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Ex Vivo Gene Therapy for Skeletal Regeneration in Cranial Defects Compromised by Postoperative Radiotherapy

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ABSTRACT

Because radiation remains a common postoperative treatment for head and neck cancers, it is critical to determine whether new bone-regenerative approaches are effective for healing craniofacial defects challenged by therapeutic doses of radiation. The objective of this study was to determine whether the deleterious effects of radiotherapy could be overcome by *ex vivo* gene therapy to heal craniofacial defects. Rat calvarial critical-sized defects were treated with either an inlay calvarial bone graft or syngeneic dermal fibroblasts transduced *ex vivo* with an adenovirus engineered to express bone morphogenetic protein 7 (BMP-7), a morphogen known to stimulate bone formation. Two weeks postoperatively, either no radiation or a single 12-Gy radiation dose was delivered to the operated area and the tissue was harvested 4 weeks later. None of the inlay bone grafts healed at the wound margins of either the radiated or nonradiated sites. In contrast, bone was successfully regenerated when using an *ex vivo* gene therapy approach. More bone formed in the nonradiated group as determined by the percentage of defect surface covered (87 ± 4.1 versus 65 ± 4.7%; $p = 0.003$) and percentage of defect area filled by new bone (60 ± 5.9 versus 32 ± 2.7%; $p = 0.002$). Although the effects of radiation on the wound were not completely overcome by the gene therapy approach, bone regeneration was still successful despite the radiation sensitivity of the fibroblasts. These results indicate that BMP-7 *ex vivo* gene therapy is capable of successfully regenerating bone in rat calvarial defects even after a therapeutic dose of radiation. This approach may represent a new strategy for regenerating skeletal elements lost due to head and neck cancer.

OVERVIEW SUMMARY

Future reconstructive approaches will depend on the development of tissue-regenerative strategies using protein, cell, or gene therapy. Many advances have been made in tissue engineering, but whether these techniques will be successful in wounds compromised by radiotherapy is still unknown. This issue is particularly important when considering the application for craniofacial or mandibular reconstruction, because these defects most commonly arise from cancer surgery, for which postoperative radiotherapy is often required. Previous studies have shown that protein therapy alone, using bone morphogenetic protein (BMP), does not heal irradiated bone defects. In this study, we determined whether *ex vivo* adenoviral gene therapy using rat fibroblasts genetically engineered to express BMP-7 could heal critical-size calvarial defects treated with a therapeutically equivalent radiation dose. Our results show that, despite significant radiosensitivity of the transplanted fibroblasts, bone can be successfully regenerated by an *ex vivo* gene therapy approach. This approach alone does not completely overcome the detrimental effects of radiation, however, as less effective healing was measured at 4 weeks postradiation compared with the well-healed gene therapy-treated nonradiated defects.

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INTRODUCTION

ACQUIRED CRANIOFACIAL and mandibular defects are physically, emotionally, and financially devastating to patients. Significant bone defects requiring reconstructive surgery most commonly arise from resection of cancers and bone grafting is usually necessary. As opposed to other skeletal defects that require bone grafts, head and neck cancer defects are unique because these wounds are contaminated with oral bacteria and are likely to be treated with pre- or postoperative radiotherapy. Because of these wound characteristics, nonvascularized, autologous bone grafts have a failure rate that approaches 50% when placed in the primary setting (Lawson *et al.*, 1982). The success rate is better when the graft is placed months or years later, but subsequent tissue scarring and fibrosis limit the functional and aesthetic outcomes of secondary reconstruction (Adamo and Szal, 1979).

The success of primary reconstruction is improved if microsurgical techniques are used to immediately restore circulation to the bone graft (Urken *et al.*, 1998). These grafts, known as free tissue transfers, can also include soft tissue for reconstruction of accompanying mucosal or cutaneous defects. Free tissue transfer has gained acceptance for reconstructing oromandibular and major craniofacial defects arising from ablative cancer surgery because of the disappointing results of nonvascularized bone grafting for these indications. This approach has some limitations, however, that restrict its universal use. Microvascular reconstruction requires the expert skills of a surgeon specifically trained in this technique and requires several additional hours of operative time. Some patients are not medically capable of tolerating this additional surgery. Despite the surgeon's best efforts, there is a 5–10% failure rate due to clot formation in the reconnected blood vessels (Urken *et al.*, 1998, 2001). There are multiple defect- and patient-related factors that are important for surgical planning, but the need for bone restricts the flap choices to the scapula, iliac crest, fibula, or radial sites. Finally, harvest of bone from these donor sites can cause significant donor site morbidity such as chronic pain, paresthesias, extremity movement problems, gait disturbances, abdominal wall hernia, and limb ischemia.

