Human Bone Morphogenetic Protein 2–Transduced Mesenchymal Stem Cells Improve Bone Regeneration in a Model of Mandible Distraction Surgery

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Background: Bone morphogenetic proteins (BMPs) are actively involved in ossification, and BMP-2 participates throughout the entire process. Gene therapy for bone regeneration using adenovirus-expressing BMPs has been successful in small mammals, but it has not been satisfactory in large mammals.

Methods: We generated a 3-component implant (3C graft) comprising autologous mesenchymal stem cells (MSCs), ex vivo transduced with an adenovirus vector–expressing BMP-2 and embedded in a demineralized human bone matrix (DBM).

Results: In vitro studies demonstrated vector-induced osteogenesis; osteoblast population and mineralization of the extracellular matrix were greater in the vector-transduced cultures than in the controls (nontransduced MSCs stimulated with osteogenic media were used as positive controls, and nontransduced MSCs served as a negative control). The 3-component grafts were used to fill osteotomies created by bone distraction surgery in mongrel dogs. Control groups comprised dogs with bone distraction alone and dogs with nontransduced MSC grafts. The radiography follow-up, performed 10 weeks after distraction, demonstrated a remarkable reduction in the consolidation period compared with controls. Postmortem mandibles submitted for anatomic and histologic analyses showed improved remodeling and bone maturation in the 3C-grafted dogs. Inflammatory infiltrates were not observed in any of the treated areas, and no liver toxicity was detected.

Conclusions: We demonstrated acceleration of osteogenesis in a dog model for bone distraction by using an implant of BMP-2 modified MSCs. These results are helpful for future clinical trials of mandible bone distraction.

Key Words: Osteogenesis, mesenchymal stem cells, BMP-2, adenoviral vector

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Bone morphogenetic proteins (BMPs) belong to the transforming growth factor β superfamily. Their main function is to promote bone formation by directing cell differentiation of mesenchymal stem cells (MSCs) to osteoblasts and osteocytes, which in turn build and renew the extracellular matrix.[1] More than 20 BMPs have been described so far,[2] and BMP-2, BMP-4, BMP-6, and BMP-9 are the main osteogenic inducers.[3] Bone morphogenetic proteins have been tested successfully in orthotopic and heterotopic rodent models of bone regeneration induced either by gene transfer or local infusion of recombinant proteins in vivo,[3,4] with outstanding results. However, gene therapy experiments have failed in large mammals, probably because of the immune responses against the adenoviral vector and its transgene product.[5] Cultured MSCs have proven osteoinductive activity.[6] These progenitor cells can differentiate more rapidly into bone-synthesizing cells if they are maintained in three-dimensional structures such as polymers, ceramics, or bone-derived matrices.[7,8] Some authors have reported that a residual amount of BMPs retained in bone-derived matrices is advantageous for bone implants.[9,10] Interestingly, the first osteogenic inducer described was demineralized bone matrix (DBM).[11] Accordingly, DBM can serve for 2 purposes in tissue engineering: as a scaffold support matrix and to stimulate bone induction. Bone distraction surgery is used frequently by surgeons to repair bone deformations.[12,13] In this surgery, the borders of a generated osteotomy are separated mechanically with a device implanted in the borders of the bone lesion until a desired distance is achieved, followed by a consolidation period of 12 to 16 weeks for complete restoration.[14]

We report on the development of a 3-component graft (3C graft) made up of autologous MSCs transduced ex vivo with adenovirus-expressing BMP-2 (Ad–CMV–BMP-2) and embedded in DBM. This engineered graft significantly decreased the consolidation period in an experimental model of bone distraction surgery in mongrel dogs.

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We present evidence of improved bone regeneration in terms of anatomic structure and radiographic follow-up of the healing tissue.

