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Poly(lactide-co-glycolide)/hydroxyapatite delivery of BMP-2-producing cells: a regional gene therapy approach to bone regeneration

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Abstract

Currently, functional treatment of fracture non-unions and bone loss remains a significant challenge in the field of orthopaedic surgery. Tissue engineering of bone has emerged as a new treatment alternative in bone repair and regeneration. Our approach is to combine a polymeric matrix with a cellular vehicle for delivery of bone morphogenetic protein-2 (BMP-2), constructed through retroviral gene transfer. The objective of this study is to develop an osteoinductive, tissue-engineered bone replacement system by culturing BMP-2-producing cells on an osteoconductive, biodegradable, polymeric-ceramic matrix. The hypothesis is that retroviral gene transfer can be used effectively in combination with a biodegradable matrix to promote bone formation. First, we examined the in vitro attachment and growth of transfected BMP-producing cells on a PLAGA–HA scaffold. Second, the bioactivity of the produced BMP in vitro was evaluated using a mouse model. It was found that the polymer-ceramic scaffold supported BMP-2 production, allowing the attachment and growth of retroviral transfected, BMP-2-producing cells. In vivo, the scaffold successfully functioned as a delivery vehicle for bioactive BMP-2, as it induced heterotopic bone formation in a SCID mouse model.

Keywords: Gene therapy; Biodegradable polymer; Bone morphogenetic protein; Delivery system; Bone repair

1. Introduction

Bone is one of the most commonly repaired organs of the body [1]. Currently, functional treatment of fracture non-unions and bone loss associated with trauma, cancer and revision total joint arthroplasty remains a significant challenge in the field of orthopaedic surgery. Existing treatment modalities include autogenous, allogeneic and synthetic bone grafts, with autograft being the clinically preferred choice of bone grafting material. Autografts are not limited by risks of disease transfer and histo-incompatibilites, two major disadvantages commonly associated with allografts. However, autograft tissue is of limited supply. Synthetic grafts based on metals and some ceramics have markedly different mechanical properties when compared to human bone tissue, and this incompatibility often results in implant failure and consequently, revision surgery. Moreover, long-term implant stability is poor, and synthetic grafts routinely fail to meet the demands of an aging yet still active population.

Due to the aforementioned limitations associated with available bone grafts, tissue engineering of bone has emerged as a new treatment alternative in bone repair and regeneration. Tissue engineering can be defined as the application of biological, chemical, and engineering principles towards the repair, restoration or regeneration of tissues using cells, factors, and biomaterials alone or in combination [2]. Using biodegradable, polymeric materials with known biocompatibility, several researchers have fabricated porous, bioresorbable scaffolds for bone regeneration [3–5]. In our laboratory, we have produced biodegradable poly(lactide-co-glycolide)–hydroxyapatite

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(PLAGA–HA) composites, which have been shown to support the attachment, growth, and phenotypic expression of rat osteoblasts in vitro [5–8]. A porous implant promotes the in-growth of bone tissue into the matrix, and it functions by providing initial structural support while promoting implant integration with surrounding bone. As the polymer matrix degrades, new bone tissue gradually infiltrates the structure and eventually, the implant is completely replaced by bone and is capable of supporting physiological level loads.

In addition to the use of biodegradable scaffolds, cells, factors and other biological moieties can be added to the matrix to promote and expedite bone formation. A number of different growth factors, including bone morphogenetic proteins (BMPs), transforming growth factor β, platelet-derived growth factor, fibroblast growth factor and insulin growth factor have been shown to stimulate bone growth, collagen synthesis, and fracture repair both in vitro and in vivo [9–13]. In particular, BMPs are osteoinductive proteins originally identified in demineralized bone [14]. Wang et al. first isolated and purified recombinant human BMP-2A in 1990, and it was shown to induce cartilage and bone formation [15]. Moreover, BMP-2 and 7 have been reported to be osteoinductive, and when delivered with a carrier substance, they have elicited the healing of bone defects in a variety of animal models [15–18].

