JOURNAL OF BONE AND MINERAL RESEARCH
Volume 15, Number 9, 2000

Long-Term Evaluation of Bone Formation by Osteogenic Protein 1 in the Baboon and Relative Efficacy of Bone-Derived Bone Morphogenetic Proteins Delivered by Irradiated Xenogeneic Collagenous Matrices

U. RIPAMONTI,[1] B. VAN DEN HEEVER,[1] J. CROOKS,[1] M.M. TUCKER,[2] T.K. SAMPATH,[2] D.C. RUEGER,[3] and A.H. REDDI[4]

ABSTRACT

To investigate the long-term efficacy of irradiated recombinant human osteogenic protein 1 (hOP-1) in bone regeneration and morphogenesis, hOP-1 was combined with a bovine collagenous matrix carrier (0, 0.1, 0.5, and 2.5 mg hOP-1/g of matrix), sterilized with 2.5 Mrads of γ-irradiation, and implanted in 80 calvarial defects in 20 adult baboons (*Papio ursinus*). The relative efficacy of partially purified bone-derived baboon bone morphogenetic proteins (BMPs), known to contain several osteogenic proteins, was compared with the recombinant hOP-1 device in an additional four baboons. Histology and histomorphometry on serial undecalcified sections prepared from the specimens harvested on day 90 and day 365 showed that γ-irradiated hOP-1 devices induced regeneration of the calvarial defects by day 90, although with reduced bone area compared with a previous published series of calvarial defects treated with nonirradiated hOP-1 devices. One year after application of the irradiated hOP-1 devices, bone and osteoid volumes and generated bone tissue areas were comparable with nonirradiated hOP-1 specimens. Moreover, 365 days after healing regenerates induced by 0.5 mg and 2.5 mg of irradiated hOP-1 devices showed greater amounts of bone and osteoid volumes when compared with those induced by nonirradiated hOP-1 devices. On day 90, defects treated with 0.1 mg and 0.5 mg of bone-derived baboon BMPs, combined with irradiated matrix, showed significantly less bone compared with defects receiving irradiated devices containing 0.1 mg and 0.5 mg hOP-1; 2.5 mg of partially purified BMPs induced bone and osteoid volumes comparable with the 0.1-mg and 0.5-mg hOP-1 devices. Control specimens of γ-irradiated collagenous matrix without hOP-1 displayed a nearly 2-fold reduction in osteoconductive bone repair when compared with nonirradiated controls. These findings suggest that the reduction in bone volume and bone tissue area on day 90 may be caused by a reduced performance of the irradiated collagenous matrix substratum rather than to a reduction in the biological activity of the irradiated recombinant osteogenic protein. This is supported by the results of in vitro and in vivo studies performed to determine the structural integrity of the recovered γ-irradiated hOP-1 before application in the baboon. Recoveries by high-performance liquid chromatography (HPLC) and sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE)/immunoblot analyses indicated that doses of 2.5–3 Mrads of γ-irradiation did not significantly affect the structural integrity of the recovered hOP-1. Biological activity of the recovered hOP-1 was confirmed in vitro by showing induction of alkaline phosphatase activity in rat osteosarcoma cells (ROS) and in vivo by de novo endochondral bone formation in the subcutaneous space of the rat. These findings in the adult primate indicate that a single application of γ-irradiated hOP-1 combined

[1]Bone Research Laboratory, Medical Research Council/University of the Witwatersrand, Johannesburg, South Africa.
[2]Creative BioMolecules, Hopkinton, Massachusetts, U.S.A.
[3]Stryker Biotech., Hopkinton, Massachusetts, U.S.A.
[4]Center for Tissue Regeneration and Repair, University of California Davis, School of Medicine, Sacramento, California, U.S.A.

with the irradiated xenogeneic bovine collagenous matrix carrier is effective in regenerating and maintaining the architecture of the induced bone at doses of 0.5 mg/g and 2.5 mg/g of carrier matrix. (J Bone Miner Res 2000;15:1798–1809)

Key words: bone morphogenetic proteins, osteogenic protein-1, bone induction, collagenous matrices, γ-irradiation, primates

INTRODUCTION

BONE REGENERATION in clinical contexts requires three key components: an osteoinductive signal; an insoluble substratum, which delivers the signal and acts as a scaffold for new bone formation; and host cells capable of differentiation into bone cells in response to the osteoinductive signal. The signals responsible for osteoinduction are conferred by the family of the bone morphogenetic proteins (BMPs). BMPs are members of a superfamily of morphogens that include the transforming growth factor βs (TGF-βs), the growth/differentiating factors (GDFs), and cartilage-derived morphogenetic proteins (CDMPs).[1–6] In addition, the BMPs show significant amino acid identities with developmentally critical regulatory genes such as decapentaplegic (DPP) and 60A in Drosophila, Vegetal (Vg-1) in Xenopus and activins and inhibins.[1–4] A striking and discriminating feature of BMPs is their ability to induce de novo cartilage and bone formation in extraskeletal (heterotopic) sites, recapitulating embryonic bone development.[1–4] Originally, the osteogenic potential of BMPs was shown by reconstituting dissociatively extracted demineralized bone matrix with purified solubilized proteins.[7] This was followed by molecular cloning and expression of several recombinant human BMPs (BMP-2 to BMP-6, osteogenic protein 1 [OP-1] and OP-2.[8–11] Recombinant human BMP-2, BMP-4, and OP-1 (BMP-7) singly initiate endochondral bone formation in the subcutaneous space of the rat when combined with insoluble collagenous bone matrix, the inactive residue obtained after dissociative extraction of bone matrix with 4 M guanidinium-HCl.[12–14] In addition to BMPs/OPs, other related signaling proteins display heterotopic bone inductive activities in the rodent subcutaneous assay, including recombinantly produced DPP and 60A,[15] gene products expressed early in Drosophila development, and GDF-5 (CDMP-1),[16] a BMP/OP-related protein that may be critical during skeletogenesis, as suggested by mutations of the GDF-5 gene in brachypodism affected mice[17] and humans.[18]

The presence of several related but different BMPs with osteogenic activity points to multiple interactions during both embryonic development and bone regeneration in postnatal life. The fact that a single BMP/OP initiates bone formation does not preclude the requirement and interactions of other morphogens deployed synchronously and sequentially during the cascade of bone formation by induction.[1–4] The apparent redundancy of BMP/OP family members may have biological and therapeutic relevance in bone induction, which may proceed via the combined action of several BMPs/OPs, resident within the natural milieu of the extracellular matrix of bone.

The necessity of the insoluble substratum (collagenous matrix) in the induction of tissue morphogenesis and regeneration by an osteogenic signal (BMPs/OPs) illustrates the critical importance of the extracellular matrix for cell recruitment, attachment, proliferation, and differentiation.[1,3,7] Although the therapeutic use of recombinant BMPs/OPs requires sterilization of both soluble signal and insoluble substratum combined to produce an osteogenic device, comprehensive studies on the therapeutic efficacy of bone formation by irradiated osteogenic proteins and irradiated matrices are lacking. Here we report on the characterization and biological activity of hOP-1 after irradiation and on the long-term evaluation of bone regeneration by the irradiated hOP-1 device in calvarial defects of adult baboons. Moreover, we compared the relative inductive efficacy of partially purified baboon BMPs, known to contain several BMPs/OPs in addition to as yet poorly characterized mitogens, with the regenerates induced by the single and recombinant hOP-1 device in the same primate model.

MATERIALS AND METHODS

Preparation of the osteogenic devices

Mature recombinant human OP-1 is a glycosylated 36-kDa homodimer of 139 amino acid residue chains. Stock solutions of hOP-1 were prepared in 50% ethanol, 0.01% trifluoroacetic acid, and protein concentration determined by absorbance readings at 280 nm using an extinction coefficient of 2.0 for a 1.0-mg/ml solution. Demineralized bone matrix, prepared from diaphyseal segments of bovine cortical bones, was dissociatively extracted in 4 M guanidinium-HCl[7] and the resulting inactive insoluble collagenous matrix was treated with 0.1 M acetic acid at 55°C for 1 h, washed with distilled water, and dried. Aliquots of carrier matrix (1 g) were combined with 0.1, 0.5, and 2.5 mg of hOP-1 and lyophilized to produce the hOP-1 device. Bovine collagenous matrix was prepared with liquid vehicle without hOP-1, lyophilized, and used as control. The hOP-1 devices were packaged in borosilicate glass vials and sealed under vacuum. The devices were then sterilized at ambient temperature with γ-radiation (Cobalt-60 source) using an irradiation dose of approximately 0.3 Mrads/h for a total of 2.5–3.0 Mrads. The irradiation was performed at a contract facility (Isomedix, Northborough, MA, U.S.A., or Radiation Technologies, Inc., NJ, U.S.A.). This dose of irradiation was selected because 2.5 Mrads is accepted by the medical device industry and the Food and Drug Administration (Rockville, MD, U.S.A.) as the minimum required dose to sterilize med-

