (46 µg) treated implant harvested at 21 days sectioned perpendicular to the long axis of the implant. Note the thin rim of bone formation (B) surrounding the implant (I). (Original magnification, ×20.)

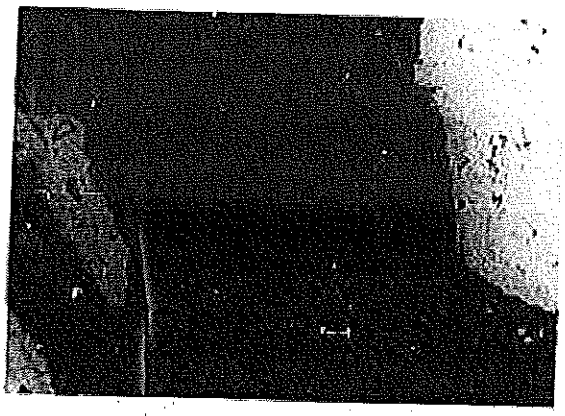

Fig 7. Backscatter scanning electron micrograph of recombinant human bone mophogenetic protein 2 (46 µg) treated implant harvested at 21 days sectioned perpendicular to the long axis of the implant. Note the thin trabecula (T) of bone formation separated from the hydroxyapatite coating and the thin layer of bone (B) on the hydroxyapatite (H) surface in direct apposition. (Original magnification, ×100.)

Orthotopic osseous locations inherently provide four essential elements to enhance biologic fixation: osteoconduction, osteoinduction, a source of osteogenic cells, and structural integrity.[10] To most reliably assay the osteoinductive capacity of recombinant human bone morphogenetic protein 2, the authors used a relatively inhospitable nonosseous ectopic location (a muscle pouch) comparatively devoid of its own osteoconductive elements or structural integrity inherent in an orthotopic location. In this environment, recombinant human bone morphogenetic protein 2 enhanced bone formation in direct contact with an implant independent of a hydroxyapatite surface. Because bone formation occurred in this nonosseous ectopic assay, it could be expected to form bone in a more hospitable orthotopic osseous location in association with an implant. However, implant stability and fixation ultimately would depend on the quality and quantity of tissue ingrowth.

Histologic observations of bone formation in the current study are consistent with the process and timing of endochondral ossification as described originally by Urist[34] and others.[23,26,35] Implants harvested at 7 days with or without the addition of recombinant human bone morphogenetic protein 2 or hydroxyapatite had a preponderance of fibrous tissue. Comparisons of recombinant human bone morphogenetic protein 2 treated implants from Day 7 to Day 21 showed a similar sequence of endochondral ossification, including early fibrocartilage and membranous bone formation, with bone formation documented at 21 days.

Previous studies comparing low (1.4 μg) and high dose (11 μg) recombinant human bone morphogenetic protein 2 showed histologic endochondral bone formation in a dose related manner in a rat segmental femoral defect model.[42] Both doses (23 μg or 46 μg) used in the current study are greater than the known minimal dose required to induce ectopic bone formation. The authors chose a relatively high dose of recombinant human bone morphogenetic protein 2 to ensure adequate residual activity after evaporation occurred during implant drying before implantation. Admittedly, the residual dosage of recombinant human bone morphogenetic protein 2 was not known. However, there is no known systemic effect of ectopically or systemically placed recombinant human bone morphogenetic protein 2 in even larger doses.[37] There were no statistical differences in the amounts or relative tissue components in the nodules formed at either 7 or 21 days. The authors' results were independent of the two doses chosen.

Experimentally, the capacity of hydroxyapatite to act as a delivery system of osteoinductive agents to the local environment has been shown. Most recently, Szivek et al[32] used recombinant human bone morphogenetic 7 in combination with calcium phosphate coatings to accelerate bone bonding in an in vivo model. Strates et al[29] found hydroxyapatite to provide an effectively large surface for the subperiosteal delivery of transforming growth factor beta. Stevenson et al[28] showed the capacity of partially purified osteogenin as a coating to a ceramic implant to induce bone formation in an orthotopic segmental defect model after implantation. Others have shown similar findings with bone morphogenetic protein.[33]

In three of nine implants coated with hydroxyapatite without recombinant human bone morphogenetic protein 2, bone formation was shown radiographically and histologically at 21 days. That hydroxyapatite alone has the capacity to form bone without the addition of an osteoinductive agent has been shown by Horisaka et al.[14] Thus, hydroxyapatite may act as a potential adsorptive surface to help sustain or immobilize locally produced osteoinductive factors.[28,29] This rationale may help explain why bone formed around some of the hydroxyapatite coated implants without the addition of recombinant human bone morphogenetic protein 2.

As an implant surface coating, hydroxyapatite has been challenged because of concerns regarding the shear strength, durability at the hydroxyapatite prosthesis interface, and contributions to osteolysis.[1,5] Recent experimental

models using hydroxyapatite with uncemented components have shown durable intermediate term results.[11] The authors' findings, although not statistically significant, suggest that hydroxyapatite in combination with recombinant human bone morphogenetic protein 2 may tend to improve the amount of apposition and formation of mature bone elements near the implant surface. More importantly, hydroxyapatite was not a requisite factor for bone formation in this model.

The use of partially purified nonrecombinant osteoinductive proteins to enhance fixation of orthotopically placed cementless implants has been described.[21,38] Recently, Kawai et al[15] used concentrated demineralized bone extract from bovine cortical bone to show bone formation in direct contact with a titanium sponge carrier after implantation in a mouse thigh muscle pouch. Partially purified nonrecombinant osteogenin (BMP-3) and transforming growth factor beta have been studied in similar models showing effective osteoinductive potential.[16,27,30] The benefits of recombinant human osteoinductive proteins include their availability, purity, and relative potency.

There are several limitations to this study. This is an unloaded ectopic implant model, and the authors were unable to demonstrate implant fixation and stabilization from bony ingrowth as one might expect had a weightbearing orthotopic site been used. However, the initial goal was to assess whether recombinant human bone morphogenetic protein 2 would continue to form bone when applied to a porous titanium implant surface with or without hydroxyapatite. Bone formation was shown by radiographic and histomorphometric analysis. In addition, because of the small size of the implants, it was difficult to create a truly porous surface identical to several implants in use clinically for cementless arthroplasty.

Recombinant human bone morphogenetic protein 2 applied directly to a titanium porous coated implant with or without hydroxyapatite induces endochondral ossification, as evidenced by contact radiography, histologic examination, and backscatter scanning electron microscopic study beginning as early as 7 days. There were no clear differences between the two doses chosen (23 μg or 46 μg) for absolute or relative amounts of bone formation. The clinical significance of this in vivo study is that recombinant human bone morphogenetic protein 2, independent of the presence of hydroxyapatite, can enhance bone formation and apposition around implants from its direct application to the implant surface. Weightbearing orthotopic animal studies are needed to help establish the role of recombinant human bone morphogenetic protein 2 in enhancing stability of the bone to implant interface. However, the intramuscular pouch continues to provide an excellent in vivo site to assay the activity of osteoinductive growth factors.

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