Clinically, a fracture non-union site has already been compromised by poor bone stock, marginal vascularity and extensive formation of scar tissue. Thus, a single exposure to an exogenous growth factor may not be sufficient to stimulate and sustain adequate bone growth. Moreover, Lucas et al. showed that the incorporation of a water-soluble BMP mixture into a synthetic polymer matrix promoted chondrogenesis and osteogenesis when implanted ectopically in vivo. However, when the BMP mixture was administered alone, this effect was not found [19]. These results suggest that the biological activity of BMP may be influenced by properties of the carrier system utilized for its delivery.

One of the major challenges facing the development of BMP-synthetic scaffolds is developing methods to successfully incorporate sufficient amount of BMPs in the matrix without compromising its bioactivity, as these proteins are highly sensitive to thermal processing, sterilization, and prolonged exposure to solvents and chemicals. Our approach is to combine a polymeric matrix with a cellular vehicle for BMP delivery, constructed through retroviral gene transfer. The objective of this study is to develop an osteoinductive, osteoconductive, tissue-engineered bone replacement system by combining BMP-2 producing cells with an osteoconductive, biodegradable, polymeric-ceramic matrix.

In addition to being a supporting structure, this system would function as a delivery vehicle of osteoinductive BMP-2 for bone regeneration. In a previous study, a BMP-2-producing cell line using retroviral gene transfer was successfully propagated, and these cells were found to induce bone formation in vivo [20]. The current study will first examine the in vitro attachment and growth of transfected BMP-producing cells on a PLAGA–HA composite scaffold. Next, this study will evaluate the bioactivity of the produced BMP-2 in vivo using a SCID mouse model. Our hypothesis is that retroviral gene transfer can be used effectively in combination with a biodegradable matrix to promote bone formation. In particular, we hypothesize that a PLAGA–HA composite will support cell attachment, growth and BMP-2 production in vitro and will serve as a delivery vehicle for bioactive BMP-2 in vivo.

2. Materials and methods

2.1. Fabrication of poly(lactide-co-glycolide)/ hydroxyapatite (PLAGA–HA) composites

The co-polymer poly(lactide-co-glycolide) (PLAGA, lactide : glycolide ratio = 50 : 50, M_w = 50,000; American Cyanamid) was dissolved in methylene chloride to produce a polymer weight to solvent volume ratio of 0.5 g/ml. Hydroxyapatite particles (HA, Howmedica, East Rutherford, NJ), 74–104 μm in size were added to the polymer solution, resulting in a PLAGA : HA weight ratio of 1:1. This composite mixture was cast into a chilled Teflon mold and placed in a -70°C freezer for 48 h. The PLAGA–HA composite sheet was then dried for 24 h, and lyophilized for an additional 24 h in order to completely remove any residual solvent. After lyophilization, a #7 cork borer was used to bore the film into composite discs of 8 mm in diameter and 2 mm in thickness. The discs were sterilized under UV light for 24 h on each side. The discs were characterized by scanning electron microscopy (SEM) and energy dispersive X-ray analysis.

2.2. Preparation of retroviral vector and BMP-2-producing cell line

The cDNA for BMP-2 (Genetics Institute, Cambridge, MA) was introduced into a retroviral vector containing the Neomycin resistant gene (MSRαTKNeo). A 293 T cell hyper-expression system was used to produce the retrovirus, where these cells were transfected with both the BMP-2 construct and a retrovirus packaging vector to produce helper-free retroviral stocks.

The retroviral stocks were subsequently used to infect W-20 cells, a murine stromal cell line (a gift from S. Tsai, Hutchinson Cancer Center, Seattle, WA). W-20 cells were selected for this study because they expressed the common markers for the osteoblastic phenotype, i.e., increased alkaline phosphatase activity and osteocalcin

synthesis when exposed to exogenous recombinant BMP-2 in tissue culture. The cells were cultured at 37°C and 5% CO_2 in Dulbeco's modified Eagle's medium (DMEM, GIBCO BRL, Grand Island, New York) supplemented with 10% heat-activated fetal calf serum (Omega, Tarzana, CA).

In particular, a retrovirus stock (Retro-BMP-2, retroviral titer $= 1 \times 10^6$) was used to infect the W-20 cells. W-20 cells were plated in 10-cm dishes and were infected with the BMP-2 retrovirus or with the BMP-2 retroviral vector alone (negative control) for 4 h. This infected cell line was then selected for resistance to the Neomycin analog G-418.