On the basis of multicenter randomized clinical trials, human recombinant protein therapy using commercially available bone morphogenetic proteins (BMPs) is now an acceptable alternative to autologous bone grafts for limited orthopedic indications (Friedlaender *et al.*, 2001; Burkus *et al.*, 2002). This treatment is still experimental, however, as a substitute for bone grafting in the craniofacial and mandibular regions. There have been some promising results when used for alveolar ridge (Howell *et al.*, 1997) and sinus floor augmentation (Boyne *et al.*, 1997), but the size of these defects is not comparable to those resulting from ablative cancer surgery or major trauma. Preclinical studies have shown successful healing of critical-sized mandibular (Toriumi *et al.*, 1991) and calvarial defects (Sato and Urist, 1985) in canines, and of mandibular defects in monkeys (Boyne *et al.*, 1999; Marukawa *et al.*, 2002), using BMP delivered on biodegradable carriers. There is also a case report of a patient that had a 6-cm lateral mandibular discontinuity defect from an ameloblastoma resection reconstructed with a BMP bioimplant rather than by vascularized free tissue transfer (Moghadam *et al.*, 2001). Although these findings are promising, the wound environment in these animal models and the case report was optimized to provide an ideal milieu for bone regeneration. This is not customary for patients with ablative head and neck surgical defects. The local vascular and mesenchymal cell environment is likely to be adversely affected by adjuvant radiation therapy, microbiological contamination, and advanced patient age. The defects tend to exceed critical size and also include the overlying periosteum and soft tissues. These patients tend to have a significant tobacco exposure history and comorbidities such as diabetes and peripheral vascular disease, which also adversely affect wound healing.

There is limited information about the ability of osteoinductive protein therapy to successfully heal wounds compromised by pre- or postoperative radiotherapy. There is concern that a single exposure to an exogenous growth factor may not provide an adequate osteoinductive response in the cells within the wound microenvironment to overcome the negative effects of radiotherapy. Animal studies investigating the effects of preoperative radiotherapy validate this concern (Khouri *et al.*, 1996; Wurtzler *et al.*, 1998). These studies model the scenarios of bone reconstruction for osteoradionecrosis and for patients who fail primary treatment with radiotherapy and subsequently require salvage surgery that requires resection of bone. Animal studies investigating the effects of postoperative radiotherapy are lacking but especially important because cancer that is invading bone at the time of presentation is optimally treated by surgical resection and postoperative radiotherapy. This is in accordance with guidelines from the National Comprehensive Cancer Network and consistent with the view of most head and neck oncologists that primary radiotherapy alone cannot provide acceptable local control rates when bone is invaded by tumor.

The use of gene therapy to deliver a gene or gene product is a powerful and potentially effective method to enhance regeneration of skeletal tissues that are compromised by postoperative radiotherapy. In fact, *ex vivo* (Krebsbach *et al.*, 2000) and *in vivo* (Alden *et al.*, 2000; Lindsey, 2001) gene therapy approaches have already been successfully used to heal experimental craniofacial defects in animal models. The *ex vivo* approach has many advantages including direct delivery of the osteoinductive gene to the desired site, targeting of cells for gene delivery, supplying of cells that directly participate in the osteoinductive process, and, potentially, control of the rate or extent of gene expression by using inducible delivery vectors (Scaduto and Lieberman, 1999). Prior studies performed in our laboratory showed that *ex vivo* gene therapy using fibroblasts transduced with an adenovirus expressing BMP-7 (AdCMVBMP-7) successfully healed 9-mm rat calvarial critical-sized defects (Krebsbach *et al.*, 2000). We have also shown that a fraction of these genetically modified fibroblasts convert to osteoblasts, and thus directly participate in the bone-regenerative process (Rutherford *et al.*, 2002). The use of an adenovirus vector in *ex vivo* gene therapy for bone regeneration provides a continuous, yet transient, period of growth factor production, thus enabling a more robust osteoinductive stimulus than protein therapy. Therefore, we hypothesized that an *ex vivo* gene therapy approach using an adenoviral vector containing the cDNA for BMP-7 would successfully heal craniofacial defects despite their being severely compromised by postoperative radiotherapy.

MATERIALS AND METHODS

Generation of recombinant adenovirus

AdCMVBMP-7 was constructed by Cre–*lox* recombination as previously described (Franceschi *et al.*, 2000). Briefly, a full-length mouse BMP-7 cDNA was cloned into pAdlox to produce pAdlox BMP-7 (Hardy *et al.*, 1997). pAdlox and $\psi 5$ virus were cotransfected into CRE8 cells. A plaque assay was used to purify virus from the cell lysate and serial dilutions were used to infect 293 cells. Positive plaques were purified by CsCl gradient ultracentrifugation. Purified virus was stored in glycerol phosphate-buffered saline and titered by the method described above.

Preparation of rat dermal fibroblasts and adenoviral infection

Operations were performed under anesthesia with ketamine (50 μg/g) and xylazine (5 μg/g). A paramedian skin incision approximately 2 cm in length was made on the dorsum and a subcutaneous pocket was bluntly created. Harvested dermis was placed in complete medium (Dulbecco's modified Eagle's medium [DMEM] supplemented with penicillin, streptomycin, and 10% fetal calf serum) and fibroblast strains were generated from primary explants. The incisions were closed with surgical staples. Cells from passages 4–12 were harvested, counted with a hemocytometer, and frozen for later use or replated, transduced, and seeded directly onto gelatin sponges (Gelfoam; Upjohn, Kalamazoo, MI). The transduction was performed *ex vivo* for 24 hr, using a multiplicity of infection (MOI) of 1000 PFU/cell. Cells were 80–90% confluent at the time of transduction.