METHODS

Autologous MSCs Transduced Ex Vivo With Adenovirus-Expressing BMP-2

This vector was kindly provided by Dr. Christopher Evans and has been described elsewhere.[15] This ΔE1/E3 type 5 adenovirus vector contains the cytomegalovirus enhancer/promoter sequence (CMV) controlling the expression of the coding sequence for human BMP-2.

Isolation of MSCs and DBM

The autologous MSCs were isolated from humeral epiphyseal biopsy specimens of recipient dogs under sedation and local anesthesia. These biopsy specimens were grounded and digested with 0.25% trypsin solution plus 1 mg/mL collagenase, and MSCs were recovered by centrifugation and separated by flask adherence in Dulbecco modified Eagle medium containing 20% fetal bovine serum, 0.1% gentamicin, 1% amphotericin B, and 1% ampicillin-kanamycin. Cells were incubated at 37°C, 5% CO_2, and 95% humidity atmosphere. The medium was changed at the third day and incubated until 70% confluence (about 7–10 days). Adherent cells forming fibroblastoid colonies were visualized 5 days after seeding. Sometimes, an additional passage was required to obtain about 1.5×10^7 cells in total. The DBM was processed and lyophilized in a cGMP facility at Universidad Autónoma de Nuevo León (UANL).

Immunophenotyping

Cells were harvested at the first passage with phosphate-buffered saline (PBS) + EDTA and incubated for 15 minutes at 37°C, with scraper, and were washed once with PBS; 1×107 cells were counted, placed in PBS, and were incubated with monoclonal antibodies against CD271(LNGFR)-PE (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), MSCA-1(W8B2)–APC (Miltenyi Biotec GmbH), and CD45-FITC (Beckman Coulter, Brea, CA) epitopes at 4°C. Cells were washed with PBS supplemented with 0.5% bovine serum albumin and 2 mM EDTA and resuspended. Phenotyping was performed in a cytometer CyAn ADP model from Dako (Carpenteria, CA).

In Vitro Studies of Human BMP-2 Transduction in Cultured Dog MSCs

Mesenchymal stem cells (1×10^5 cells) were transduced for 3 hours with Ad–CMV–BMP-2 at 100 MOIs (multiplicities of infection) in 6-well plates. After transduction, cells were washed 3 times with PBS, and media was completely replaced. Nontransduced cells were used as negative controls, and nontransduced cells treated with 100 nM dexamethasone, 50 μM vitamin C, and 10 mM β-glycerol-phosphate (osteogenic media) were used as positive controls.

Type I collagen and osteocalcin were detected by Western blot analysis at day 32. Briefly, 100 μg of total protein extract in Laemmli buffer was subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane; blocking was performed with nonfat milk 5% Tris-buffered saline Tween 20. Primary rabbit polyclonal anti–human osteocalcin (dilution 1:1000) (Santa Cruz Biotechnology Inc, Santa Cruz, CA) and rabbit polyclonal anti–human type 1 collagen immunoglobulin G (IgG) (dilution 1:100) (Abcam plc, Cambridge, UK) were used to detect osteocalcin and type 1 collagen, respectively. Bound antibodies were detected with horseradish peroxidase conjugated with goat anti–rabbit IgG antibodies (1:3000) (Santa Cruz Biotechnology Inc) and donkey anti–rabbit IgG, respectively (by cross reaction), and revealed with diaminobenzidine.

To test in vitro bone generation, the same sets of experimental groups mentioned above were implemented including Ad–CMV–BMP-2–transduced dog MSCs at MOI = 100. Cells were incubated during 32 days and fixed in a solution of methanol-acetone in 1:1 ratio for the different experimental groups. Histological analyses for cell morphology, collagen deposition, and mineralization were performed at days 16 and 32 using Masson trichrome and von Kossa stains. Immunohistochemistry for type I collagen was performed to analyze the collagen composition of the extracellular matrix.