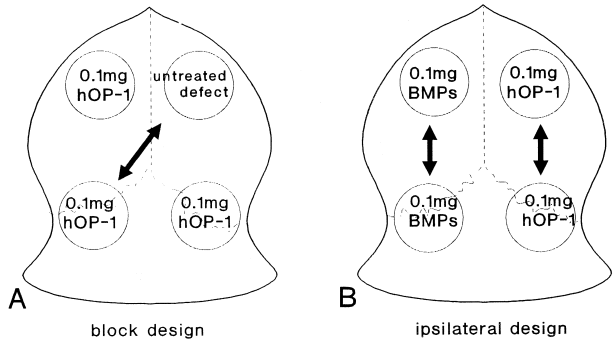

FIG. 1. Calvarial model and implantation design in 24 adult male baboons. In 20 animals (12 and 8 for tissue harvest on day 90 and day 365, respectively), (A) a block design was used to allocate three identical doses of the irradiated device (either 0, 0.1, 0.5, or 2.5 mg/g of bovine collagenous matrix in triplicate). Remaining defects (n = 20) were left untreated and sequentially alternated in each animal (arrow). (B) An ipsilateral design was used in the remaining four animals to investigate the relative efficiency of bone-derived BMPs (0.1, 0.5, and 2.5 mg) delivered by irradiated bovine collagenous matrix (n = 10). Remaining contralateral defects (n = 6) were implanted with 0.1 mg and 0.5 mg of hOP-1 irradiated device.

ical supplies.[19–21] For this collagen-based device, 1.5 Mrads was determined to be the minimum dose required to achieve a 10^{-6} sterility assurance level.[22] In addition to inactivating bacteria, 2.5 Mrads γ-irradiation has been shown to reduce viral titers by 3–5 logs using model virus systems.[23]

Baboon acid–demineralized bone matrix was extracted in 4 M guanidinium-HCl,[7] and partial purification was achieved by sequential chromatography of the protein extract on heparin-Sepharose, hydroxyapatite, and Sephacryl S-200 columns, washed and eluted as described.[19,20] To increase specific osteogenic activity of the preparation, Sephacryl S-200 fractions were chromatographed on a second heparin-Sepharose affinity column (20-ml bed volume). The recovered 500-mM NaCl step-eluted fraction was concentrated, exchanged with 5 mM HCl to a final concentration of 1 mg/ml protein (7.5 mg total amount), and sterilized by filtration (0.22 μm; Millex; Millipore Corp., Bedford, MA, U.S.A.). Aliquots were combined with 25 mg of rat insoluble collagenous matrix and assayed for osteogenic activity in the subcutaneous space of the rat as described.[24–26] Implants were harvested on day 12 and osteogenic activity in the rat was assessed by measuring alkaline phosphatase activity, calcium content, and histology. For preparation of devices, bone-derived BMPs in 500 μl of 5 mM HCl were added to 1 g of irradiated bovine collagenous matrix per sample at doses of 0.1 (n = 4), 0.5 (n = 4), and 2.5 mg (n = 2) and lyophilized.

Characterization and biological activity of hOP-1 device after γ-irradiation

To determine the recovery of the recombinant morphogen from the collagenous matrix, hOP-1 was eluted from the matrix with 8 M urea buffer, and the integrity and yield of the recovered protein was assessed by reversed-phase high-performance liquid chromatography (rpHPLC) in acetonitrile gradient. The recovered hOP-1 also was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblot analysis using antibodies specific for hOP-1.[27] The biological activity of the proteins recovered from γ-irradiated and nonirradiated collagenous matrices was assayed using rat osteosarcoma (ROS) 17/2.8 cells cultured as described.[28] The alkaline phosphatase activity induced by hOP-1 recovered from irradiated and nonirradiated devices was compared with the activity induced by an hOP-1 standard.[14,28] To assess the in vivo osteogenic activity of the hOP-1 device after γ-irradiation, 3 doses of OP-1 (0.5, 1, and 2.5 μg) were combined with 25 mg of bovine collagenous matrix as carrier and sterilized with 2.5 Mrads of γ-irradiation. The pellets were implanted in the subcutaneous space of Long-Evans rats at bilateral sites over the pectoralis fascia.[14,26] Nonirradiated hOP-1 devices were used as positive controls. Implants were harvested on day 12 and assayed for tissue alkaline phosphatase activity, calcium content, and histology.[14,26]

TABLE 1. EFFECT OF γ-IRRADIATION OF THE RECOVERY OF hOP-1 FROM COLLAGENOUS MATRIX[a]

Irradiation	*hOP-1 recovery (mg/g collagenous matrix)*	*hOP-1 recovery (%)*
None (control)	2.13 ± 0.18	85%
2.5 Mrads	1.69 ± 0.24	67%

[a] The 2.5 mg of recombinant hOP-1 was combined with 1 g of bovine collagenous matrix and sterilized with 2.5 Mrads of γ-irradiation. The recombinant protein was eluted from irradiated and nonirradiated hOP-1 devices with 8 M urea buffer and analyzed by rpHPLC as described in the Materials and Methods section. Determinations were done in triplicate and are expressed as mean and SD.

Primate model for tissue induction

Twenty-four clinically healthy adult Chacma baboons (*Papio ursinus*), with a mean weight of 34.8 ± 3.1 kg, were selected from the primate colony of the University of the Witwatersrand, Johannesburg. Comparative histomorphometric studies between iliac crest biopsy specimens of humans and *Papio ursinus* showed a remarkable degree of similarity.[29] This makes the adult male baboon ideally suited for the study of comparative bone physiology and repair with relevance to man.[29] Criteria for selection, housing conditions and diet were as described.[30] Research protocols were approved by the Animal Ethics Screening Committee of the university, and conducted according to the *Guidelines for the Care and Use of Experimental Animals* prepared by the university, and in compliance with the *National Code for Animal Use in Research, Education and Diagnosis in South Africa*.[31] The orthotopic calvarial model in the baboon has been described in detail.[32–34] On

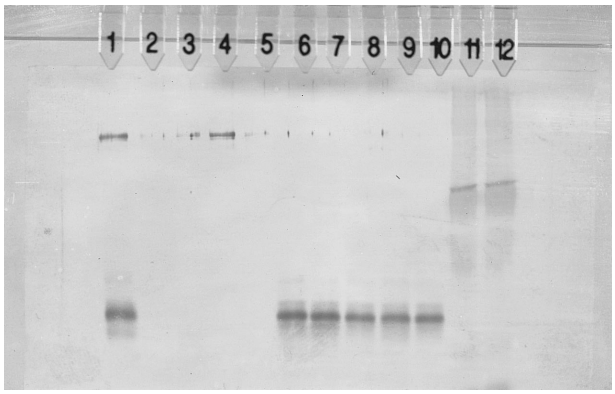

FIG. 2. Immunoblot analysis of recovered hOP-1 eluted from hOP-1 devices: effect of irradiation. Aliquots of collagenous matrix combined with hOP-1 were sterilized with 0.5–0.6, 1.5–1.8, and 2.5–3.0 Mrads of γ-irradiation. Proteins were eluted with 8 M urea buffer and analyzed for structural integrity by SDS/PAGE followed by immunoblot analysis and compared with doses of hOP-1 standard. Lane 1: hOP-1 standard, 0.5 ng; lane 2: nonirradiated collagenous matrix; lane 3: collagenous matrix, 0.5–0.6 Mrads; lane 4: collagenous matrix, 1.5–1.8 Mrads; lane 5: collagenous matrix, 2.5–3.0 Mrads; lane 6: nonirradiated hOP-1device, 1 ng; lane 7: hOP-1 device, 0.5–0.6 Mrads, 1 ng; lane 8: hOP-1 device, 1.5–1.8 Mrads, 1 ng; lane 9: hOP-1 device, 2.5–3.0 Mrads, 1 ng; lane 10: hOP-1 standard, 0.9 ng; lanes 11 and 12: molecular weight standard.

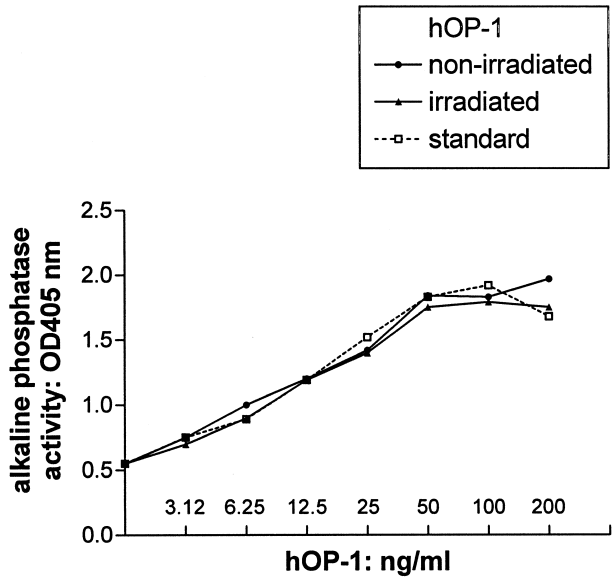

FIG. 3. Stimulation of alkaline phosphatase activity in ROS 17/2.8 cells by hOP-1. Confluent cells, cultured as described,[28] were treated with doses of hOP-1 eluted from irradiated and nonirradiated hOP-1 devices or with an hOP-1 standard. After removal of culture medium, washed cell layers were sonicated in 500 μl of extraction buffer (0.15 M NaCl and 3 mM $NaHCO_3$) containing 1% Triton X-100. Samples were assayed for alkaline phosphatase activity with *p*-nitrophenyl phosphate as substrate in 0.05 M glycine-NaOH buffer, pH 9.3, and absorbance was measured at 405 nm after stopping the reaction with 100 μl of 0.1 M NaOH.[21,23] The hOP-1 concentrations were based on rpHPLC recoveries, as described in the Materials and Methods section and the Results section.

