Recycled Dentin Root Matrix for a Carrier of Recombinant Human Bone Morphogenetic Protein

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KEY WORDS
Recycled root matrix
Bone morphogenetic protein (BMP)
Recombinant human bone morphogenetic protein (rhBMP-2)

INTRODUCTION
Dentin matrix is osteoinductive and rich in bone morphogenetic protein (BMP). BMP is a low molecular weight hydrophobic glycoprotein found in the organic matrix of both bone and dentin. BMP has the same physicochemical characteristics in dentin as in bone. Classified as a local hormone, or morphogen, BMP forms a concentration gradient pattern leading cell development along a pathway of bone in orthotopic and heterotopic sites.

Bone BMP and dentin BMP are assayed by implantation in heterotopic
sites as partially purified, partially insoluble native (rhBMP/NCP) and as highly purified soluble recombinant (recombinant human bone morphogenetic protein [rhBMP-2]) buffered to about pH 6. To retain the morphogen at the site of implantation long enough for the host-bed cells (of perivascular mesenchymal type) to gather, soluble rhBMP-2 is adsorbed to a carrier or to a delivery system. The delivery system may also provide an adhesive substratum. In our experiments, the partially demineralized matrix (PDM) provided the matrix for adsorption of rhBMP-2. The antigen-extracted, autolysed, delipidized allogeneic dentin matrix (AAAM) provided a substratum with minimal antigenic substances. The host mice were either normal (Swiss Webster strain) or athymic mice that were deficient in the α/β T-cells, and immunogenic reactions to BMP. However, autogeneic fresh bone has the disadvantage of being immunogenic in normal mice. Three sets of 4-week-old male Swiss Webster mice were recipients of 1, 2, and 5 μg of rhBMP-2 in glycine-buffered solutions. The solutions were instilled in the open half of disassembled no. 5 gelatin capsules, were reassembled without any other carrier, and were then implanted in the hindquarter muscles of the mice. The same number of implants were placed in athymic mice. The number of animals in each treatment group and the quantity of induced bone development is shown in Table 1.

<table>
<thead>
<tr>
<th>Implants</th>
<th>1 μg</th>
<th>2 μg</th>
<th>5 μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>rhBMP-2 solution in gelatin capsules</td>
<td>0.0%</td>
<td>10%</td>
<td>10%</td>
</tr>
<tr>
<td>PDM in gelatin capsules</td>
<td>3%</td>
<td>3%</td>
<td>3%</td>
</tr>
<tr>
<td>PDM + rhBMP-2 in capsules</td>
<td>61 ± 1.8%</td>
<td>83 ± 11%</td>
<td>104 ± 16%</td>
</tr>
<tr>
<td>AAAD in gelatin capsule</td>
<td>3%</td>
<td>3%</td>
<td>3%</td>
</tr>
<tr>
<td>AAAD + rhBMP-2 in capsules</td>
<td>78 ± 6.9%</td>
<td>100 ± 10%</td>
<td>100 ± 10%</td>
</tr>
</tbody>
</table>

*rhBMP-2, recombinant human bone morphogenic protein. PDM, partially demineralized root dentin matrix. AAAD, autolysed, antigen-extracted root matrix.

**Materials and Methods**

rhBMP-2 (batch 2D11024) was provided by Dr Vicki Rosen, and Dr John Wozney (Genetics Institute, Boston, Mass). Biological activity was verified in adult rats using intramuscular implants composed of rhBMP-2 and 6 M guanidine hydrochloric acid (GuHCl)-deactivated rat bone matrix.

**Implants of rhBMP-2 in a glycine buffer solution in gelatin capsules**

Three sets of 4-week-old male Swiss Webster mice were recipients of 1, 2, and 5 μg of rhBMP-2 in glycine-buffered solutions. The solutions were instilled in the open half of disassembled no. 5 gelatin capsules, were reassembled without any other carrier, and were then implanted in the hindquarter muscles of the mice. The same number of implants were placed in athymic mice. The number of animals in each treatment group and the quantity of induced bone development is shown in Table 1.

**Carriers for rhBMP-2**

Root dentin matrix was prepared from human teeth donated by outpatients at the University of California School of Dentistry. Immediately after extraction, the roots were trimmed and cleaned of all extraneous material and sterilized in 70% alcohol. The roots were partially demineralized with 0.6 N hydrochloric acid for 24 hours at 2°C and were then cut into 0.5-g blocks. PDM, including cementum, was washed in cold water and lyophilized. PDM was also converted into antigen-extracted autolysed matrix (AAAD) by methods employed for human use in previous reports. The PDM and AAAD root matrices were cut into 1.0- to 2.0-mm3 blocks. Briefly stated, antigen was also extracted with chloroform methanol, the soluble noncollagenous proteins were autolysed and digested in 0.1 M phosphate-buffered saline, and bone collagen was converted to insoluble bone gelatin in 8 M lithium chloride.