Each gelatin sponge was designed to be a 9 $\times$ 9 mm disk. The sponges were prewetted in complete medium and air bubbles were removed by applying gentle pressure on the sponge between two pieces of sterile filter paper. Two million transduced cells were collected, suspended in 50 μl of collagen (2.5 mg/ml, rat tail collagen, type I; BD Biosciences, Bedford, MA), and loaded onto each sponge by capillary action. All procedures involving animals were performed in accordance with protocols approved by the Unit for Laboratory Animal Medicine (University of Michigan, Ann Arbor, MI).

Repair of craniotomy defects with genetically modified fibroblasts or autologous inlay calvarial bone grafts

Fischer rats weighing approximately 200 g were anesthetized with ketamine (50 μg/g) and xylazine (5 μg/g). A linear scalp incision was made from the nasal bone to the occiput and full-thickness flaps were elevated. The periosteum overlying the calvarial bone was completely resected. A trephine was used to produce a 9-mm craniotomy defect centered on the sagittal sinus and the wounds were copiously irrigated with Hanks' balanced salt solution (HBSS) while drilling. The calvarial disk was removed carefully in order to avoid injury to the underlying dura or brain. For rats undergoing bone graft surgery, the calvarial disks were copiously irrigated with HBSS and placed in the proper orientation back into the craniotomy defects. For rats undergoing treatment with *ex vivo* gene therapy, gelatin sponges previously loaded with transduced syngeneic fibroblasts were placed into the defects. The sponges filled the entire defect and touched the bone edges around the entire periphery. The incisions were closed with 5-0 Vicryl suture (Ethicon/Johnson & Johnson, Sommerville, NJ) and the rats recovered from anesthesia under a heat lamp.

Animal irradiation

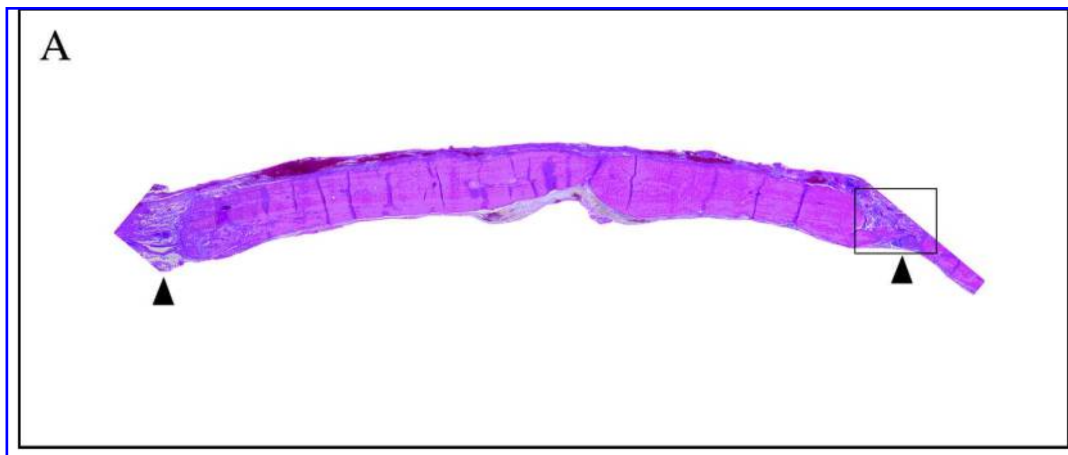
For rats in the radiation treatment group, a single 12-Gy dose from a cobalt source (^{60}Co) was delivered to $D_{\max}$ at a source-to-skin distance of 80 cm in an 11.47-min exposure. Radiation was delivered exclusively to the surgical site. The pharynx and the rest of the body were shielded by a combination of beam collimation and Cerrobend blocks (Fields *et al.*, 2000). Rats were anesthetized with ketamine (50 μg/g) and xylazine (5 μg/g) during the radiation treatment. The radiation dose was given 2 weeks after the surgical procedure. Side effects from the radiation were monitored afterward, and included weight loss, wound-healing problems, tissue necrosis, alopecia, ocular problems, and unusual behavior.

Tissue harvest and histology

Calvaria were harvested 4 weeks after surgery in nonradiated rats and 6 weeks after surgery in radiated animals (4 weeks after radiation). This experimental design maintained a consistent time interval to allow for bone regeneration after the last insult to the defect. The rats were euthanized with CO_2 gas. Skin was separated from the underlying skull, and the amount of wound healing was grossly estimated. Calvaria were harvested with a dental drill equipped with a carbide cutting burr, maintaining a minimal 2-mm margin peripheral to the craniotomy site. The harvested bone was irrigated with sterile saline and fixed in buffered zinc formalin (Z-Fix; Anatech, Battle Creek, MI) overnight. Radiographs were taken of each specimen after fixation. The tissue was rinsed in water and then decalcified in 10% formic acid for 14–17 days. After decalcification, the tissue was divided in the coronal plane immediately in the center of the defect and embedded in paraffin. Five-micron sections were made and placed on 10 slides with 3 sections per slide. The tissue was deparaffinized, hydrated, and the first, fifth, and tenth slides were stained with hematoxylin and eosin.