Matrix Scaffold and MSC Engrafting

Mesenchymal stem cells (1×10^5 cells) were transduced for 3 hours with Ad-CMV-GFP (green fluorescent protein) at MOI of 100 plaque-forming units/cell in T-25 cm^2 plate. After transduction, cells were washed 3 times with PBS, and media was completely replaced. Mesenchymal stem cells were incubated for 24 hours and then were harvested and seeded at 4×10^5 MSCs per well with 20 mg of DBM in a 96-well plate cast. Implants were incubated at 37°C, 5% CO_2/95% humidity atmosphere, and observed at 24, 48, and 72 hours.

In Vivo Studies

Healthy mongrel dogs weighing 20 to 25 kg used in these experiments were provided by the Municipal Antirabic Service and were adapted, maintained, and treated in the animal facilities of the School of Medicine, UANL, following the Mexican ordinances for the treatment of experimental animals (Norma Oficial Mexicana 062-ZOO-1999). The protocol was submitted and accepted by the Internal Review Board of the School of Medicine, UANL. To create the 3C grafts, 1.5×10^7 cultured dog MSCs were transduced for 3 hours at MOI of 81.1 PFU/cell and then were washed. Twenty-four hours later, cells were harvested with 0.25% trypsin solution, resuspended in 150 μL of serum-free Dulbecco modified Eagle media, and embedded in a similar volume of powder DBM in a 96-well plate cast. The control graft with MSCs was mock transduced with PBS and prepared according to the same protocol. Dogs were allocated in 3 groups (n = 3 each) for the mandible bone distraction and graft experiments because of the limited availability of healthy dogs for this study. Two groups received a graft (3C graft for group 1 and control graft for group 2), whereas the last did not receive any implant (group 3).

Bone Distraction Surgery

Distraction surgeries were performed under general anesthesia with ketamine-pentobarbital. A total corticotomy was performed surrounding the mandible, preserving bone marrow (around 3–5 mm deep), and an external distractor was placed creating a 1.0-cm defect. The mandible borders of the osteotomy were fixed with bone distractor devices. Grafts, constituted by 2 previously molded disks (0.37-cm^2 area by 1.0-cm thickness), were washed in PBS to remove vector excess before implantation and were engrafted inside the osteotomy defect. The borders of the osteotomy were covered with periosteum. Bone distraction was performed at 1 mm/d for 10 days.

Postsurgical Follow-Up

Imaging follow-up of the operated area was done by radiography every 2 weeks after the last distraction, during 10 weeks. For biosafety purposes, liver function tests were performed before and 2 weeks after surgery to analyze any possible toxicity associated to the use of adenoviral vectors. These included prothrombin time and the concentrations of albumin, globulin, total bilirubin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, lactic dehydrogenase, and γ-glutamyl transpeptidase.

Postmortem Studies

In the 10th week, dogs were killed by intravenous administration of pentobarbital-KCl solution. The treated bone areas of the mandible were recovered, fixed in 10% formaldehyde for 5 days, and demineralized in 5% HCl for 10 days. Paraffin-embedded sections were counterstained with hematoxylin and Masson trichrome stains to study bone formation, architecture, and inflammation.

Statistical Analysis

Data resulted from triplicate measurements. Parametric data were analyzed for significance using Student *t*-test with the SPSS version 13 software (SPSS-UK Ltd, St Andrews House, UK) and considering $P \leq 0.05$ for a significant difference.

RESULTS

Immunophenotyping

The MSC phenotype was confirmed by cytofluorometric analysis. The MSC isolated pool was constituted by a population of 68.20%, 76.49%, and 10.87% of CD271, MSCA-1, and CD45 cells, respectively. We conclude that percentages of MSCs positive for these markers were sufficient to confirm the phenotype of these cells for subsequent experiments.