**Root AAAD powders**

Two hundred milligrams of root AAAD were pulverized in a milling machine (Spex Ind, Metuchen, NJ) to a particle size of 100–200 μm. Samples weighing 70 mg, with and without 1, 2, or 5 μg of rhBMP-2, respectively, were implanted in normal and athymic mice (C Rivers Lab, North Wilmington, Mass).

**Histologic methods**

The mice were sacrificed at 21 days. The hindquarters were x-rayed, and tissues were fixed in 10% neutral formalin, decalcified in ethylenediaminetetra-acetic acid (EDTA), sectioned in paraffin, and stained by hematoxylin,
RECYCLED DENTIN ROOT USED AS CARRIER OF rhBMP-2

The Student’s t-test was applied to each carrier. The data were reported as the mean value and standard deviation. Sample sizes of groups 2, 3, and 4 were too small for statistical evaluation. The paired Student’s t-test for unusual sample sizes was used to compare group 1 (rhBMP-2 solution in gelatin capsules) with group 2 (AAAD and rhBMP-2 in gelatin capsules).

RESULTS

The PDM and AAAM were implanted either en bloc or as powders in gelatin capsules in hindquarter muscles in both normal and athymic mice.

Normal mice

In the control group of normal mice, blocks of human PDM and AAAD dentin implanted in muscle caused no muscle to be replaced by new bone development. Implantation of 1 mg of rhBMP-2 alone was absorbed without inducing bone development, while the response to implantation of 2 mg of rhBMP-2 was bone replacement of muscle at a rate of 42%. The response to implantation of 5 mg of rhBMP-2 was 59.7% replacement by new bone.

rhBMP-2-PDM composites

As a result of implantation of 1 mg of rhBMP-2 plus PDM, the rate of muscle replacement by new bone was 61%. Bone replaced muscle at a rate of 83% with 2 mg of rhBMP-2 plus PDM, and 100% replacement occurred with 5 mg of rhBMP-2 plus PDM. Without rhBMP-2, PDM induced no new bone development.

rhBMP-2-AAAD composite

Implantation of 1 µg of rhBMP-2 using AAAD as a carrier produced bone replacement of the mouse hindquarter muscles at rates of 78 to 100%. Implantation of 2 and 5 µg of rhBMP-2 with AAAD as a carrier both produced the same rates of replacement as the 1-µg trial (78–100%; Table 1). Without rhBMP-2, 70 mg of AAAD tooth matrix produced histologically detectable replacement at a rate of about 3%; that is, only slight endogenous native BMP activity that was hardly detectable in radiographs (Figs 1–4). Implants of root matrix dentin were rapidly resorbed and replaced by new bone at rates proportional to the quantity of exogenous rhBMP-2 in the implant. Bone development increased with increases of rhBMP from 1 to 2 to 5 µg and was associated with progressive replacement of old matrix. With implantation of fine powders, the rates of the hindquarter replacement of muscle by bone were 40% with 1 and 2 µg of rhBMP-2 and 63% with 5 µg of rhBMP-2 (Table 2). The percentage of unab- sorbed matrix decreased in proportion to the area of new bone. Implantation of 2 µg of rhBMP-2 with both PDM and AAAM carriers showed statistically significant equivalent responses (Figs 3–5).

HISTOLOGICAL OBSERVATIONS

Implants of 2 µg of rhBMP-2 without a carrier other than the gelatin capsules

eosin, and azure II for histologic evaluation.

The areas of new bone, which displaced muscle, and the old unabsorbed matrix particles (Tables 1, 2) were measured with a grid micrometer on radiographs and on histological sections. The data were obtained by random point analysis computed as described in detail by Kawai and Urist7 and calculated as follows:

\[
\text{% new bone} = \frac{\text{area of new bone}}{\text{area of hindquarter muscle}} \times 100
\]
produced no bone, but implants of 2 to 5 μg of rhBMP-2 induced considerable bone development. The rhBMP-2-PDM and -AAAD composites induced similar quantities of bone in both athymic and normal mice (Figs 6–9). While there were significant differences in quantities of bone induced, normal mice showed slight macrophage, lymphocyte, plasma cell, and reticulocyte reactions suggestive of a cell-mediated immune response. Without exogenous BMP, AAAD and PDM root matrix alone implanted in normal mice produced little or no histological evidence of cartilage development or bone development. A thin membrane of fibrous tissue covered the AAA and PDM root surfaces (Figs 10–12).