each side of the calvaria, two full thickness defects, 25 mm in diameter, were created with a craniotome under saline irrigation.[32–34] After determination of the structural integrity and biological activity of the γ-irradiated hOP-1, a block design was used to allocate the position of the irradiated hOP-1 device in 80 calvarial defects in 20 adult male baboons (Fig. 1A). In each animal, three defects were implanted with an identical dose of hOP-1 in conjunction with the collagenous matrix as carrier. The remaining defect was left untreated, to determine whether hOP-1 had the ability to influence the untreated calvarial site at a distance from implantation. Thus, 15 defects in 5 baboons were implanted with 0.1 mg hOP-1, 15 defects with 0.5 mg hOP-1 and 15 defects with 2.5 mg hOP-1/g of collagenous matrix as carrier. In addition, 15 defects in 5 baboons were implanted with irradiated collagenous matrix without hOP-1 as control. To determine the relative efficacy of bone-derived partially purified baboon BMPs delivered by irradiated bovine collagenous matrix, experiments were performed in the remaining 4 baboons with a modified implantation design (Fig. 1B) in that in each animal, the two ipsilateral defects were implanted with doses of bone-derived BMPs (0.1, 0.5, and 2.5 mg/g of irradiated bovine collagenous matrix). Remaining defects ($n = 6$) were implanted with 0.1 mg and 0.5 mg of irradiated hOP-1 per device.

Tissue harvest, histology, and histomorphometry

Anesthetized animals were killed with an intravenous overdose of sodium pentobarbitone, 16 animals on day 90 and 8 animals on day 365 after surgery. Bilateral carotid perfusion and harvest of specimens with surrounding calvaria were as described.[32–34] Specimen blocks were cut along the sagittal one-fourth of the implanted defects, dehydrated in ascending grades of ethanol, and embedded, undecalcified, in a polymethyl methacrylate resin (K-Plast; Medim, Buseck, Germany). Undecalcified serial sections, cut at 7 μm (Polycut-S; Reichert, Heidelberg, Germany), were stained, free-floating, with Goldner's trichrome or with 0.1% toluidine blue in 30% ethanol. Goldner's trichrome–stained sections were examined with a Provis AX70 research microscope (Olympus Optical Co., Japan) equipped with a calibrated Zeiss Integration Platte II (Oberkochem, Germany) with 100 lattice points for determination by the point-counting technique,[35] of mineralized bone, osteoid, and residual collagenous matrix volumes (in %). Sections were analyzed at 40×, superimposing the Zeiss graticule over five sources[36] selected for histomorphometry and defined as follows: two anterior and posterior interfacial regions (AIF and PIF), two anterior and posterior internal regions (AIN and PIN), and a central region (CEN).[32–34] This technique allows the histomorphometric evaluation of the distribution of bone regeneration across

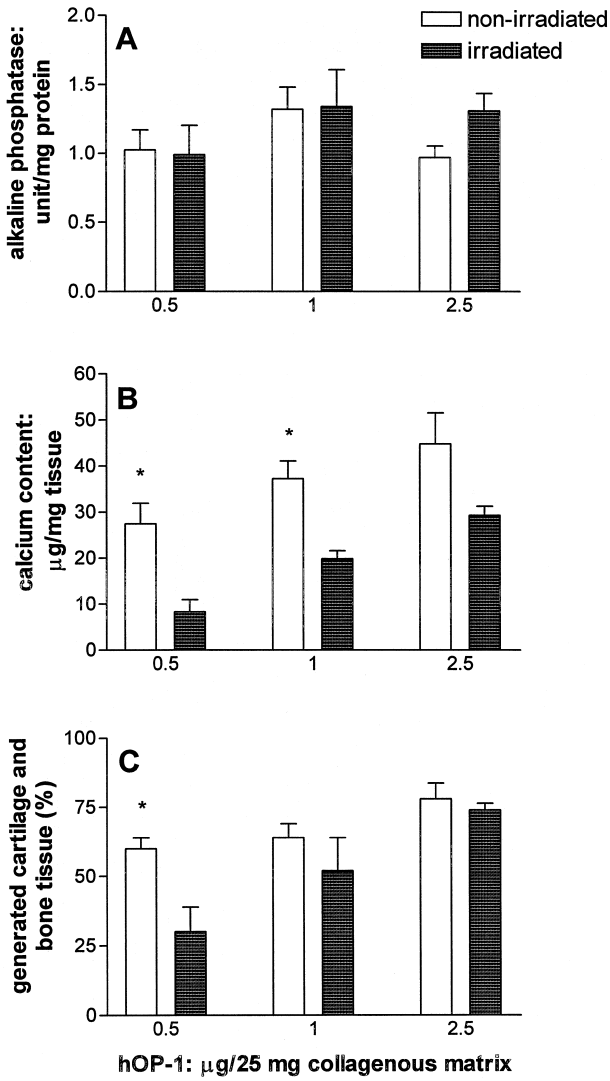

FIG. 4. In vivo biological activity of nonirradiated and irradiated hOP-1 devices. Doses of hOP-1, combined with 25 mg of bovine collagenous matrix as carrier were sterilized with 2.5 Mrads of γ-irradiation. Nonirradiated (control) and irradiated hOP-1 devices were implanted in the subcutaneous space of Long-Evans rats at bilateral sites over the pectoralis fascia. Generated tissues were removed on day 12 and subjected to (A) alkaline phosphatase activity and (B) calcium content determination. The alkaline phosphatase activity of the supernatant after homogenization of implants was determined with 0.1 M *p*-nitrophenyl phosphate as substrate (pH 9.3) at 37°C for 30 minutes.[26] Alkaline phosphatase is expressed as units of activity per milligram protein. Protein concentration in the supernatant was measured by the method of Lowry et al.[49] The calcium content of the acid-soluble fractions of the pellets was determined by colorimetric assay.[14] (C) Newly formed cartilage and bone (%) were examined on 1-μm sections stained with toluidine blue after fixation in Bouin's fluid and embedding in Historesin plastic medium (Reichert-Jung). Histomorphometric analysis was as described in the Materials and Methods section for baboon calvarial specimens. Values represent the mean ± SEM of four to five specimens per group; *$p < 0.05$ versus nonirradiated specimens.

the defects.[32–34] Each source represented a field of 7.84 mm^2. The cross-sectional area (in mm^2) of newly generated bone tissue (mineralized bone, osteoid, and marrow)[36] in each calvarial defect was measured using a computerized image analysis system (Flexible Image Processing System; Council for Scientific and Industrial Research, Pretoria, South Africa) connected to a capturing video-camera (WV-CP410/G Panasonic; Panasonic, Osaka, Japan).[33] Morphometry (volumes and areas) was performed on four sections per implant, representing four parasagittal levels, approximately 2 mm apart from each other.[33]

Statistical analysis

The data were analyzed with the Statistical Analysis System.[37] An F test was performed using the General Linear Models procedure for an analysis of variance with multiple interactions.[32] Comparison of mean values was obtained using a Duncan's multiple-range test on the dependent variables included in the analysis. The significance probability value associated with the F value for each class variable was accepted as significant at $p < 0.05$.

RESULTS

Characterization of the hOP-1 device

The amount of hOP-1 recovered to assess the effect of irradiation of hOP-1 after elution from the 2.5-mg hOP-1 device is shown in Table 1. Chromatographic profiles obtained from rpHPLC of eluted hOP-1 from nonirradiated and irradiated collagenous matrices indicated that structurally intact hOP-1 could be recovered from hOP-1 devices sterilized by 2.5 Mrads of γ-irradiation (not shown). The structural integrity of the irradiated and recovered protein was confirmed by SDS/PAGE followed by immunoblot analysis, indicating that gamma irradiation does not significantly alter the immunoreactivity and the electrophoretic mobility of hOP-1 (Fig. 2). The biological activity of hOP-1 recovered from irradiated and nonirradiated devices was assessed using ROS 17/2.8 cells and induced levels of alkaline phosphatase activity comparable with that of the hOP-1 standard (Fig. 3). The effect of 2.5 Mrads of γ-irradiation on the in vivo biological activity of hOP-1 was assessed in the rat subcutaneous assay and the data are summarized in Fig. 4. Implantation of γ-irradiated hOP-1 devices resulted in a histologically reproducible pattern of endochondral bone differentiation comparable with that of tissues generated by nonirradiated hOP-1 devices and with comparable tissue alkaline phosphatase activity (Fig. 4A). γ-Irradiated specimens yielded less calcium when compared with nonirradiated samples (Fig. 4B) and contained lower amounts of newly generated cartilage and bone at the lowest dose of irradiated hOP-1 used (Fig. 4C).