Histomorphometry and polarized light microscopy

Computer-assisted bone histomorphometry was performed with a Nikon Eclipse E800 microscope (Nikon, Melville, NY) and Image-Pro Plus 4.1 software (Media Cybernetics, Silver Spring, MD). The third section on the fifth slide, which approximated the midline cut, was used for each rat. Wound healing was quantified by measuring the percentage of defect surface covered by bone (length of bone regenerated across the defect divided by total length of defect) and the percentage of defect area filled by new bone (area of bone regenerated in the defect divided by total area of the defect). The amount of osteosynthesis at the wound margins was also qualitatively assessed. The data for the percentage of defect surface covered by bone were analyzed by an unpaired t test, and the percentage of defect area filled by new bone was analyzed by an un-



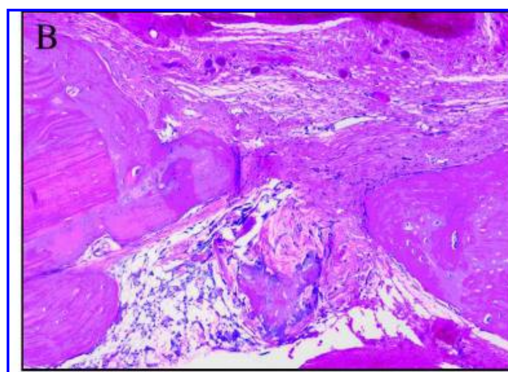
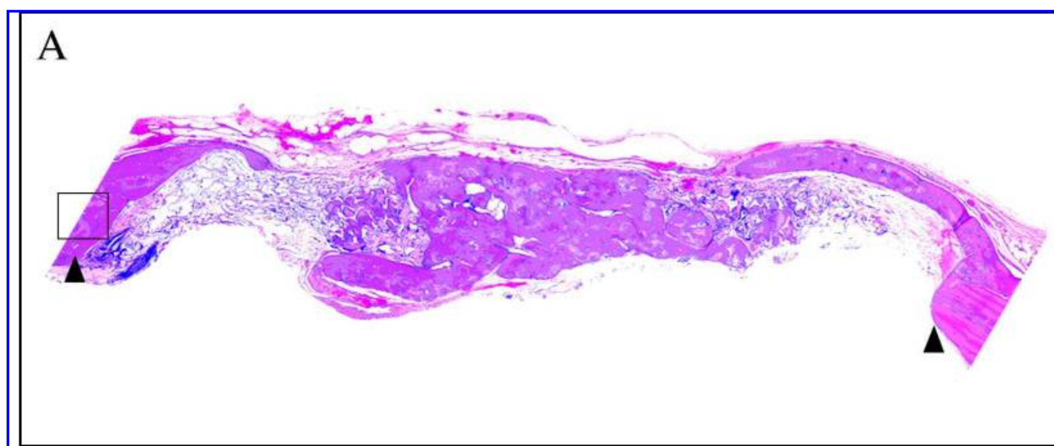


FIG. 1. Inlay calvarial bone grafts fail to heal critical-size calvarial defects independent of radiation treatment. (**A**) Nonirradiated cranial defect treated with an autologous inlay calvarial bone graft. Solid arrowheads designate the surgical margins. Original magnification, ×4. (**B**) Right wound margin corresponding to the boxed area in (**A**), revealing fibrous union. Original magnification, ×40.



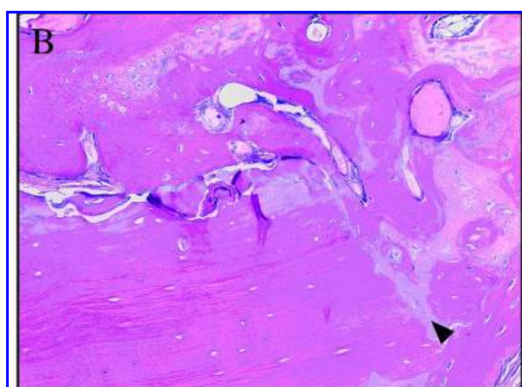
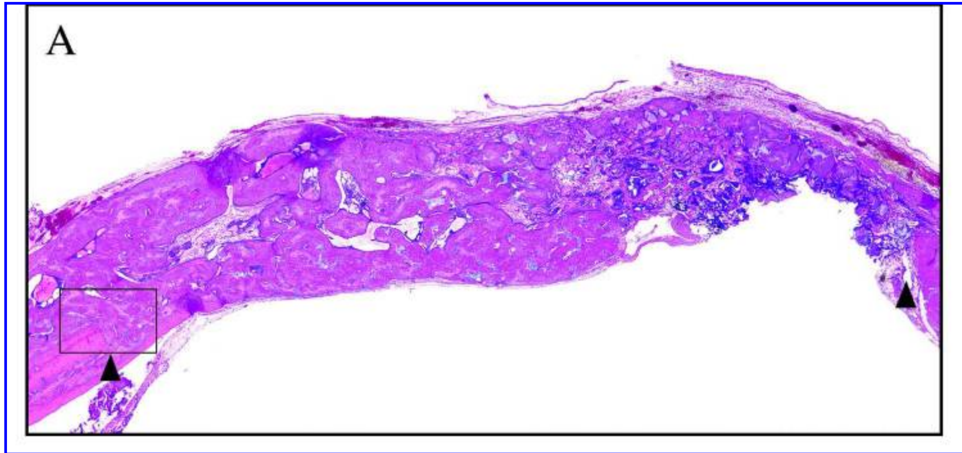