**rhBMP-2-PDM and rhBMP-2-AAAD composites implanted in normal mice**

Within 3 weeks, new bone deposits were remodeled and filled with hematopoietic marrow. In some places, histological sections showed direct bone-to-carrier attachments with no intervening fibrous or inflammatory connective tissue. The volume of bone formation was directly proportional to the dose levels of rhBMP-2 in the ranges of 1, 2, and 5 μg per 0.07 g of (PDM or AAAD) carrier (Figs 13, 14).

**AAA tooth and PDM-rhBMP-2 composite implants in the athymic nude mice**

Figures 18 and 19 show the histologic reactions to composites of 2 μg of rhBMP in athymic mice. Both implants caused large deposits of new bone, including active bone marrow, as well as direct appositional bone deposition on the tooth surfaces of the implants in both the AAAD and the PDM composite subjects.

Table 2 summarizes observations of the carrier function of AAAD pulverized to increase the surface area of absorption of rhBMP-2. Without rhBMP-2, AAAD powder was rapidly absorbed and was without any osteoinductive effects. With 1, 2, or 5 μg of rhBMP-2 per 50 mg of AAAD powders, however, 40, 44, and 63% of the muscle matrix particles were displaced by bone, respectively. The reduction of
Table 2

<table>
<thead>
<tr>
<th>Implant</th>
<th>% New Bone</th>
<th>% Unabsorbed Matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAAM powder</td>
<td>0.0</td>
<td>66.7 ± 14.2</td>
</tr>
<tr>
<td>with 2 mg of rhBMP-2</td>
<td>0.0</td>
<td>66.7 ± 14.2</td>
</tr>
<tr>
<td>AAAM (10 mg) with 1 mg of rhBMP-2</td>
<td>40.5 ± 6.4</td>
<td>32.7 ± 13.6</td>
</tr>
<tr>
<td>AAAM (10 mg) with 2 mg of rhBMP-2</td>
<td>43.9 ± 4.1</td>
<td>23.7 ± 2.8</td>
</tr>
<tr>
<td>AAAM (10 mg) with 5 mg of rhBMP-2</td>
<td>63.3 ± 4.2</td>
<td>13.3 ± 4.0</td>
</tr>
</tbody>
</table>

*AAAM, Antigen-extracted, autolysed, delipidized allogenic dentin matrix. rhBMP-2, recombinant human bone morphogenic protein.

The rate of replacement by bone associated with the implanted powder in the absence of rhBMP-2 was 66.7%. In the composite with 1 μg of rhBMP-2 plus AAAM, the reduction rate was 32%, but dropped to 23% with 2 μg of rhBMP-2 with AAAM, and to only 3% with 5 μg of rhBMP-2 with AAAM.

This striking reciprocal relationship of the volume of new bone to the absorption of the AAAD carrier is shown in Figs 20 and 21.

Discussion

The experiments on human adult extracted teeth presented here were designed to determine whether root matrix, including small patches of cementum may (1) induce heterotopic bone development and/or (2) serve as a carrier for rhBMP-2 induced bone development. A literature review showed that the following substances have also been investigated for carrier properties: human collagen, atelocollagen, albumin, osteonectin, osteocalcin, bone matrix, blood clot, polylactic polyglycolic acid polymers, calcium sulfate, hydroxyapatite, β-tricalcium phosphate, and titanium oxide. Although endogenous BMP has been found in large quantities in dentin in normal young adult mammals, dentin matrix seems not to have been evaluated as a carrier of exogenous BMP. BMP activity declines in bone with aging, but whether or not BMP also declines in dentin with time and/or with periodontal disease requires investigation by quantitative methods. The surprising findings in our study were the large quantity of induced bone development and the complete resorption and replacement of root dentin matrix as compared with bone matrix development induced by the carriers, listed above.

The quantity of unresorbed matrix after 21 days was less than previously observed with implantation of either crown dentin or cortical bone matrix. Microscopic remnants of unresorbed root dentin matrix powders were osteointegrated, that is, embedded in bone trabecular like old bone in...
FIGURE 11. Photomicrograph of the implant shown in Fig 10. Note muscle (top), adipose tissue (middle), and root dentin (bottom), with surface coat of cementum. Hematoxylin, eosin, and azure II (magnification ×100).