In vitro Histology of the Cultured MSCs

Masson trichrome staining of Ad–CMV–BMP-2–transduced MSCs, MSCs grown in osteogenic media, and nontransduced MSCs showed secretion of type I collagen in the first 2 groups 16 days after transduction/stimulation. Cells grown in osteogenic media formed cell aggregates around collagen fibers. Production of type I collagen fibers was notorious in the Ad–CMV–BMP-2–transduced MSCs at day 32 (Fig. 1A). These observations were corroborated by anticollagen immunohistochemistry, which reveals remarkable extracellular accumulation of type I collagen fibers in the vector-transduced cells (Fig. 1C). The von Kossa staining also showed better calcification in -2–transduced cells at day 32 after transduction (Fig. 1B). To determine the in vitro engrafting of the cells in the DBM, Ad-CMV-GFP–transduced MSCs casted in 96-well plates demonstrate a relatively homogeneous engrafting of the cells, which was clearly evident at

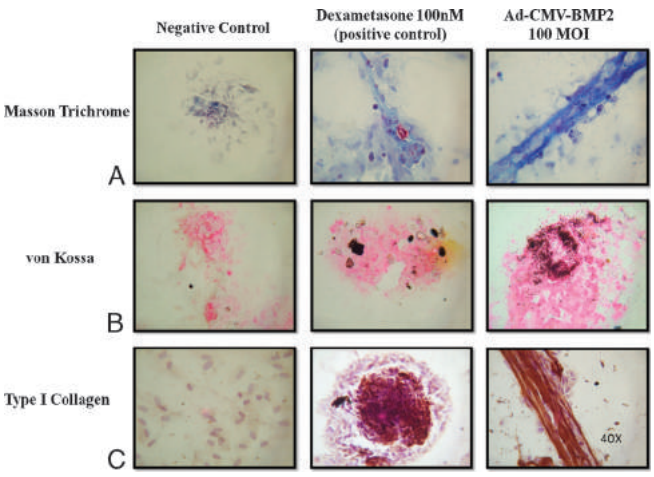

FIGURE 1. In vitro extracellular matrix production. A, Masson trichrome staining of MSCs transduced with Ad–CMV–BMP-2 showed active production of collagen fibers. B, Von Kossa staining showed increased mineralization in Ad–CMV–BMP-2–transduced in comparison with the positive and negative controls. C, Immunohistochemistry for type I collagen demonstrates formation of extracellular fibers in MSCs treated with Ad–CMV–BMP-2.

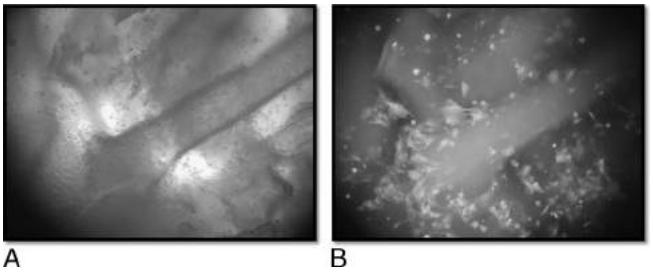

FIGURE 2. Demineralized bone matrix scaffold and MSC in vitro engrafting at 72 hours (magnification ×10). Casted MSCs transduced with Ad-CMV-GFP at 100 MOIs and embedded in the DBM scaffold. A, White light micrograph showing the DBM particles distribution into the matrix. B, Green fluorescent protein fluorescence micrograph. Several adherent cells with cytoplasmic projections engraft most of the DBM particle surface in relatively large areas of the same scaffold illustrated in A.

72 hours after implant generation (Fig. 2). A quantitative study to corroborate the accumulation of type I collagen in Ad–CMV–BMP-2–transduced cells was performed by Western blot analysis, as referred in the next section.

Western Blot Analyses

Osteocalcin and type I collagen were assessed in the same in vitro experimental groups described above at day 32. A densitometric analysis using GAPDH expression for assay normalization was performed (Phoretix 1D software, TOTALLAB, Newcastle upon Tyne, UK). Protein bands were measured in 3 independent experiments to calculate means and SEs. Increased production of osteocalcin was noticed in the BMP-2–transduced MSCs and in the cells cultured in osteogenic media at days 8, 16, and 32, without a significant difference between the vector-transduced and the osteogenic media–induced cells ($P = 0.35$). The presence of type I collagen was patent just in the vector-transduced MSCs at day 32 (Fig. 3).