Morphology of calvarial regeneration

Ninety days and 365 days after surgery, untreated defects showed minimal osteogenesis whether adjacent to defects treated with hOP-1 devices or to defects treated with col-

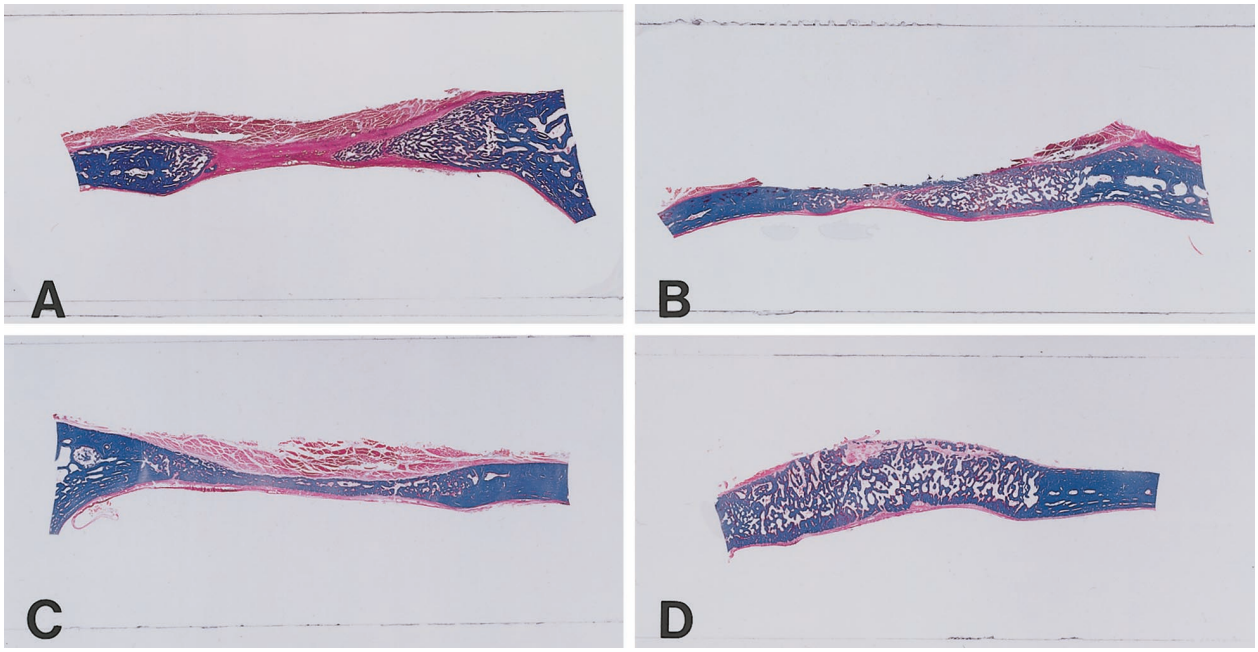

FIG. 5. Low power photomicrographs of calvarial specimens harvested on day 90. Undecalcified sections at 7 μm stained with Goldner's trichrome (original magnification $\times$2.5). (A) Irradiated bovine collagenous matrix without hOP-1 (control). (B and C) Regeneration of bone across the defects with doses of (B) 0.1 mg and (C) 0.5 mg of irradiated hOP-1 device. Newly formed trabeculae with thick osteoid seams (B), bone remodeling and the beginning of the formation of both pericranial and endocranial cortices (B and C). (D) Extensive induction of bone in a defect treated with 2.5 mg hOP-1; thick trabeculae of newly formed and mineralized bone connected to solid blocks of corticalized bone above the dura.

lagenous matrix alone (data not shown). On day 90, defects treated with bovine collagenous matrix without OP-1 (control) showed limited bone formation in continuity with the severed calvaria and complete dissolution of the implanted matrix (Fig. 5A). Defects treated with devices containing 0.1 mg and 0.5 mg hOP-1 resulted in bone regeneration across the defects (Fig. 5B), although the regenerated bone tissue appeared thinner than the original calvaria (Fig. 5C). Doses of 2.5 mg hOP-1/g of collagenous matrix induced a more pronounced osteogenic response, with numerous trabeculae covered by continuous osteoid seams facing newly generated marrow (Fig. 5D). On day 365, devices with 0.1, 0.5, and 2.5 mg hOP-1 induced complete bone regeneration, with reconstruction of the internal and external cortices of the calvaria (Fig. 6).

Macroscopic examination on day 90 showed areas of ossification beneath the fascia of the temporalis muscle, bilaterally, in animals that were treated in triplicate with the 2.5-mg hOP-1 device. In two animals, discrete flat ossicles, loose beneath the fascia, and ossification along the previously sutured fasciae and underlying muscle were observed. A third animal showed extensive ossification in the form of thick plates of newly formed bone covering almost the entirety of the temporalis muscle, bilaterally (Fig. 7A). Histological analysis showed formation of cortical and trabecular bone covered by thick osteoid seams (Figs. 7B and 7C). The finding of heterotopic osteogenesis above the temporalis muscle when the higher dose of the hOP-1 device was used in triplicate in the same animal may be the result of desorption of the recombinant protein from the surface of the carrier matrix, followed by diffusion of hOP-1 along the length of the surgical wound of the temporalis muscle during healing. However, only minor heterotopic flat ossicles were found macroscopically in the fasciae of animals from which tissues were harvested 1 year after the application of the higher dose of hOP-1.

On day 90, 0.1, 0.5, and 2.5 mg of bone-derived baboon BMPs delivered by irradiated bovine collagenous matrix induced new bone formation across the defects (Fig. 8), with newly formed and mineralized trabeculae being covered by continuous osteoid seams. Defects treated with 0.1 mg and 0.5 mg hOP-1 devices, which were harvested ipsilaterally to the defects treated with bone-derived BMPs, showed bone regeneration comparable with that of the previous series harvested on day 90 (Fig. 8D).

Morphometry: Effect of hOP-1 and bone-derived BMP doses on bone induction

Volume fractions (with levels of significance) of bone and osteoid in defects treated with the irradiated hOP-1 device are presented in Table 2. On day 90, 0.1, 0.5, and 2.5 mg hOP-1 devices induced greater amounts of bone and osteoid when compared with irradiated bovine matrix without hOP-1 (control; $p < 0.05$, Table 2), with the 2.5 mg dose showing the greater amount of bone when compared with 0.1 mg and 0.5 mg hOP-1 specimens ($p < 0.05$, Table 2). On day 365, the 0.1-, 0.5-, and 2.5-mg hOP-1 devices

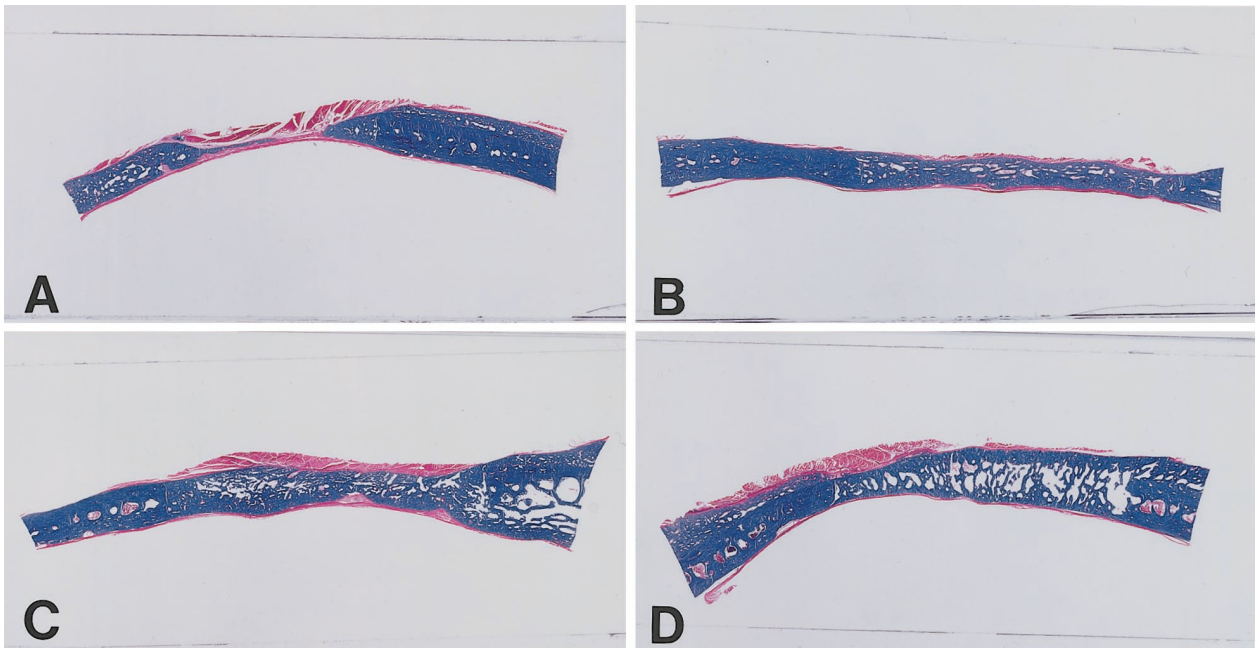

FIG. 6. Low-power photomicrographs of specimens of irradiated hOP-1 device harvested on day 365. Undecalcified sections at 7 μm stained with Goldner's trichrome (original magnification $\times$2.5). (A) Collagenous matrix without hOP-1 (control). (B–D) Complete reconstruction of defects with doses of (B) 0.1 mg, (C) 0.5 mg, and (D) 2.5 mg of the irradiated hOP-1 device. Maintenance of the generated bone tissue 1 year after a single application of hOP-1 and remodeling of the regenerates with doses of 0.1 mg and 0.5 mg hOP-1. (D) Reconstruction of both pericranial and endocranial cortices, with intervening trabeculae facing large areas of newly generated marrow in a defect treated with 2.5 mg of hOP-1.