FIGURE 12. Photomicrograph of an implant of a composite of 5 μg of rhBMP-2 and PDM 3 weeks after the operation. Note muscle (top right), bone and bone marrow (middle), cementum, human dental root PDM (bottom) (magnification ×100).

FIGURE 13. Photomicrograph of a composite of root dentin (AAAM and 5 μg of rhBMP-2 per 30 g body weight) 3 weeks after implantation in a normal mouse. Note muscle (top), new bone and bone marrow (middle), cementum and dentin (bottom) (magnification ×100).

FIGURE 14. Photomicrograph of implant of the PDM control in athymic mouse. Note muscle (top), granulation tissue (middle), and root dentin (bottom) (magnification ×100).

FIGURE 15. Photomicrograph of PDM coated with cementum implanted in an athymic mouse. Note muscle (top), root dentin (bottom) with deposit of new bone developed at the interface (arrow).

FIGURE 16. Photomicrograph of an AAAM control showing the fibrous tissue envelope between muscle and root dentin in an athymic mouse.
new bone in an autogeneic hom structural graft.\textsuperscript{22}

Extensive investigations of growth factors and BMP on shavings of cementum and dentin have been reported; rhBMP-2 induced connective tissue proliferation with attachments to cementum and bone.\textsuperscript{20,24} Sigurdsson \textit{et al}.\textsuperscript{25} demonstrated that rhBMP-2 induced bone formation and periodontal bone regeneration in dogs. Arzate \textit{et al}.\textsuperscript{26} isolated a partially purified cementum protein (with a molecular weight of 55 kDa) that stimulated chondrogenesis and mineralization in cultures of mouse embryo forelimb buds. In that study, progenitor cells (presumably derived from periodontal ligaments and alveolar bone) differentiated into cartilage and bone. BMP expression was transient in both embryonic development and organogenesis of bones and teeth, as observed by \textit{in situ} hybridization or immunohistochemical methods.\textsuperscript{26,27}

The quantity of endogenous BMP in dysfunctional teeth was very small or nil compared with the large deposits of bone induced by 2 $\mu$g of exogenous BMP. The antigenicity of recombinant BMP compared with native dentin BMP remains to be determined by quantitative immunohistochemical methods.\textsuperscript{28,29} Previous experiments with demineralized cortical bone matrix as a carrier\textsuperscript{17,18} showed no qualitative difference in response to partially purified native hBMP and rhBMP-2.

Microscopically, implants of pulverized root with cementum on one surface of some particles or on no surfaces or implants of cementum shavings in-
duced bone formation occurring in apposition to both cementum and non-cementum surfaces. Wozney et al., Sommerman et al., Sommerman et al., and Kagayama et al. (1997) made GuHCl and EDTA extracts of bovine cementum and other tissues known to contain BMP and observed stimulation of human gingival fibroblasts in vitro. Miki et al., King et al., Nishimura et al., Kinoshita et al., Vanio et al., and Wang et al. observed an increase in DNA synthesis in culture media with and without supplements of BMP and other growth factors.

Conover and Urist separated BMP from collagenase digests of gelatinized, demineralized dentin matrix of adult rabbits; the undigested residual matrix was rapidly resorbed and demonstrated very high levels of BMP activity. Fractionation of the collagenase resistant proteins produced a native BMP (with molecular weight of 30 and 23 kDa). Previously, Butler et al. isolated an insoluble noncollagenous BMP from a soluble phosphoprotein of dentin in rats, guinea pigs, and rabbits. Kawai and Urist described water-insoluble BMPs with molecular weights of 15 to 28 kDa isolated from a pulverized mixture of enamel, dentin, and cementum of unerupted bovine teeth. The isoinductive activity of these BMPs was slightly higher than that of the BMPs isolated from cortical bone.

Present observations on root-matrix-induced bone development were derived from discarded dysfunctional teeth deficient in endogenous BMP. Some of the teeth were so decayed that only the root structure with a thin coat of cementum remained available for use as a carrier. Exogenous rhBMP-2 adsorbed to a pulverized root PDM or AAAM proved to be as osteoinductive as autogeneic bone. Whether or not a large periodontal alveolar bone defect may be repaired using composites of rhBMP and PDM or AAAM warrants special investigation.

**ACKNOWLEDGMENTS**

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**REFERENCES**