In Vivo Studies

The radiographic follow-up demonstrated complete consolidation in mandibles treated with the 3C graft in the sixth week after

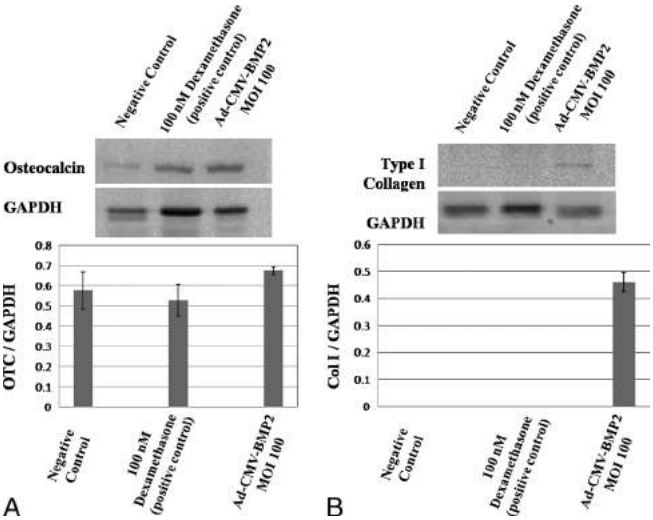

FIGURE 3. Western blot analyses for in vitro expression of osteocalcin and type I collagen. A, Osteocalcin and type I collagen expression was measured at day 32 after stimuli. A densitometric analysis using GAPDH expression for assay normalization (Phoretix 1D software) showed that osteocalcin expression was similar in dexamethasone stimulated and Ad–CMV–BMP-2–transduced MSCs. B, Type I collagen expression was restricted to the Ad–CMV–BMP-2–transduced MSCs.

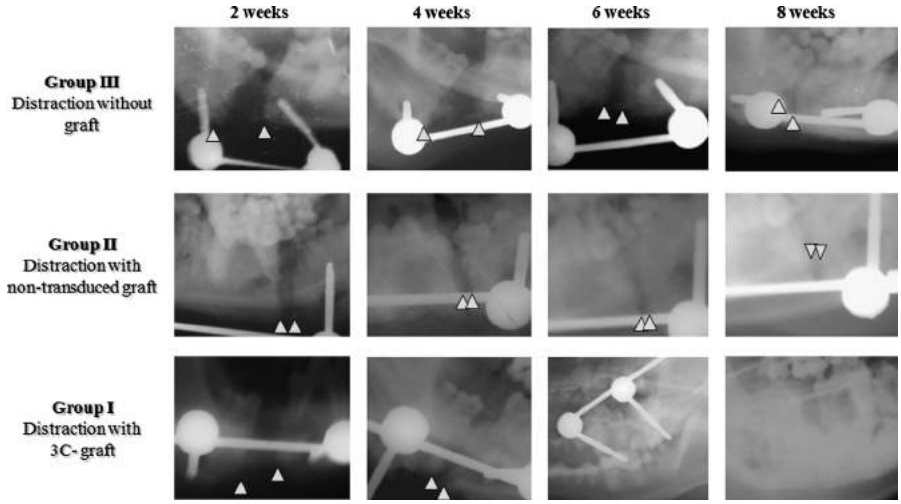

FIGURE 4. Radiographic evaluations at sixth and eighth weeks after surgery. Bone consolidation is observed in the 3C graft implanted group at week 6. Open defects are patent in the jaws of control treatment groups. Arrowheads: surgical edges of the bone distraction.

distraction, whereas the mandibles in the 2 other groups showed partial consolidations in the eighth week. These results indicate a remarkable reduction in the time needed for consolidation in the 3C-grafted group (Fig. 4).