FIG. 3. BMP-7-transduced fibroblasts successfully induce bone formation when treated with postoperative radiation therapy. (**A**) Cranial defect treated with rat dermal fibroblasts transduced with AdCMVBMP-7 seeded within a gelatin sponge approximating the size of the defect. Woven-appearing bone was successfully regenerated, but it was not as well organized as compared with gene therapy-treated, nonradiated defects. Solid arrowheads designate the surgical margins. Original magnification, ×4. (**B**) Left wound margin corresponding to the boxed area in (**A**), revealing osteointegration. Solid arrowhead designates the surgical margin. Original magnification, ×100.



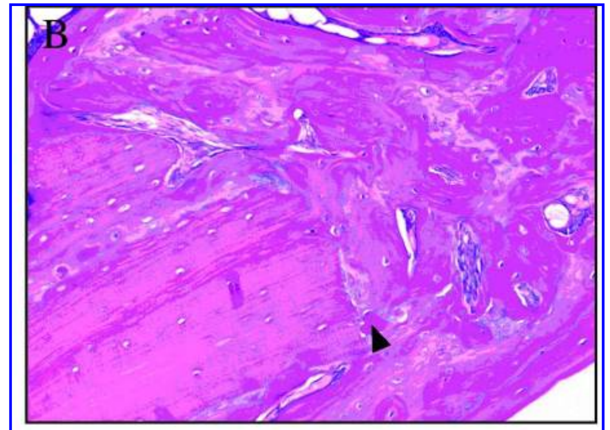
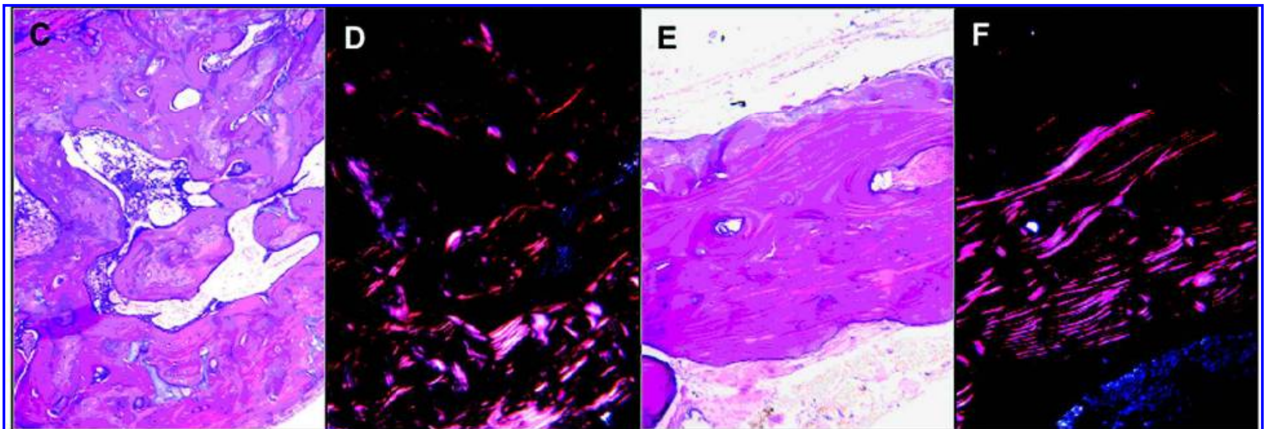


FIG. 2. BMP-7-transduced fibroblasts heal calvarial critical-size defects not treated with radiation therapy. (**A**) Cranial defect repair with rat dermal fibroblasts transduced with AdCMVBMP-7 seeded within a gelatin sponge approximating the size of the defect. Confluent bone nearly crosses the entire defect. The solid arrowheads designate the surgical margins. Original magnification, ×4. (**B**) Left wound margin corresponding to the boxed area in (**A**), revealing osteointegration. Solid arrowhead designates the surgical margin. Original magnification, ×100. (**C**) Central portion of the regenerated bone at 1 month, with marrow-appearing spaces admixed with the new bone. Original magnification, ×100. (**D**) Polarized light microscopy through the central portion of regenerated bone at 1 month. Collagen fibers are organized in a pattern consistent with woven bone. Original magnification, ×100. (**E**) Central portion of the regenerated bone at 6 months, with remodeling of bone to a lamellar appearance and Haversian canal formation. Original magnification, ×63. (**F**) Polarized light microscopy through the central portion of regenerated bone at 6 months. Collagen fibers are organized in a pattern consistent with lamellar bone. Original magnification, ×63.



paired t test with Welch correction (GraphPad InStat version 3.0a for the Macintosh; GraphPad Software, San Diego, CA). Data are presented as means ± SEM.