2.3. Culturing of BMP-2 producing cells on PLAGA–HA matrix

W-20 cells transfected with the retroviral BMP-2 gene were plated on the PLAGA–HA composites at the density of 3×10^5 cells per disc, and grown in Dulbeco's Modified essential medium (DMEM) supplemented with 10% fetal calf serum and cultured at 37°C and 5% CO_2. The cells were grown on the composite discs for up to one week. Cell attachment morphology and growth behavior were examined using SEM.

2.4. Western blot to assess BMP-2 production

W-20-BMP-2-producing cells were plated on the PLAGA–HA composites, and grown in DMEM plus 10% fetal calf serum. After 24 h of culture, the supernatants (20 ml) were collected and then purified with 90 μl of heparin-sepharose beads. The beads were incubated for 3 h at room temperature, and were then centrifuged and resuspended in phosphate-buffered saline. The beads were boiled at 100°C for 5 min. Loading buffer was then added to the beads, and after centrifugation, the extract was loaded onto a 10% polyacrylamide gel.

Transfer to nitrocellulose was performed over 6 h at 200 mA following polyacrylamide gel electrophoresis. Filters were incubated in blocking buffers (10% non-fat dry milk, 1% 1.0 M *Tris* buffer at pH 7.4, and 3% 5.0 M NaCl) at room temperature for 30 min. Blots were then incubated overnight at 4°C with 1 μg/ml anti-BMP-2 mouse monoclonal antibody (Genetics Institute Cambridge, MA). After three washes with a solution consisting of 1% 1.0 M *Tris* buffer (pH 7.4), 10% 5.0 M NaCl and 0.1% Tween-20, the filters were incubated with a sheep anti-mouse HRP conjugated secondary antibody (Amersham, Inc.). The filters were then rinsed three times in 10% 1.0 M *Tris* buffer (pH = 7.4), 10% 5.0 M NaCl, and 0.1% Tween-20. The filters were immersed for one minute in an equal volume of the enhanced chemiluminescence detection reagents 1 and 2 (Amersham, Incorporated), and then exposed to X-ray film.

2.5. In vivo bone induction at a heterotopic site

Institutional Animal Care and Use Committee (IACUC) approval was obtained before beginning all animal studies. W-20 BMP-2-producing cells (3×10^5) were plated on the composite discs and grown in DMEM with 10% fetal calf serum for 7 days prior to implantation. In vivo bone formation was assessed in a heterotopic, rodent assay system. Severe combined immune deficient (SCID) mice were anesthetized with an intramuscular injection of ketamine (1.5 mg) and xylazine (0.3 mg) and were prepared for aseptic surgery. A 2-cm incision was made on the lateral aspect of both thighs. The quadriceps musculature was identified, and a 1.5 cm incision was made. The implant was inserted into the pocket in the quadriceps muscles of each leg. The incisions were closed with wound clips. Three experimental groups were implanted, W-20-BMP-2-producing cell cultured with the PLAGA–HA composite ($n = 5$), W-20-non-transfected (negative control, $n = 2$) cultured with the PLAGA–HA composite, and PLAGA–HA composite loaded with 3 μg of rhBMP-2 protein ($n = 2$). The animals were sacrificed one month after implantation, and the samples were analyzed radiographically and histologically.

2.6. Radiography and histology

At the time of sacrifice, the quadriceps muscles were removed. Radiographs of the specimens were taken. For histology, the samples were fixed in 40% ethanol at 4°C. Following dehydration and cleaning, the samples were embedded in poly(methylmethacrylate), and subsequently cut into 5–6 μm sections. The sections were mounted on glass slides, and treated with modified Goldner's trichrome stain.

3. Results

3.1. Cell attachment and production of BMP-2

SEM examination of cells grown on the PLAGA–HA composites revealed extensive cellular attachment and growth on the discs during the 1-week period. Cells retained the customary spindle-shaped morphology, and appeared to spread over the surface of the composite (Fig. 1).