Macroscopic postmortem analysis of the 3C-grafted mandibles performed 10 weeks after distraction showed a bone with almost normal aspect, in which the adhering surrounded tissue was easy to remove from the regenerated periosteum. Extensive areas of fibrous callus in the engrafted area were noticed in all nontransduced MSC-grafted jaws. Incomplete consolidation was evident in the nongrafted group, in which the mandibles showed the expected osteum callus usually observed during the evolution of distracted bone defects (Fig. 5A). Analysis of the hematoxylin-stained tissues from the treated areas showed reconstitution of mature bone in the mandibles treated with the 3C grafts, comprising periosteum, lamellar bone, and bone marrow. The extensive areas of fibrotic tissue were confirmed in the nontransduced MSC-grafted animals, and abundant and disperse cores of osteoblastic activity were observed. Regenerative lesions in the nongrafted tissues were characterized by large areas of osteoblastic activity and few trabecular zones. Masson trichrome staining confirmed the presence of newly formed bone marrow and trabecular bone, mainly in the 3C-grafted jaws, areas of diffuse osteoblastic activity in nongrafted mandibles, and active cores of bone synthesis in jaws treated with the nontransduced MSC grafts (Fig. 5B).

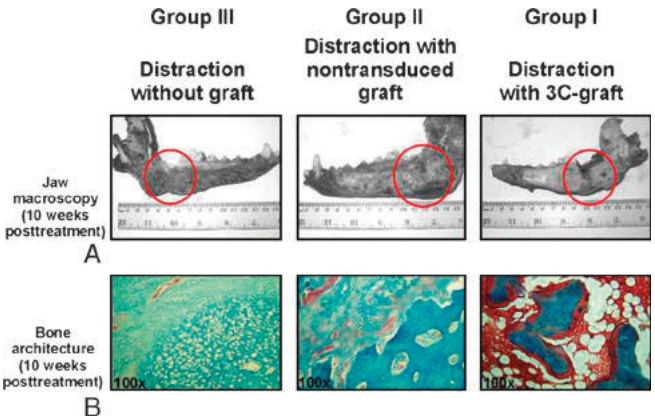

FIGURE 5. Postmortem studies. A, Macroscopic studies. Nonimplanted jaws show normal bone contraction in the distracted area with notable indentations in nonimplanted mandibles. The mandible implanted with the nontransduced graft shows extensive areas of fibrosis. The 3C-grafted bone demonstrates complete ossification and displays trabecular bone without fibrosis. B, Microscopic studies (Masson staining). Bone tissue samples from the distraction zone demonstrate widespread immature osteoblastic activity in nonimplanted jaws, cores of osteogenic activity mixed with fibrotic areas in the mandible implanted with nontransduced MSCs, and adequate bone remodeling, with lamellar and spongy bone structures, and minimal fibrosis in the mandible treated with the 3C graft.

Safety Studies

No signs of systemic or liver toxicity were detected in the blood tests in any of the treated groups during the study (Table 1).

DISCUSSION

Bone morphogenetic proteins, particularly BMP-2 and BMP-7, scaffold biomaterials, and MSCs are being tested alone or in combination for use in bone regeneration in fractures, vertebrae fusion, and distraction surgeries in different experimental models with variable results.[16] Human recombinant BMP-2 and BMP-7 are currently US Food and Drug Administration–approved drugs for restricted clinical uses,[17] but the short half-life limits their use in bone healing. Bone

TABLE 1. Laboratory Toxicity Test Performed Before and 2 Weeks After Mandible Surgery