Polarized light microscopy was performed on tissue specimens from rat calvarial critical-sized defects treated with BMP-7-transduced fibroblasts harvested at 1 month ($n = 1$) and at 6 months ($n = 1$) postoperatively. A Leitz Aristoplan optical microscope (Leica Microsystems, Wetzlar, Germany) was used and electronic image capture was performed with a SPOT-RT digital camera (Diagnostic Instruments, Sterling Heights, MI).

Radiation sensitivity

A limiting dilution assay was performed to determine the radiation sensitivity of rat dermal fibroblasts (Grenman *et al.*, 1989). This technique was used because rat dermal fibroblasts do not tend to form colonies *in vitro.* Briefly, single-cell suspensions of fibroblasts (passage 11) were radiated in complete medium. Radiation doses of 0, 6, and 12 Gy were used. For each radiation dose, cells were then plated into 96-well plates at concentrations of 6, 12, 18, and 24 cells/well in 200 μl of complete medium. For each cell concentration, the central 60 wells were plated with cells and the peripheral 36 wells received 200 μl of distilled deionized H_2O. The plates were kept in an incubator at 37°C in a water vapor-saturated room air atmosphere containing 5% CO_2. The plates were examined with an inverted phase-contrast microscope at 17 days. A well was considered positive if a colony in it reached a size of 32 cells or more. The plating efficiency was calculated according to the formula PE = –ln(negative wells/total wells)/number of cells plated per well (Thilly *et al.*, 1980). The fraction survival was determined by dividing the plating efficiency at each radiation dose by the plating efficiency for 0 Gy. The experiment was repeated twice.

RESULTS

Tolerance of rats to radiotherapy

All rats that received radiation experienced a weight loss of 10–15 g during the first week after treatment, but then gained weight appropriately during the subsequent weeks before tissue harvest. The radiation caused mild alopecia of the scalp but no skin-healing problems or infections occurred. No rats experienced overt signs of pharynx or ocular toxicity from the radiation.

Autologous bone grafts fail to heal craniotomy defects independent of radiation treatment

At the time of tissue harvest, none of the inlay calvarial bone grafts in nonradiated ($n = 5$) or radiated ($n = 5$) craniotomy defects presented gross evidence of healing at the wound margins. The surfaces of these grafts were flush with the surrounding bone, but were mobile. Histology revealed intact bone grafts with viable osteocytes, but fibrous union with minimal osteogenesis at the wound margins (Fig. 1).

BMP-7-transduced fibroblasts heal calvarial defects not treated with radiation therapy

At the time of tissue harvest, bone healing was estimated as nearly 100% by gross inspection ($n = 8$) for the craniotomy defects treated with fibroblasts genetically modified to express BMP-7. Confluent bone spanned across the entire defect and, in many cases, the exact wound margins were difficult to identify because of excellent healing at the edges. This new bone was not mobile. Histology revealed confluent bone spanning the defect, with excellent osteointegration at many of the wound margins (Fig. 2A and B). The regenerated bone appeared woven when harvested 1 month after surgery (Fig. 2C and D), but this bone remodeled to form lamellar bone when harvested 6 months later (Fig. 2E and F). No overt immune or inflammatory response was seen histologically in the *ex vivo* gene therapy-treated animals. Histomorphometric analysis revealed that the mean defect surface covered by bone was 87 ± 4.1% and the mean defect area filled by new bone was 60 ± 5.9%.

BMP-7-transduced fibroblasts successfully induce bone formation when treated with radiation therapy after transplantation

Fibroblasts genetically modified to express BMP-7 successfully induced new bone formation in craniotomy defects subsequently treated with a therapeutically equivalent dose of radiation ($n = 10$). This *ex vivo* gene therapy approach, however, was less effective compared with the results for nonradiated animals. At the time of tissue harvest, bone healing was estimated as 50–100% and islands of new bone were more common than confluent areas spanning the entire defect. Histology revealed woven-appearing bone formation that was not as well organized or healed at the wound edges as compared with nonradiated *ex vivo* gene therapy-treated defects (Fig. 3). Histomorphometric analysis revealed that the mean defect surface covered by bone was 65 ± 4.7% and the mean defect area filled by new bone was 32 ± 2.7%. Statistical analysis confirmed that this group experienced significantly less bone regeneration when compared with defects not treated with radiation (Fig. 4).

In vitro *radiation sensitivity of rat dermal fibroblasts*

A limiting dilution assay was performed in order to determine the radiation sensitivity of rat dermal fibroblasts. The fraction survival for a single 12-Gy radiation dose was a 2-log kill ratio (Fig. 5). Despite this finding, *ex vivo* gene therapy using fibroblasts still provided a robust osteoinductive signal that stimulated good bone regeneration in these radiated defects.

DISCUSSION

This animal model was used to investigate whether an *ex vivo* gene therapy approach to regenerate bone in critical-size defects would be successful if a therapeutic dose of postoperative radiation was given. A single dose of 12 Gy was used because of logistic problems in delivering fractionated radiation to rats, an estimation that this single dose for a rat was equivalent to a fractionated 60-Gy dose for a human, and prior studies that have shown that this is a safe dose for cranial irradiation in rats (Remsen *et al.*, 1995). No rats experienced cutaneous wound dehiscence or ocular toxicity, and effective shielding of the snout prevented mucosal toxicity.