The presence of BMP-2 was detected in the culture supernatant. Western Blot analysis of the supernatant demonstrated the continued production of Rh-BMP-2 by cells attached to the PLAGA–HA composites, as seen in the positive staining in the gel shown in Fig. 2.

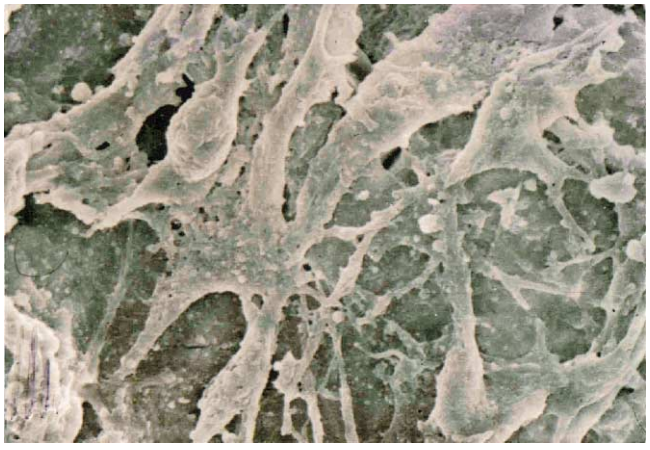

Fig. 1. Scanning electron micrograph of W-20-BMP-2-producing cells grown on poly(lactide-co-glycolide)-hydroxyapatite (PLAGA–HA) composites. Note that the cells adapted osteoblast-like morphology and were found throughout the matrix surface.

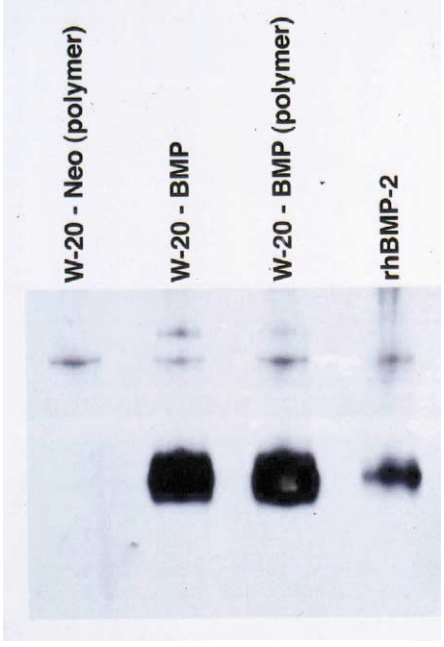

Fig. 2. BMP-2 production confirmed by western blot analysis of the supernatant collected from in vitro culture of transfected W-20 cells on PLAGA–HA composites. W20-BMP-2-producing cells grown on PLAGA–HA composite (W-20-BMP (polymer)) exhibited a pronounced band, while the negative control (W-20-Neo (Polymer)), W-20 cells not transferred with BMP-2 vector did not express the protein when grown on the polymer.

3.2. Radiographic and histological analyses

Radiographs and histological analysis of the in vivo samples confirmed the presence of bone formation in two out of five W-20-BMP-2-producing cells-composite constructs studied. Fig. 3 compares the radiographs of the three types of implants studied. Specifically, Fig. 3a is of W-20-BMP-2-producing cell grown on PLAGA–HA composite (Fig. 3a), and in Fig. 3b, W-20-non-transfected cells grown on PLAGA–HA composite, and in Fig. 3c, PLAGA–HA composite loaded with 3 μg of rhBMP-2 protein**.** Heterotopic bone formation was observed on the composites seeded with W-20-BMP-2 producing cells (Fig. 3a) and the group loaded with rhBMP-2 (Fig. 3c). The negative control (non-transfected cells plus composite) did not induce any bone formation.

The radiographic results were confirmed by histological analysis, where sections of the BMP-2 producing cell combined with PLAGA–HA composite showed positive evidence of heterotopic bone formation (Fig. 4a). Bone was found to form surrounding the PLAGA–HA composite disc, represented by a thin mineralized layer located on the surface of the composite. In contrast, sections taken from the negative control (Fig. 4b) did not reveal any bone formation around the implant.

