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Biologic effect and immunoisolating behavior of *BMP-2* gene-transfected bone marrow-derived mesenchymal stem cells in APA microcapsules

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Abstract

We investigated the encapsulation of *BMP-2* gene-modified mesenchymal stem cells (MSCs) in alginate-poly-L-lysine (APA) microcapsules for the persistent delivery of bone morphogenic protein-2 (BMP-2) to induce bone formation. An electrostatic droplet generator was employed to produce APA microcapsules containing encapsulated *β-gal* or *BMP-2* gene-transfected bone marrow-derived MSCs. We found that X-gal staining was still positive 28 days after encapsulation. Encapsulated *BMP-2* gene-transfected cells were capable of constitutive delivery of BMP-2 proteins for at least 30 days. The encapsulated *BMP-2* gene-transfected MSCs or the encapsulated non-gene transfer MSCs (control group) were cocultured with the undifferentiated MSCs. The gene products from the encapsulated BMP-2 cells could induce the undifferentiated MSCs to become osteoblasts that had higher alkaline phosphatase (ALP) activity than those in the control group ($p < 0.05$). The APA microcapsules could inhibit the permeation of fluorescein isothiocyanate-conjuncted immunoglobulin G. Mixed lymphocyte reaction also indicates that the APA microcapsules could prevent the encapsulated *BMP-2* gene-transfected MSCs from initiating the cellular immune response. These results demonstrated that the nonautologous *BMP-2* gene-transfected stem cells are of potential utility for enhancement of bone repair and bone regeneration *in vivo*.

Keywords: Alginate-poly-L-lysine (APA) microcapsules; Mesenchymal stem cells (MSCs); Bone morphogenic proteins (BMPs); Immune protection

Bone morphogenic protein-2 (BMP-2) is a member of the transforming growth factor-β superfamily and has been reported to be a potent inducer of osteogenic differentiation *in vitro* [1–3] and of endochondral bone formation *in vivo* [4,5]. We have previously described a *BMP-2* gene therapy strategy mediated by bone marrow-derived mesenchymal stem cells (MSCs) or adipose tissue-derived stromal cells (ADSCs) to enhance bone regeneration. Delivery of the engineered cells *in vivo* resulted in new bone formation in ectopic sites and the repair of bone defects in rats, goats, and canines [6–8]. Both the engrafted cells and the host cells could be induced to differentiate to bone-forming cells by autocrine or paracrine secretion of recombinant human BMP-2 (rhBMP-2) from the engrafted cells.

In the above study, the autologous cells were engineered to produce BMP-2 proteins *in vivo*. Because the isolation and proliferation of MSCs from bone marrow is time-consuming, the clinical application of autologous cell-based *BMP-2* gene therapy cannot be used for most fracture or trauma patients who need emergency surgery. Nonautologous cells are a better choice but carry the risk of inducing immunologic rejections. Adenovirus vectors also could induce activation of both the innate and the adaptive parts of the recipients' immune system when applied *in vivo*, which may limit gene expression *in vivo* [9,10].

Many researchers have proposed that transplantation of microencapsulated universal cell lines would be useful for

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the delivery of bioactive molecules *in vivo* [11]. The microcapsule membrane is fabricated so that its permeability permits the exit of the recombinant product and allows glucose and other nutrients to diffuse freely through the membrane but excludes the larger immune mediators such as complement, lymphocytes, and macrophages responsible for the destruction of allogeneic cells [12]. This immunoisolation ensures that the same recombinant encapsulated cell line can be implanted into different affected individuals to deliver the desired product, potentially reducing the cost and increasing the safety of gene therapy. This approach has been applied traditionally in allogeneic or xenogeneic tissue implants, such as islet cells for treatment of diabetes, hepatic cells for liver failure, and chromaffin cells for pain control. The efficacy of this approach for treating classical diseases has been proved in several murine models, including dwarfism [13], lysosomal storage disease [14], hemophilia B [15], and cancer [16]. Recently, some reports have indicated that microcapsules could create a three-dimensional microenvironment that would provide a niche for stem cell growth [17–20].