showed greater amounts of bone when compared with control ($p < 0.05$, Table 2). Although doses of 0.1 mg and 0.5 mg hOP-1 generated comparable amounts of bone on day 90, on day 365 greater amounts of bone were found in specimens treated with 0.5 mg hOP-1 ($p < 0.05$ vs. 0.1 mg hOP-1, Table 2). Histomorphometric data of the present series of 80 calvarial defects were compared with previously published results using identical doses of nonirradiated hOP-1 devices[33] (Table 2). On day 90, on average, less bone formed in calvarial defects implanted with the irradiated hOP-1 device, including controls (Table 2). However, osteoid volumes generated by irradiated hOP-1 devices were found to be significantly greater ($p < 0.05$ vs. nonirradiated hOP-1, Table 2), with the exclusion of the 2.5-mg dose of hOP-1 (6.0 vs. 6.1%, respectively). On day 365, doses of 0.5 mg and 2.5 mg of irradiated hOP-1 showed greater amounts of bone when compared with equivalent doses of hOP-1 delivered by nonirradiated bovine matrix, and with a significant increase in bone volume between day 90 and day 365 ($p < 0.05$, Table 2).

Volume fractions of tissue components in calvarial defects treated with bone-derived baboon BMPs are shown in Table 3. Specimens generated by combining doses of baboon BMPs with irradiated bovine collagenous matrix showed substantial osteoid, comparable with osteoid volumes generated by irradiated hOP-1 devices (Table 3). Doses of 0.1 mg and 0.5 mg of baboon BMPs showed significantly less bone on day 90 when compared with doses of 0.1 mg and 0.5 mg of irradiated hOP-1 devices ($p < 0.05$, Table 3). The 2.5-mg baboon BMPs, in conjunction with irradiated bovine bone matrix, generated a comparable bone volume with the 2.5-mg hOP-1 device (Tables 2 and 3). Separate analysis of the irradiated 0.1-mg and 0.5-mg hOP-1 devices implanted in the two series of animals showed equal or comparable amounts of bone and osteoid volumes (Table 3). Greater amounts of residual collagenous matrix were found in specimens treated with 0.1 mg and 0.5 mg of baboon BMPs when compared with specimens of hOP-1 devices (Table 3).

Computer-generated data of cross-sectional areas (in mm^2) of specimens treated with the irradiated hOP-1 device on day 90 and day 365 are shown in Fig. 9. On day 90, irradiated hOP-1 devices generated less bone tissue area when compared with nonirradiated hOP-1 devices (Fig. 9A). On average on day 90, irradiation of both OP-1 and collagenous matrix resulted in regenerates with reduced tissue area when compared with normal calvaria (mean cross-sectional area, 60.8 $\pm$ 3.1 mm^2),[33] with the exception of the 2.5-mg dose of irradiated hOP-1 device (Fig. 9A). On day 365, defects treated with 0.5 mg and 2.5 mg of irradiated hOP-1 devices showed a significant increase compared with day 90 ($p < 0.05$), with remodeling that resulted in levels of bone tissue area comparable with normal calvaria (Fig. 9B). The 0.5-mg dose of hOP-1 showed the highest increase in bone tissue area from day 90 to 365, approaching levels of bone tissue area comparable with the 2.5-mg dose of hOP-1 (Fig. 9B).

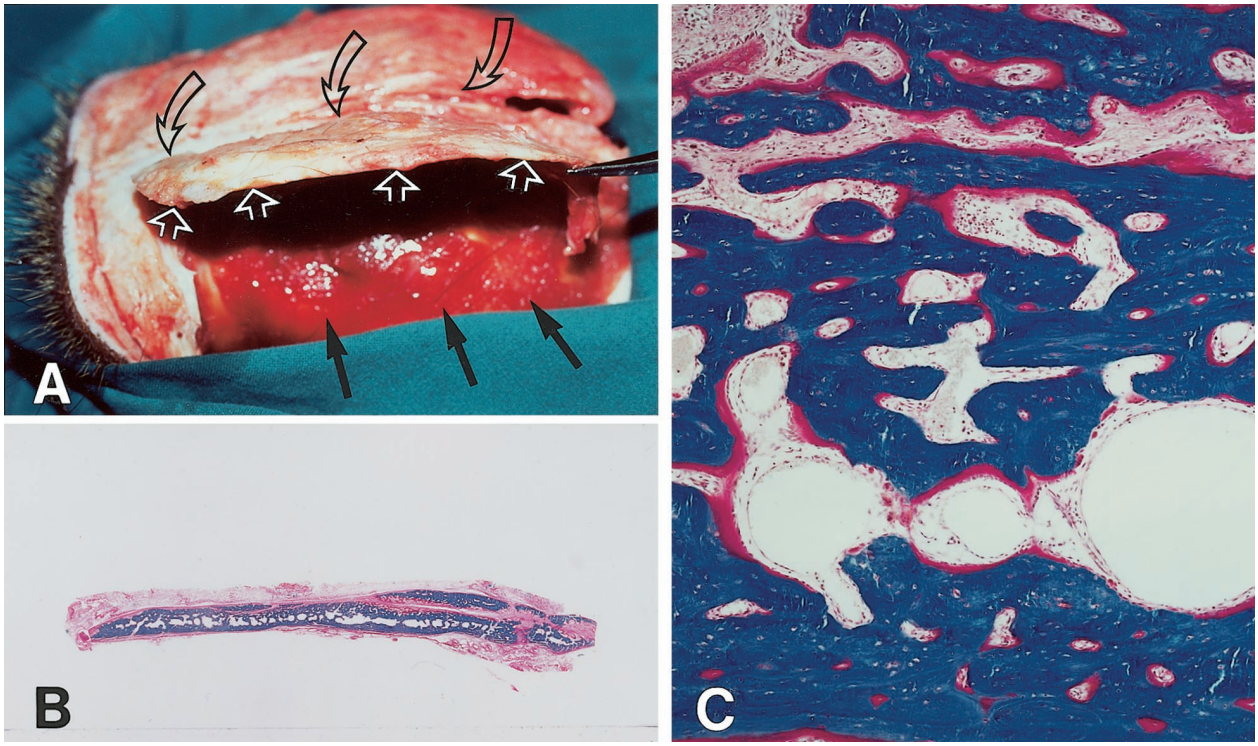

FIG. 7. (A) Autoptic preparation of a baboon calvaria before harvest of three defects that were treated with the 2.5-mg hOP-1 device. Thick plates of bone (white arrows) had formed between the temporalis fascia and the muscle, covering anterior-posteriorly and laterally, most of the underlying temporalis muscle (closed black arrows). The bone plates were united via the fascia to the temporalis crest bilaterally (open black arrows). (B) Low-power photomicrograph of the newly formed bone (frontal section) showing corticalization with formation of a pseudodiploic space. (C) Higher magnification of previous section; layers of mineralized bone covered by osteoid seams surrounding large central vascular spaces. Undecalcified section at 7 μm stained with Goldner's trichrome (B and C, original magnification, $\times$2.5 and $\times$30, respectively).