Despite receiving a therapeutic dose of postoperative radiation, BMP-7 *ex vivo* gene therapy successfully regenerated bone in rat calvarial critical-size defects. Although the detrimental

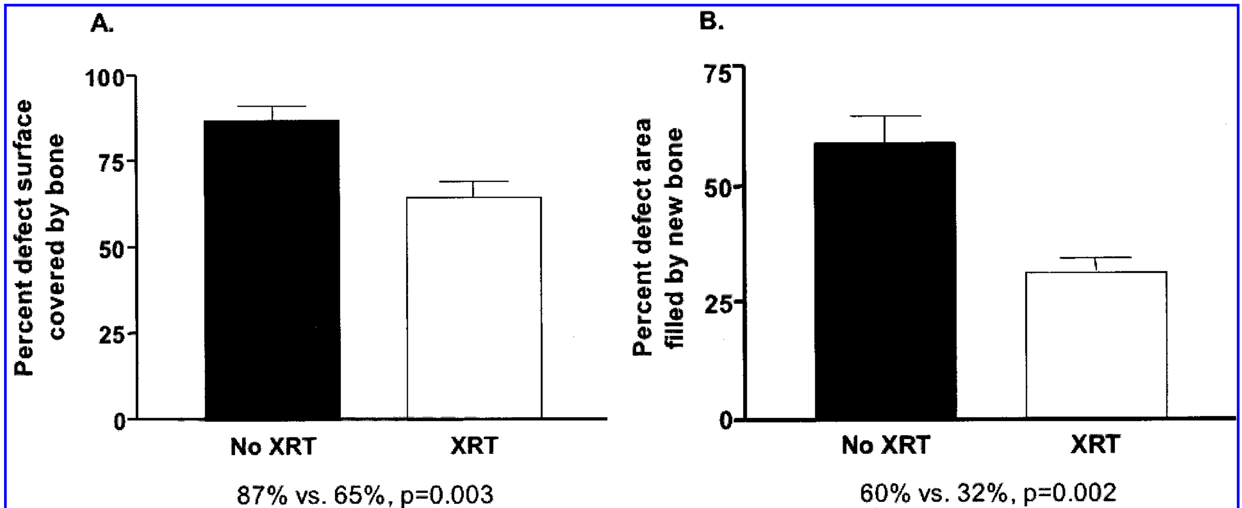


FIG. 4. Histomorphometric analysis of new bone formation in calvarial defects. The bar graphs compare *ex vivo* gene therapy-treated defects that did not receive radiation with *ex vivo* gene therapy-treated defects that received postoperative radiation. Quantification was performed by computer-assisted bone histomorphometry. (**A**) Percentage of defect surface covered by bone. (**B**) Percentage of defect area healed. XRT, radiation therapy.

effects of radiation were not completely overcome by the gene therapy approach, this regeneration was remarkable given the significant radiation sensitivity of the transplanted fibroblasts used to deliver the gene into the wound. The control group received an inlay bone graft because this is an accepted treatment option in patients and prior studies have shown that fibroblasts genetically modified to express *lacZ* did not show any evidence of bone regeneration when placed in critical-size rat calvarial defects (Krebsbach *et al.*, 2000). The inlay autologous bone grafts did not heal in this defect model independent of radiation exposure. This may be related to not providing rigid fixation for these grafts (Abbott *et al.*, 1994; DeLuca *et al.*, 1997) or may be due to removal of the periosteum (Hopper *et al.*, 2001). Excellent healing in the gene therapy-treated defects still occurred despite nonrigid scaffold fixation and periosteum resection.

Patients with large craniofacial or mandibular defects resulting from ablative surgery will benefit greatly if regenerative medicine can successfully be applied for bone regeneration. Potential advantages over current treatments involving vascularized or nonvascularized autologous bone grafting include avoidance of donor site morbidity, decreased operative time, decreased surgical complications, and custom fabrication of the reconstructed segment to recapitulate the three-dimensional shape of the resected bone. The surgeon would also be able to choose from a greater variety of flaps for soft tissue reconstruction, rather than being limited to the fibula, iliac crest, scapula, or radial donor sites.

Radiation therapy, administered either pre- or postoperatively, is an integral part of comprehensive treatment for advanced-stage head and neck malignancies, and thus the nega-