The most commonly applied materials to prepare the microcapsules is the alginate-poly-L-lysine (APA) system [12,17,18]. In our study, *BMP-2* gene-modified rat MSCs were encapsulated in APA microcapsules. Both the activity of secreted BMP-2 proteins and the immunoisolation behavior of the microcapsules were investigated *in vitro*.

Materials and methods

Cell culture and transfection of mesenchymal stem cells with BMP-2 gene. Bilateral femurs of Sprague–Dawley rats were recovered by dissection, and the soft tissues were detached aseptically. Metaphyses from both ends were resected, and bone marrow was collected by flushing the diaphyses with 10 mL of α-minimal essential medium ([α-MEM] Gibco Life Technology, Gaithersburg, MD, USA) containing 10% (volume to volume) heat-inactivated fetal bovine serum ([FBS] Hyclone, Logan, UT, USA) and antibiotics (penicillin, 100 U/mL; streptomycin, 100 μg/mL). Single-cell suspensions were obtained by drawing bone marrow into a syringe through a 22-gauge needle. Confluent MSCs were released by 5-min exposure to a solution of 0.05% trypsin containing 0.02% ethylenediaminetetraacetic acid (EDTA) and passaged at a 1:3 split. Passage 2 MSCs were incubated with the lysate of the recombinant adenovirus carrying the human BMP-2 gene (Adv-BMP-2) or the recombinant adenovirus carrying the bacterial β-galactosidase gene (Adv-β-gal, served as the reporter gene) at 100 multiples of infection at 37 °C overnight. The Adv-BMP-2 and Adv-β-gal were constructed by *in vivo* homologous recombination in 293 cells between the expression vector pAC-CMVBMP-2 and the fragment of Ad5 genomic DNA as previously described [21]. Infected cells were continuously cultured in complete α-MEM with FBS after removal of transfection medium.

Microencapsulation of recombinant mesenchymal stem cells. Sodium alginate of low viscosity (M type) and poly-L-lysine (PLL) with a molecular weight in the range of 15,000–30,000 amu were obtained from Sigma (St. Louis, MO, USA). The sodium alginate was purified according to the method proposed by de Vos et al. [22]. APA microcapsules were produced by a special high-voltage electrostatic microcapsule generator, as previously described [23], with minor modifications. Briefly, a suspension of cells at a concentration of 3×10^6/mL was mixed with 1.5 g/mL alginate in a syringe and extruded as droplets through a 27-gauge needle with a syringe pump. The gelled droplets were collected in a 1.1% $CaCl_2$ solution. The outer alginate layer was cross-linked with PLL for 6 min and then coated with alginate. The core of unpolymerized alginate was dissolved with sodium citrate for 5 min to yield microcapsules with the cells suspended within. All the above washing procedures were performed at 4–10 °C. The collected microcapsules were cultured in the dish at 37 °C for further determination.

X-gal staining of the encapsulated cells. X-gal staining was used to indicate the viability of the Adv-β-gal-infected MSCs in APA microcapsules at 4, 7, 14, and 28 days. The microcapsules were transferred to a 15-mL tube and fixed with 0.5% glutaraldehyde. After being washed three times with phosphate buffer solution, the microcapsules were treated at 37 °C overnight with X-gal staining agents (Shanghai Sangon, Shanghai, China) that contained 1 mg/mL X-gal, 35 mM $K_3Fe(CN)_6$, 35 mM $K_4Fe(CN)_6{\cdot}3H_2O$, and 2 mM $MgCl_2$.