DISCUSSION

Information concerning the efficacy of irradiated osteogenic devices in nonhuman primates is an important prerequisite for clinical applications. A series of in vitro and in vivo studies were performed to determine the structural integrity and biological activity of the recoverable hOP-1 after γ-irradiation before preclinical application in calvarial defects of the adult baboon. Recoveries from rpHPLC and SDS/PAGE and immunoblot analysis indicated that doses of 2.5–3 Mrads of γ-irradiation did not significantly affect the structural integrity of hOP-1, although less hOP-1 could be recovered from the irradiated collagenous matrix. This possibly reflects some hOP-1 inactivation caused by cross-linking to the collagenous matrix. Biological activity of γ-irradiated hOP-1 was confirmed in vitro by assessing its induction of alkaline phosphatase activity in ROS cells, and in vivo by evaluating its induction of de novo endochondral bone formation in the subcutaneous space in the rat. Lower doses of γ-irradiated hOP-1, that is, 0.5 μg and 1 μg, generated less cartilage and less bone tissue of lower calcium content than nonirradiated controls. A single application of γ-irradiated recombinant morphogen in conjunction with the xenogeneic bovine collagenous matrix induced regeneration of large calvarial defects of the adult baboon. Comparison of the data with a previous series of calvarial defects treated with nonirradiated hOP-1 devices prepared with an identical collagenous matrix as carrier[33] showed that γ-irradiation resulted in reduced bone volume and reduced generated bone tissue area on day 90, as evaluated by histomorphometry. Control specimens of γ-irradiated collagenous matrix without hOP-1 showed a near 2-fold reduction in osteoconductive bone repair when compared with nonirradiated controls. These data suggest that less bone volume and bone tissue area on day 90 obtained with irradiated hOP-1 devices compared with nonirradiated devices is caused by, at least in part, a reduced performance of the irradiated substratum of the collagenous matrix, although optimal experiments to show this potentially reduced performance would have to be designed to compare the activity of γ-irradiated hOP-1 delivered by both irradiated and nonirradiated collagenous matrix. The operational reconstitution of a soluble signal (hOP-1) with an insoluble substratum (the collagenous matrix) underscores the critical role of the collagenous matrix for the induction of tissue morphogenesis and regeneration.[1,7,25] The importance of

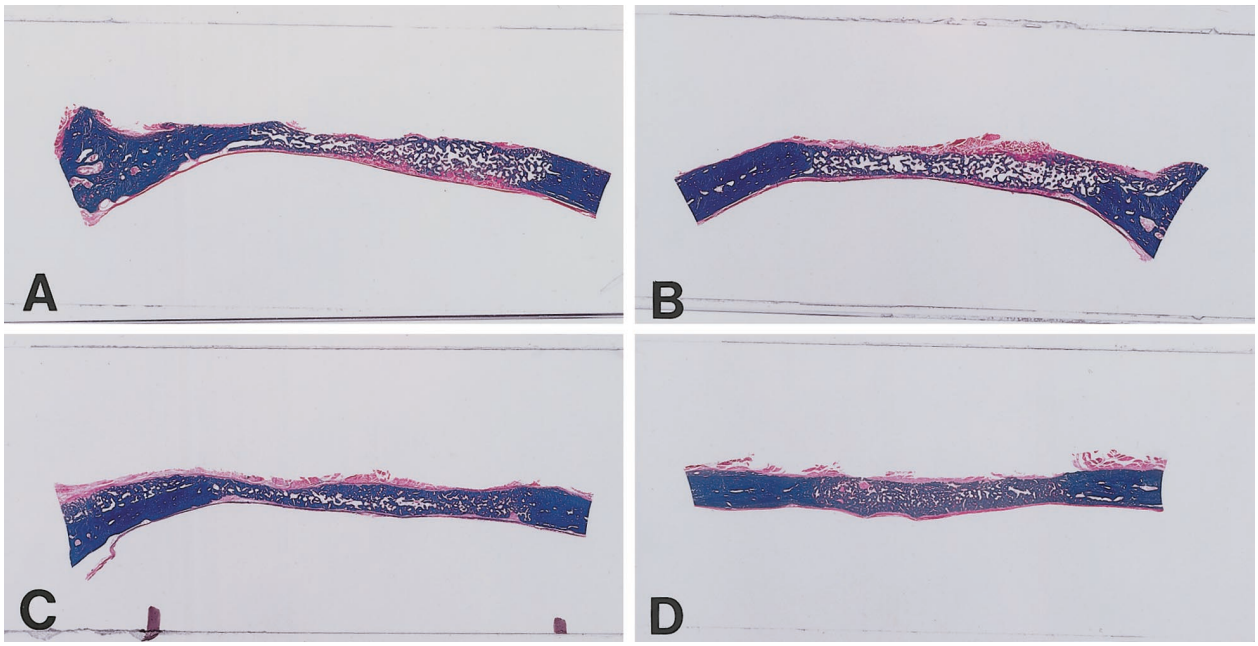

FIG. 8. Low-power photomicrographs of defects treated with bone-derived baboon BMPs in conjunction with irradiated bovine collagenous matrix as carrier and harvested on day 90. Undecalcified sections at 7 μm stained with Goldner's trichrome (original magnification, $\times$2.5). (A and B) Regeneration of bone across defects implanted with (A) 0.1 mg and (B) 0.5 mg of baboon BMPs, with trabeculae of newly formed bone facing newly generated marrow. (C and D) Induction of bone and remodeling with more compact structure in defects treated with (C) 2.5 mg of baboon BMPs and (D) 0.5 mg of irradiated hOP-1 device.

TABLE 2. EFFECT OF GAMMA IRRADIATION AND hOP-1 DOSES ON BONE INDUCTION BY hOP-1 DEVICES IMPLANTED IN 80 CALVARIAL DEFECTS PREPARED IN 20 ADULT BABOONS[a]

Days	*hOP-1 (mg)*	*Bone (%)*	*Osteoid (%)*	*Matrix (%)*
90	0.0	25.7 ± 2.9 (30.6 ± 2.6)	3.8 ± 0.5 (2.5 ± 0.2)	0.0 (4.2 ± 0.9)
	0.1	52.9 ± 1.6* (60.1 ± 1.1)	5.9 ± 0.2*,‡ (3.1 ± 0.2)	1.5 ± 0.4 (0.1 ± 0.05)
	0.5	48.4 ± 1.4* (60.8 ± 2.8)	4.9 ± 0.2*,‡ (2.9 ± 0.3)	0.3 ± 0.1 (0.3 ± 0.1)
	2.5	58.1 ± 1.7† (70.0 ± 0.9)	6.0 ± 0.3* (6.1 ± 0.3)	0.0 (0.0)
365	0.0	32.5 ± 2.8 (36.0 ± 6.1)	1.3 ± 0.1 (0.4 ± 0.1)	0.0 (0.0)
	0.1	51.6 ± 2.1*((64.9 ± 3.9)	1.3 ± 0.1 (0.8 ± 0.2)	0.0 (0.0)
	0.5	68.7 ± 1.9†,‡ (57.3 ± 5.5)	1.8 ± 0.1‡ (0.3 ± 0.1)	0.0 (0.0)
	2.5	73.7 ± 0.8†,‡ (64 ± 4.2)	1.7 ± 0.1‡ (0.3 ± 0.1)	0.0 (0.0)

[a] Doses of hOP-1, combined with 1 g of bovine collagenous matrix as carrier per sample, were subjected to irradiation (2.5 Mrads) and applied once at time of surgery in calvarial defects prepared in 20 adult baboons. Operated sites were harvested on day 90 and day 365 after bilateral carotid perfusion,[27–29] and serial undecalcified sections, cut at 7 μm, were analyzed by histomorphometry. Volume fractions of tissue components (in %) were calculated using a Zeiss Integration Platte II with 100 lattice points superimposed over 5 sources[31] in each of the four saggital sections used for analysis as described in the Materials and Methods section. Corresponding values of bone, osteoid, and matrix volumes (in %) obtained using nonirradiated hOP-1 devices[28] are shown in parenthesis. Bone refers to mineralized bone plus osteoid. Matrix refers to the residual collagenous carrier used for local delivery of hOP-1. Values are mean ± SEM.

* $p < 0.05$ versus 0.0 mg hOP-1 (control); † $p < 0.05$ versus 0.1 mg and 0.5 mg hOP-1 on day 90 and $p < 0.05$ versus 0.1 mg hOP-1 on day 365; ‡ $p < 0.05$ versus nonirradiated hOP-1.

the collagenous matrix for cell recruitment, attachment, proliferation, and differentiation has been previously reported.[38,39] Experiments using γ-irradiated bone matrices in rodents have indicated that irradiation damages collagen cross-linking, possibly by formation of free radicals, leading to peptide bond cleavage.[40,41] These changes may affect the instructive role of the substratum in defining the local microenvironment for osteoprogenitor cells proliferation and differentiation.[40] However, it was noteworthy that 1 year after the single application of the γ-irradiated hOP-1 device, bone and osteoid volumes and generated bone tissue areas were comparable with those of nonirradiated hOP-1 specimens. In particular by 1 year, regenerates induced by 0.5 mg and 2.5 mg of γ-irradiated hOP-1 induced greater amounts of bone and osteoid volumes when compared with nonirradiated hOP-1. This may be the result of sustained

TABLE 3. RELATIVE INDUCTIVE EFFICIENCY OF BONE-DERIVED BABOON BMPs COMBINED WITH BOVINE IRRADIATED COLLAGENOUS MATRIX AND HARVESTED ON DAY 90[a]

Treatment	*Bone (%)*	*Osteoid (%)*	*Matrix (%)*
0.1 mg BMPs	41.6 ± 2.4	5.4 ± 0.3	8.0 ± 1.1†
0.5 mg BMPs	40.7 ± 2.4	5.6 ± 0.5	13.1 ± 2.4†
2.5 mg BMPs	57.3 ± 0.3*	5.3 ± 0.3	0.0
0.1 mg hOP-1	53.5 ± 2.6* (52.9 ± 1.6)	5.7 ± 0.5 (5.9 ± 0.2)	1.1 ± 0.7 (1.5 ± 0.4)
0.5 mg hOP-1	52.5 ± 4.5* (48.4 ± 1.4)	4.8 ± 0.6 (4.9 ± 0.2)	0.0 (0.3 ± 0.1)

[a] BMP fractions, purified sequentially by liquid chromatography of guanidinium-extracted proteins from acid-demineralized baboon bone matrix, were combined at doses of 0.1, 0.5, and 2.5 mg with 1 g of γ-irradiated bovine collagenous matrix as carrier per sample, and after lyophilization, applied to 10 calvarial defects prepared in four adult male baboons. Remaining defects ($n = 6$) were implanted with 0.1 mg and 0.5 mg of irradiated hOP-1 device. Specimens were harvested on day 90 and serial undecalcified sections were analyzed by histomorphometry as described in the Materials and Methods section. Corresponding morphometric data on day 90 obtained using the 0.1-mg and 0.5-mg hOP-1 doses of the previous experiment (Table 2) are shown in parenthesis. Bone refers to mineralized bone plus osteoid. Matrix refers to the residual collagenous carrier used for local delivery of BMPs on hOP-1. Values are mean ± SEM.