4. Discussion

By combining biodegradable, PLAGA–HA composites with BMP-2-producing cells transfected via an ex vivo gene-transfer strategy, we have successfully constructed a BMP-2 delivery system with demonstrated bone regeneration potential. In vitro, BMP-2-producing cells rapidly attached and adapted to the degradable PLAGA–HA scaffold. These cells continued the production of BMP-2 on the composite discs. Furthermore, the synthesized BMP-2 remained bioactive in vivo and induced heterotopic bone formation in SCID mice. The results of this study demonstrate that BMP-producing cells can be grown on a PLAGA–HA carrier, and implantation of this tissue-engineered, cell-carrier system can induce bone formation in vivo. In vitro analysis of the cell/composite system revealed that the transfected W-20 cells attached, proliferated, and produced BMP-2 on the surface of a bioresorbable carrier. Furthermore, the in vivo subcutaneous implantation of the BMP-2 cells grown on the PLAGA–HA carrier resulted in heterotopic bone formation, as active BMP-2 continued to be synthesized by the transfected cells and effectively delivered the protein to the defect site.

The primary advantage of this approach when compared to delivery of BMP alone is that the continuous production of BMP by cells grown and supported on a biodegradable matrix may provide the much needed long-term osteoinductive and chemotactic stimuli, which can result in clinically significant bone formation. Moreover, it may become possible to modulate the rate of bone synthesis. Although there is no direct evidence of cell viability after implantation, the formation of bone tissue suggests that these cells remained functional. In a recent study, Bellincampi et al. labeled rabbit fibroblasts seeded on a collagen scaffold with red fluorescent dye having a half-life of more than 100 days [21]. The labeled fibroblast-collagen construct was autogenously