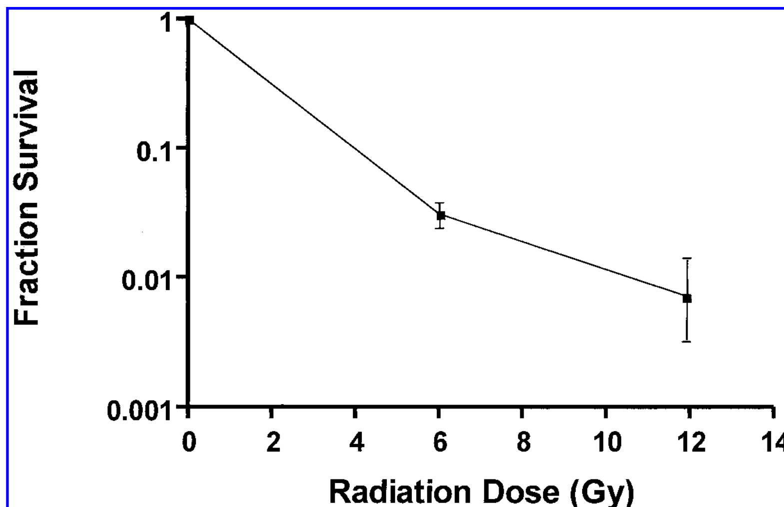


FIG. 5. Radiation survival curve of rat dermal fibroblasts measured by limiting dilution assay. There is an approximately 2-log kill ratio at a dose of 12 Gy.

tive effects of radiotherapy on the wound will need to be accounted for when devising strategies for regenerating skeletal elements lost because of head and neck cancer (Denham and Hauer-Jensen, 2002). To date, studies have been limited and have investigated only the use of osteoinductive protein therapy with BMP in previously radiated tissues. Irradiation *in vitro* up to 8 Gy did not affect BMP-2-induced osteoblast differentiation of C2C12 cells (Ikeda *et al.*, 2000), and bone growth still occurred on a BMP-2-treated hydroxyapatite disk implanted into a rabbit snout subperiosteal pocket radiated preoperatively with a fractionated dose of 20 Gy (Howard *et al.*, 1998). Unfortunately, results with orthotopic defects are not as encouraging. Using the rat calvarium, 3-mm defects treated with a preoperative 12-Gy radiation dose and subsequently with BMP-2 had successful bone regeneration but incomplete healing of the defects (Wurzler *et al.*, 1998). In a 4-mm rat mandible defect treated with a fractionated 45-Gy radiation dose and 2 weeks later with demineralized bone powder, only 39% of defects had more than 50% bone fill (Lorente *et al.*, 1992). Using a 7-mm rat calvarial defect, Khouri *et al.* showed that microvascular transfer of a muscle flap in addition to treatment with BMP-3 allowed for complete healing of the defect despite receiving a single preoperative 15-Gy radiation dose (Khouri *et al.*, 1996). Treatment with no implant, a microvascular muscle flap alone, or BMP-3 alone did not heal the defect. Collectively, these findings suggest that a well-vascularized environment with a population of responsive cells needs to be restored in addition to delivering BMP for complete wound healing to occur using osteoinductive protein therapy.

Ex vivo gene therapy is an alternative tissue-regenerative approach that may be advantageous for healing compromised wounds. *Ex vivo* gene therapy can provide a period of sustained BMP production, thus enabling the host's wound to respond to the osteoinductive stimulus in a more robust fashion compared with when a single dose is delivered by protein therapy (Scaduto and Lieberman, 1999). Although there are craniofacial defect models showing success with *in vivo* approaches (Alden *et al.*, 2000; Lindsey, 2001), an *ex vivo* gene therapy approach has several advantages including direct delivery of the osteoinductive gene to the desired site, targeting of cells for gene delivery, supplying of cells that directly participate in the reparative process, and the potential to control the rate or extent of gene expression by using inducible delivery vectors (Scaduto and Lieberman, 1999). Because of these advantages, our laboratory has used the *ex vivo* approach for delivering genes to modulate bone regeneration. Although different types of cells can be genetically modified to express BMP (Musgrave *et al.*, 2000), fibroblasts were chosen because of the ease of harvest, isolation, and cell culture. Although fibroblasts lack intrinsic responsiveness to BMP-2 (Musgrave *et al.*, 2000), these cells are capable of converting into other tissue lineages, such as osteoblasts, when genetically modified (Rutherford *et al.*, 2002). Thus, in addition to providing the osteoinductive signal, these cells may also directly participate in the reparative process. Our study did not delineate the extent to which donor or recipient cells were involved in the bone-regenerative process.

The *ex vivo* gene therapy approach led to significant bone regeneration in this severely compromised, clinically relevant setting. Despite our findings that transduced fibroblasts were extremely radiosensitive at the radiation dose studied, *ex vivo* gene therapy provided a potent osteoinductive stimulus when examined 6 weeks after surgery. This *ex vivo* approach, however, was less effective in irradiated animals compared with those not radiated. Future studies will investigate whether bone regeneration in radiated defects eventually becomes equivalent to that in nonradiated defects at later time points. Strategies for enhancing bone regeneration initiated by *ex vivo* gene therapy in radiation-compromised wounds also need to be investigated. Some promising approaches include increasing the number of genetically modified cells loaded onto the scaffold, using an osteoconductive scaffold, or a scaffold that also allows growth factors to be released in a controlled manner (Oldham *et al.*, 2000; Richardson *et al.*, 2001; Sakiyama-Elbert *et al.*, 2001), delivery of multiple genes (Peng *et al.*, 2002), improving wound vascularity, or the use of radioprotective medications (Forrest *et al.*, 2002), and anabolic hormonal therapy (Schneider *et al.*, 2003).

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