Blood Test for Toxicity Test	Before Surgery	2 wk After Surgery
Total proteins, g/dL	6.63 ± 0.50	6.17 ± 0.23
Albumin, g/dL	2.83 ± 0.57	2.53 ± 0.32
Globulin, g/dL	3.80 ± 0.55	3.63 ± 0.32
Total bilirubin, mg/dL	0.10	0.10
Aspartate transaminase, IU/L	33.00 ± 12.52	24.00 ± 4.35
Alanine transaminase, IU/L	58.33 ± 31.89	43.33 ± 35.72
Alkaline phosphatase, IU/L	60.66 ± 13.27	67.33 ± 38.03
Lactic dehydrogenase, IU/L	419.33 ± 40.96	298.66 ± 84.00
γ-Glutamyl transpeptidase, IU/L	4.90 ± 3.10	0.10

morphogenetic protein 2 gene transfer, particularly with a nonreplicant adenoviral vector, can sustain production of the morphogenetic proteins for about 4 to 6 weeks. Although measurement of transduced BMP-2 concentration was not performed, our in vitro results demonstrate sustained osteogenic activity of this factor during the 32-day observation period.

The bone healing effects of BMP-2 gene transfer have been demonstrated in most experimental reports using rodents, but testing in big mammals such as dogs and sheep remains a challenge to probe the efficacy of BMP-2 in clinical gene therapy trials. Preliminary reports argue against the use of the adenoviral vectors.[18] We minimize the animal exposure to the adenoviral vector in the implant by washing it after overnight transduction.

Our in vitro experiments performed with Ad–CMV–BMP-2–transduced MSCs showed production of type I collagen fibers and osteocalcin and accumulation of calcium salts at 32 days after transduction/stimulation. Given the possible induction effect of the DBM, we expected that a graft incorporating this material as support for the Ad-2–transduced MSCs should improve bone regeneration in a large mammal model, because other materials such as alginate counteract the bone induction once the transduced progenitor cells are implanted.[19] The radiologic follow-up showed that the 3C graft implants in our dog model of mandible distraction reduced the healing time in comparison to the normal consolidation period. The gross macroscopy and histology of the regenerated bone, 10 weeks after distraction, showed that the 3C graft–reconstructed mature bone was of almost normal morphology. These features were not achieved in the nontransduced MSCs or with the standard distraction procedure in the same time. The induction of extensive fibrosis within the MSC-embedded matrix was a remarkable observation and argues against its use in bone regeneration in our model. Engraftment of the genetically modified implant immediately after its assembling may synchronize the right times to trigger the genetic and metabolic mechanisms required to initiate and sustain the osteogenic activity.

Although we acknowledge a limitation in the number of dogs in each experimental group as a limitation of this study, several other factors explored in our mongrel dog model argue in favor of the use of the 3C graft in large mammals. This model uses immunocompetent animals with wide genetic heterogeneity and previous exposure to the nonlaboratory environment. This is supposed to be a challenge in preclinical research. It is also important to mention that the treated area is a subject of frequent and strong mechanical pressure in carnivores and, as the first portion of the digestive tract, is located in a zone of strong immunologic activity.

Histological examination did not show evidence of a relevant inflammatory reaction or lymphocytes in the implanted zones. This may be explained either by the low immune exposure of the adenoviral vector and its transgenic protein by direct intraosseous administration, as claimed,[5] or by the washing of nonreplicative adenoviral remnants in the cultures and graft before the implantation. The use of autologous MSCs avoided graft rejection. Laboratory blood tests performed before and after implantation also argue in favor of the safety of this ex vivo approach.

In conclusion, the in vitro and in vivo tests showed the feasibility of regenerating normal bone tissue in the dog (a big mammal model), reducing the consolidation time to approximately half (6 weeks). The 3C graft accelerated bone consolidation, restored normal bone tissue and architecture (as compared with surgeries without the graft or with nontransduced MSCs matrix grafts), and induced no noticeable inflammatory response in the treated area. These experiments also demonstrate that the therapeutic approach is safe, probably because the washing of vector excess in the implant before the grafting reduces the presentation of vector to the immune system.

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