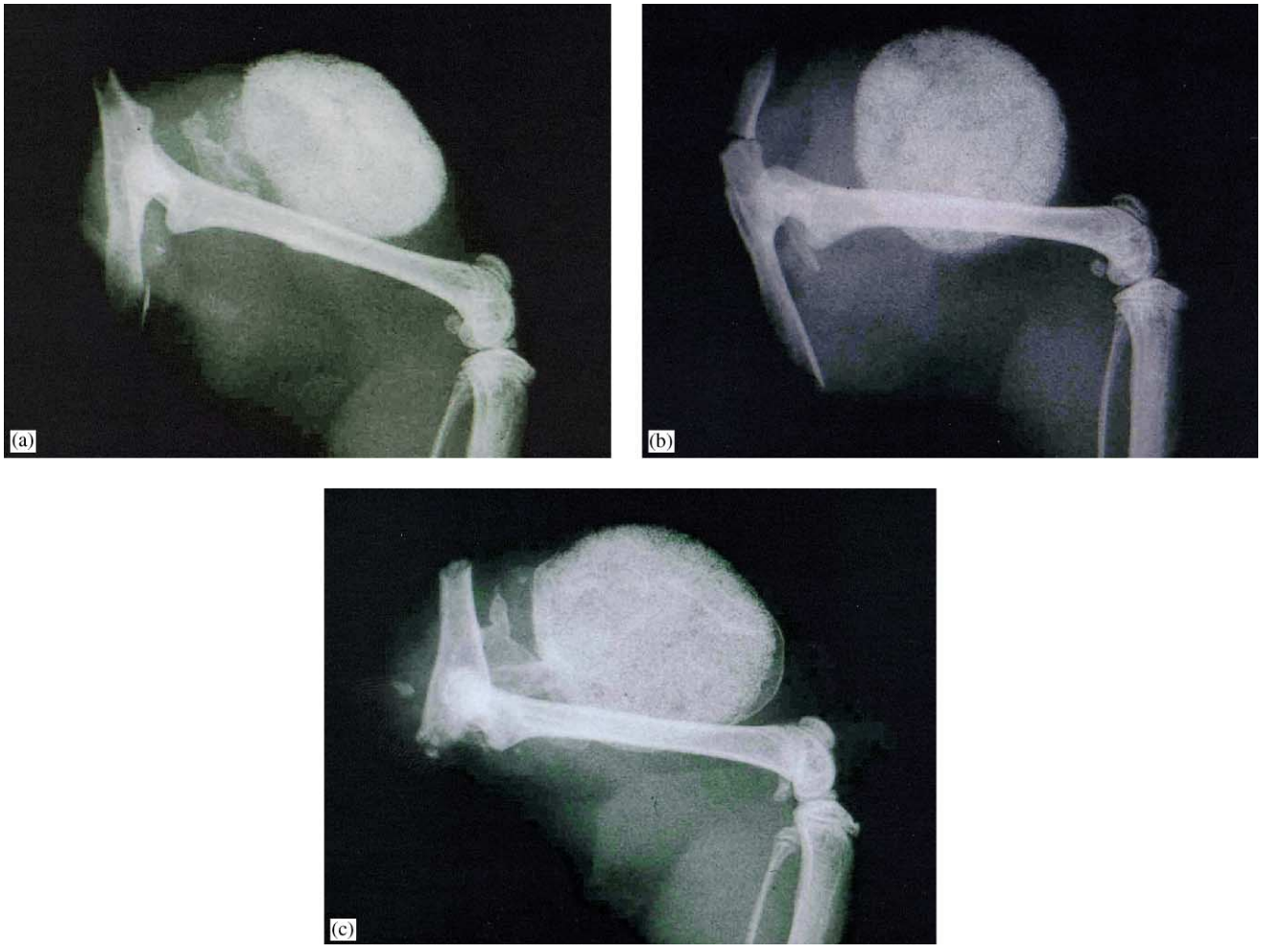

Fig. 3. (a) Radiograph of PLAGA–HA cultured with transfected BMP-2-producing cells and implanted in SCID mice for 1 month. An area of radio-density adjacent to the polymer composite is seen, representing regions of heterotopic bone formation. (b) Radiograph of PLAGA–HA with non-transfected W-20 cells implanted in SCID mice for 1 month. No regions of radio-density adjacent to the polymer composite are found. (c) Radiograph of PLAGA–HA loaded with 3 μg of the rhBMP-2 implanted in SCID mice for 1 month. An area of radio-density adjacent to the polymer composite is seen, representing regions of heterotopic bone formation.

implanted in rabbits for up to 8 weeks, in order to evaluate the fate of the seeded fibroblasts. The authors reported that these cells remained viable for as long as 6 weeks after implantation. The presence of new bone formation observed in vivo in this study suggest that the W-20 cells may remain viable, and are able to produce BMP in a sustained fashion.

Regional gene therapy involves the delivery of growth factors to a selected anatomical site, and it has the potential to be used in humans to enhance tissue formation and organ repair. One of the goals of this study was to determine whether regional gene therapy through ex vivo retroviral gene transfer could be used effectively in combination with a biodegradable matrix to promote bone formation. Based on our results, it is apparent that cells transfected with a retroviral BMP-2 construct were able to induce bone formation two weeks after implantation.

Clinically, retroviral and adenoviral vectors have both been used in gene therapy. A retroviral vector has specific advantages for ex vivo gene therapy, where isolated cells are propagated in culture, genetically modified by retroviral infection and subsequently transplanted into a recipient [22]. Adenovirus-based gene therapy has been utilized to treat bone defects [20]. Lieberman et al. demonstrated the ability of a W-20 stromal cell line infected with an adenovirus and expressing recombinant human BMP-2 cDNA to secrete biologically active BMP-2 [20]. These cells induced heterotopic bone formation when implanted into the quadriceps muscles of SCID mice.

Adenovirus-based vectors can infect both dividing and non-dividing cells, which results in a high level of transient gene expression. However, the immunogenic response to adenoviral proteins produced concurrently with the transgenic products may limit gene expression in vivo. Yang et al. found that the synthesis of adenoviral gene products stimulated an immune response to the infected cells, which resulted in a loss of therapeutic gene expression 1–2 weeks after injection