* $p < 0.05$ versus 0.1 mg and 0.5 mg bone-derived BMPs; † $p < 0.05$ versus 2.5 mg BMPs and hOP-1 devices.

osteogenesis over time in γ-irradiated specimens as shown by the presence of substantial osteoid volumes on day 90.

Doses of 0.1 mg and 0.5 mg of bone-derived baboon BMPs combined with γ-irradiated bovine collagenous matrix yielded significantly less bone but substantial osteoid volumes when compared with 0.1-mg and 0.5-mg doses of γ-irradiated hOP-1. Although the partially purified BMP preparation was not subjected to γ-irradiation, thus precluding a direct comparison with irradiated hOP-1 specimens, it is noteworthy that 2.5 mg of partially purified BMPs and 2.5 mg of hOP-1 delivered by bovine collagenous matrix induced almost identical bone and osteoid volumes by day 90. The hOP-1 specimens (2.5 mg) yielded greater bone tissue area when measured by histomorphometry (data not shown). Partially purified preparations from bone matrix are known to contain, in addition to specific BMPs/OPs, several other proteins and some as yet poorly characterized mitogens.[42] The partially purified preparation from bone matrix obtained using the chromatographic procedures described is known to contain BMP-2, BMP-3, and OP-1 but not detectable TGF-βs (N.S. Cunningham and A.H. Reddi, unpublished data, 1989). To date, more than 15 related proteins with BMP-like sequences and activity have been cloned, but little is known about their interaction during the cascade of bone formation by induction, or about the biological and therapeutic significance of this apparent redundancy. Recombinantly produced hBMP-2, hBMP-4, and OP-1 are capable of singly initiating bone formation in vivo.[12–14] It is likely that the endogenous mechanisms of bone repair and regeneration in postnatal life necessitate the deployment and concerted actions of several of the BMPs/OPs resident within the natural milieu of the extracellular matrix of bone. Whether the biological activity of partially purified BMPs is the result of the sum of a plurality of BMP activities or of a truly synergistic interaction among BMP family members deserves appropriate investigation. In addition to bone induction in postfetal life, BMPs/OPs are involved in inductive events that control pattern formation during embryonic morphogenesis and organogenesis in such disparate tissue as the kidney, eye, nervous system, lung, teeth, skin, and heart.[43] These strikingly pleiotropic effects of BMPs/OPs

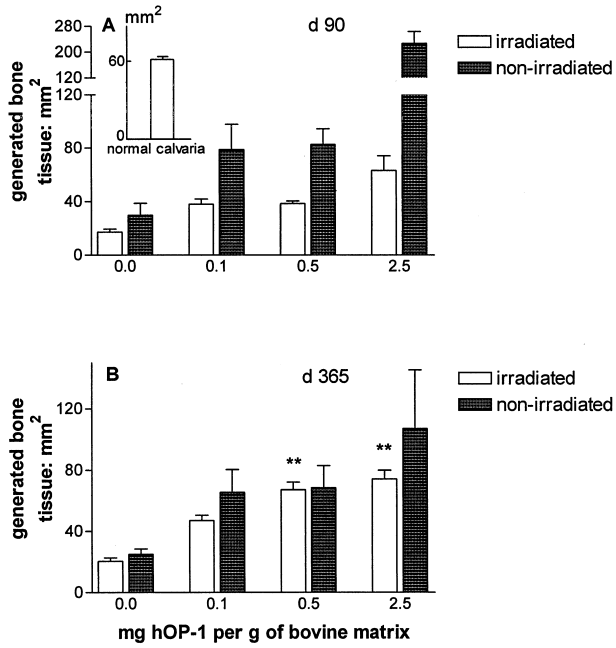

FIG. 9. Computerized analysis of new bone tissue area (mineralized bone, osteoid, and marrow) generated by doses of hOP-1 in conjunction with bovine collagenous matrix on (A) day 90 and (B) day 365. Specimens of irradiated hOP-1 device were compared with specimens of nonirradiated hOP-1 device prepared with an identical bovine collagenous matrix.[28] On day 90, doses of 0.1 mg and 0.5 mg of nonirradiated hOP-1 device showed a 2-fold increase in generated bone tissue ($p < 0.05$ vs. irradiated hOP-1 device), including collagenous matrix implanted without hOP-1 (A). On day 365, irradiated specimens showed a significant increase over 90 days ($p < 0.05$; B), approaching levels of bone tissue area induced by nonirradiated hOP-1 and comparable with the profile of normal unoperated calvaria (inset in A). *$p < 0.05$ versus nonirradiated hOP-1 specimens; **$p < 0.05$ versus irradiated specimens on day 90.

may spring from minor amino acid sequence variations in the carboxy-terminal region of the proteins,[44] as well as in the transduction of distinct signaling pathways by individual Smad proteins after transmembrane serine/threonine kinase receptor activation.[45]

In conclusion, the present findings illustrate the long-term efficacy of a single application of γ-irradiated hOP-1 delivered by a xenogeneic collagenous matrix in regenerating large defects of membranous bone of the adult primate. Ultimately, it will be necessary to gain insight into the potentially distinct spatial and temporal patterns of expression of other BMPs/OPs during morphogenesis and regeneration elicited by a single application of hOP-1. In vitro studies indicate that both hOP-1 and hBMP-2 modulate messenger RNA (mRNA) expression of related BMP family members.[46–48] In vivo studies may be useful in designing therapeutic approaches based on information of gene regulation by hOP-1.

ACKNOWLEDGMENTS

This work is supported by grants of the South African MRC, the University of the Witwatersrand, Johannesburg, and in part by a grant from the National Institutes of Health (DE 10712–01). We thank N. Ramoshebi for critical help during experiments and J. Tasker for critical reading of the manuscript.