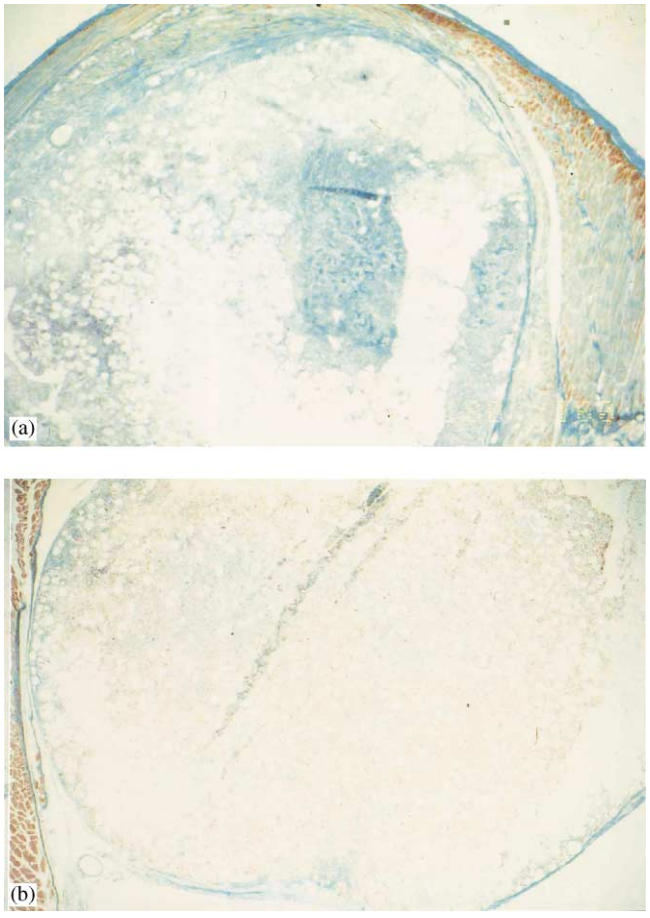

Fig. 4. (a) Histological analysis of implant of PLAGA–HA with tranfected W-20 cells revealed the formation of new bone is SCID mice after 2 weeks of implantation. (b) Histological section of implant of PLAGA–HA composite with non-transfected W-20 cells after implantation of SCID mice for 2 weeks. Note that no new bone formation is visible.

[23]. Retroviral vectors can be permanently integrated into the genome of infected cells, and consequently expressing the therapeutic gene for the life of the transfected cell. This is an attractive strategy as a therapy to treat larger bone defects, as sustained gene expression would provide continuous healing. Further investigations are needed to compare the efficiency of adenoviral and retroviral infected BMP-2-producing cells delivered using PLAGA–HA composites in order to design the optimal system for gene therapy.

The results of this study indicated that the combination of BMP-producing cells and a bioresorbable polymeric carrier might prove to be an effective alternative to conventional bone grafting techniques. The advantage of a polymeric carrier system lies in its ability to provide the critical, initial structural support for the implant system, and equally important, the necessary scaffold for osteoblastic growth and anchorage. In a previous study, Lieberman et al. examined the ability of BMP-2-producing rat bone marrow cells to induce healing of critical-size femoral defects in Lewis rats [24]. The authors found that after treatment with BMP-producing cells, bone formed after 2-month implantation demonstrated lower torsional stiffness when compared to intact femora. We believe implanting a polymer composite matrix with BMP-2-producing cells may enhance the overall mechanical stability and strength of the defect site. Composite discs were utilized in this study, however, we have fabricated 3-D, PLAGA–HA composites with mechanical properties similar to those of trabecular bone [2]. Future study will examine the ability of the BMP-2-producing cell combined with porous, 3-D polymeric composites to induce biological bone formation in a vertical size defect model.

This study is one of the first efforts in administering gene therapy through transfected cells combined with biodegradable, PLAGA–HA composite delivery systems, with the long-term goal of inducing significant and timely bone repair and formation. There remain still many questions and challenges in this approach, and we hope to address them in future studies. Specifically, the mechanical properties of the PLAGA–HA system must be evaluated as a function of biological mineralization and polymer degradation. In addition, the fate of the BMP-2-producing murine cells, the duration of BMP production in vitro as well as in vivo and potential pathogenic effects of the retroviral vector must be understood in order to optimize bone regeneration in vivo.

5. Conclusions

A polymer-ceramic scaffold supported BMP-2 production, and promoted the attachment and growth of retroviral transfected, BMP-2-producing cells. In vivo, the scaffold successfully functioned as a delivery vehicle for bioactive BMP-2, as it induced heterotopic bone formation in a SCID mouse model.

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