REFERENCES

1. Reddi AH 1992 Regulation of cartilage and bone differentiation by bone morphogenetic proteins. Curr Opin Cell Biol **4:**850–855.
2. Wozney JM 1992 The bone morphogenetic protein family and osteogenesis. Mol Reprod Dev **32:**160–167.
3. Reddi AH 1994 Bone and cartilage differentiation. Curr Opin Genet Dev **4:**737–744.
4. Centrella M, Horowitz M, Wozney JM, McCarthy TL 1994 Transforming growth factor β (TGF-β) family members and bone. Endocr Rev **15:**27–39.
5. Lee S-J 1990 Identification of a novel member (GDF-1) of the transforming growth factor-β superfamily. Mol Endocrinol **4:**1034–1040.
6. Chang SC, Hoang B, Thomas JT, Vukicevic S, Luyten FP, Ryba NJ, Kozak CA, Reddi AH, Moss M 1994 Cartilage-derived morphogenetic proteins. New members of the transforming growth factor-β superfamily predominantly expressed in long bones during human embryonic development. J Biol Chem **269:**28227–28234.
7. Sampath TK, Reddi AH 1981 Dissociative extraction and reconstitution of extracellular matrix components involved in local bone differentiation. Proc Natl Acad Sci U S A **78:**7599–7603.
8. Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA 1988 Novel regulators of bone formation: Molecular clones and activities. Science **242:**1528–1534.
9. Celeste AJ, Iannazzi JM, Taylor JA, Hewick RC, Rosen V, Wang EA, Wozney JM 1990 Identification of transforming growth factor ί family members present in bone-inductive protein purified from bovine bone. Proc Natl Acad Sci USA **87:**9843–9847.
10. Özkaynak E, Rueger DC, Drier EA, Corbett C, Ridge RJ, Sampath TK, Oppermann H 1990 OP-1 cDNA encodes an osteogenic protein in the TGF-ί family. EMBO J **9:**2085–2093.
11. Özkaynak E, Schnegelsberg PNJ, Jin DF, Clifford GM, Warren FD, Drier EA, Oppermann H 1992 Osteogenic protein-2. A new member of the transforming growth factor-ί superfamily expressed early in embryogenesis. J Biol Chem **267:**25220–25227.
12. Wang EA, Rosen V, D'Alessandro JS, Bauduy M, Cordes P, Harada T, Israel DI, Hewick RM, Kerns KM, LaPan P, Luxenberg DP, McQuaid D, Moutsatsos IK, Nove J, Wozney JM 1990 Recombinant human bone morphogenetic protein induces bone formation. Proc Natl Acad Sci USA **87:**2220–2224.
13. Hammmonds RG, Schwall R, Dudley A, Berkemeier L, Lai C, Lee J, Cunningham N, Reddi AH, Wood WI, Mason AJ 1991 Bone inducing activity of mature BMP-2b produced from a hybrid BMP-2a/2b precursor. Mol Endocrinol **5:**149–155.
14. Sampath TK, Maliakal JC, Hauschka PV, Jones WK, Sasak H, Tucker RF, White KH, Coughlin JE, Tucker MM, Pang RHL, Corbett C, Ozkaynak E, Oppermann H, Rueger DC 1992 Recombinant human osteogenic protein-1 (hOP-1) induces new bone formation in vivo with a specific activity comparable with natural bovine osteogenic protein and stimulates osteoblast proliferation and differentiation in vitro. J Biol Chem **267:**20352–20362.
15. Sampath TK, Rashka KE, Doctor JS, Tucker RF, Hoffmann FM 1993 Drosophila TGF-β superfamily proteins induce endochondral bone formation in mammals. Proc Natl Acad Sci USA **90:**6004–6008.
16. Hotten GC, Matsumoto T, Kimura M, Bechtold RF, Kron R, Ohara T, Tanaka H, Satoh Y, Okazaki M, Shirai T, Pan H, Kawai S, Pohl JS, Kudu A 1996 Recombinant human growth/differentiation factor 5 stimulates mesenchyme aggregation and chondrogenesis responsible for the skeletal development of limbs. Growth Factors **13:**65–74.
17. Storm EE, Huynch TV, Copeland NG, Jenkins NA, Kingsley DM, Lee S-L 1994 Limb alterations in brachypodism mice due to mutations in a new member of the TGF-β superfamily. Nature **368:**639–643.
18. Thomas JT, Lin K, Nandedkar M, Camargo M, Cervenka J, Luyten FP 1996 A human chondrodysplasia due to a mutation in a TGF-β superfamily member. Nat Genet **12:**315–317.
19. Reddi AH 1998 Role of morphogenetic proteins in skeletal tissue engineering and regeneration. Nat Biotechnol **16:**247–252.
20. Sterilization by Ionizing Radiation 1999 US Pharmacopeia **24**: 2145.
21. Process Control Guidelines for Gamma Radiation Sterilization of Medical Devices 1984 Association for the Advancement of Medical Instrumentation, Arlington, VA, U.S.A.
22. Sterilization of Health Care Products—Requirements for Validation and Routine Control—Radiation Sterilization 1994 ANSI/AAMI/ISO 11137, Association for the Advancement of Medical Instrumentation, Arlington VA, U.S.A.
23. Wyatt DE, Keeathley JD, Williams CM, Broce R 1993 Is there life after irradiation? Part **1:** Inactivation of Biological Contaminants. BioPharm.
24. Luyten FP, Cunningham NS, Ma S, Muthukumaran N, Hammonds RG, Nevins WB, Wood WI, Reddi AH 1989 Purification and partial amino acid sequence of osteogenin, a protein initiating bone differentiation. J Biol Chem **264:**13377–13380.
25. Ripamonti U, Ma S, Cunningham N, Yeates L, Reddi AH 1992 Initiation of bone regeneration in adult baboons by osteogenin, a bone morphogenetic protein. Matrix **12:**369–380.

26. Reddi AH, Huggins CB 1972 Biochemical sequences in the transformation of normal fibroblasts in adolescent rat. Proc Natl Acad Sci U S A **69:**1601–1605.
27. Thomadakis G, Ramoshebi LN, Crooks J, Rueger DC, Ripamonti U 1999 Immunolocalization of bone morphogenetic protein-2 and -3 and osteogenic protein-1 during murine tooth root morphogenesis and in other craniofacial structures. Eur J Oral Sci **107:**368–377.
28. Maliakal JC, Asahina I, Hauschka PV, Sampath TK 1994 Osteogenic protein-1 (BMP-7) inhibits cell proliferation and stimulates the expression of markers characteristics of osteoblastic phenotype in rat osteosarcoma (17/2.8) cells. Growth Factors **11:**227–234.
29. Schnitzler CM, Ripamonti U, Mesquita JM 1993 Histomorphometry of iliac crest trabecular bone in adult male baboons in captivity. Calcif Tissue Int **52:**447–454.
30. Ripamonti U 1991 Bone induction in nonhuman primates. An experimental study on the baboon (*Papio ursinus*). Clin Orthop **269:**284–294.
31. Public Service Department 1990 National Code for Animal Use in Research, Education, Diagnosis and Testing of Drugs and Related Substances in South Africa. Public Service Department, Pretoria, South Africa.
32. Ripamonti U, Ma S, Cunningham N, Yeates L, Reddi AH 1993 Reconstruction of the bone-bone marrow organ by osteogenin, a bone morphogenetic protein, and demineralized bone matrix in calvarial defects of adult primates. Plast Reconstr Surg **91:**27–36.
33. Ripamonti U, van den Heever B, Sampath TK, Tucker MM, Rueger DC, Reddi AH 1996 Complete regeneration of bone in the baboon by recombinant human osteogenic protein-1 (hOP-1, bone morphogenetic protein-7). Growth Factors **13:** 273–289.
34. Ripamonti U, Bosch C, van den Heever B, Duneas N, Melsen B, Ebner R 1996 Limited chondro-osteogenesis by recombinant human transforming growth factor-β1 in calvarial defects of adult baboons (*Papio ursinus*). J Bone Miner Res **11:**938–945.
35. Parfitt AM 1983 Stereologic basis of bone histomorphometry; theory of quantitative microscopy and reconstruction of the third dimension. In: Recker RR (ed.). Bone Histomorphometry: Techniques and Interpretation. CRC Press, Boca Raton, FL, U.S.A., pp. 53–87.
36. Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR 1987 Bone histomorphometry: Standardization of nomenclature, symbols, and units. J Bone Miner Res **2**:595–610.
37. Statistical Analysis System 1989 SAS/STATS User's Guide, version 6, 4th ed, vol 1. SAS Institute, Inc., Cary, NC, U.S.A., pp. 209–244.
38. Reddi AH 1974 Importance of geometry of the extracellular matrix in endochondral bone differentiation. Adv Biol Med Phys **15:**1–18.
39. Reddi AH 1984 Extracellular matrix and development. In: Piez KA, Reddi AH (eds.) Biochemistry of Extracellular Matrix. Elsevier, New York, NY, U.S.A., pp. 375–412.
40. Wientroub S, Reddi AH 1988 Influence of irradiation on the osteoinductive potential of demineralized bone matrix. Calcif Tissue Int **42:**255–260.
41. Katz RW, Felthousen GC, Reddi AH 1990 Radiation-sterilized insoluble collagenous bone matrix is a functional carrier of osteogenin for bone induction. Calcif Tissue Int **47:**183–185.
42. Hauschka PV, Mavrakos AE, Iafrati MD, Doleman SE, Klagsbrun M 1986 Growth factors in bone. J Biol Chem **261:**12665–12674.
43. Reddi AH 1997 Bone morphogenetic proteins: An unconventional approach to isolation of first mammalian morphogens. Cytokine Growth Factor Rev **8:**11–20.
44. Staehling-Hampton K, Jackson PD, Clark MJ, Brand AH, Hoffmann MF 1994 Specificity of bone morphogenetic protein-related factors: Cell fate and gene expression changes in *Drosophila* embryos induced by *decapentaplegic* bu t not *60A*. Cell Growth Differ **5:**585–593.
45. Graff JM, Bansal A, Melton DA 1996 Xenopus mad proteins transduce distinct subsets of signals for the TGFβ superfamily. Cell **85:**479–487.
46. Harris SE, Sabatini M, Harris MA, Feng JQ, Wozney JM, Mundy GR 1994 Expression of bone morphogenetic protein messenger RNA in prolonged cultures of fetal rat calvarial cells. J Bone Miner Res **9:**389–394.
47. Chen D, Harris MA, Rossini G, Dunstan CR, Dallas SL, Feng JQ, Mundy GR, Harris SE 1997 Bone morphogenetic protein 2 (BMP-2) enhances BMP-3, BMP-4, and bone cell differentiation marker gene expression during the induction of mineralized bone matrix formation in cultures of fetal rat calvarial osteoblasts. Calcif Tissue Int **60:**238–290.
48. Honda Y, Kniutsen R, Strong DD, Sampath TK, Baylink DJ, Mohan S 1997 Osteogenic protein-1 stimulates mRNA levels of BMP-6 and decreases mRNA levels of BMP-2 and -4 in human osteosarcoma cells. Calcif Tissue Int **60:**297–301.
49. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ 1951 Protein measurement with the folin phenol reagent. J Biol Chem **193:**265–275.

Address reprint requests to:
Ugo Ripamonti, M.D., Ph.D.
Bone Research Laboratory
MRC/University of the Witwatersrand
Medical School, 7 York Road
Parktown 2193, Johannesburg, South Africa

Received in original form August 13, 1999; in revised form February 4, 2000; accepted March 2, 2000.