Quantification of the BMP-2 proteins released from the encapsulated cells. Five aliquots of encapsulated BMP-2 gene-transfer MSCs were cultured in 15 mL α-MEM with 2.5×10^6 cells in each aliquot for 30 days. The same amounts of encapsulated non-gene transfer MSCs served as the control. Every 2 days, the supernatant was collected and the medium was completely changed. The concentration of BMP-2 in the supernatant was determined using a Quantikine BMP-2 Enzyme-Linked Immunosorbent Assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA). The assay was performed according to the protocol recommended by the manufacturer. BMP-2 concentration was determined by comparison with a standard curve.

Evaluation of the biologic effect of gene products secreted from the encapsulated cells. The coculture system (cell culture insert, BD, Falcon) was used to determine the biologic effect of gene products secreted from the encapsulated BMP-2 gene-transfected MSCs on the differentiation of the stem cells. The permeable membrane (pore size: 7 μm) is attached to the bottom end of a cell culture insert, which permits the diffusion of media components and gene products. The encapsulated BMP-2 gene-transfected MSCs (experimental group) or the encapsulated non-gene transfer MSCs (control group) were cocultured with the undifferentiated MSCs in the two 6-well plates with 1×10^6 encapsulated cells and 1×10^6 undifferentiated stem cells in each well. Every 2 days, 2 mL α-MEM were changed. After 10 days, the cells in the well were washed twice with Tris-buffered saline (50 mM Tris and 150 mM NaCl; pH 7.4) and lifted with a scraper. Harvested cells were lysed by sonicate. Alkaline phosphatase (ALP) activity in the cell lysate was assayed at 37 °C in buffer containing 0.1 M 2-amino/2-methyl/1-propanol, 10 mM $MgCl_2$ (pH 10.3), and 10 mM *p*-nitrophenylphosphate as a substrate. ALP enzyme activity was expressed as micromoles of *p*-nitrophenol produced per minute per milligram of protein. The protein content was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA), with bovine serum albumin as the standard.

Observation of the permeability of APA microcapsules. The APA microcapsules were added into 200 μL of 0.05% weight per volume fluorescein isothiocyanate-immunoglobulin G ([FITC-IgG] molecular weights: FITC, 390 amu; IgG, 160,000 amu) solution. The microcapsules were then shaken in solution at room temperature for 1 h and then examined by confocal laser scanning microscopy.

Evaluation of immunoisolation effect of APA microcapsules by lymphocyte proliferation assay. Briefly, 3 mL blood from F344 rat hearts was removed aseptically into a 15-mL anticoagulated tube, then resuspended in 3 mL RPMI-1640 (Sigma). The lymphocytes were separated by centrifuge at 2400 rpm with 70% Percoll (1.077) solution in 15-mL centrifuge tubes and resuspended in RPMI-1640 (10% FBS), and then counted as responding cell populations; the encapsulated Adv-BMP-2 infected MSCs or unencapsulated Adv-BMP-2-infected MSCs were counted as the stimulating cell populations. All the stimulating cells were irradiated with γ radiation of ^{60}Co at a dosage of 100 Gy to stop cellular mitosis and proliferation.

Both the responding cells and the stimulating cells or agents were mixed in the wells of 96-well plates in the following 6 groups: group 1, Adv-BMP-2-infected MSCs (1×10^5/mL) plus lymphocytes; group 2, Adv-BMP-2-infected MSCs (5×10^5/mL) plus lymphocytes; group 3, encapsulated Adv-BMP-2-infected MSCs (1×10^5/mL) plus lymphocytes; group 4, empty APA microcapsules without cells plus lymphocytes; group 5,

alginate/Ca microbeads without cells plus lymphocytes; group 6, 200 μL RPMI-1640 media plus lymphocytes. Five wells were repeated in each group with 100 μL lymphocytes (1×10^5/mL) in each well. After the third mixed incubation, 1 μCi tritiated thymidine (^{3}H-TdR) per well was added and the mixture was incubated for 16 h. Cells were collected on type 49 fibrous glass filter papers to assay the scintilla counts per minute (CPM) of ^{3}H-TdR in each specimen with liquid scintigraphy instrument (Wallac 1450, Wallac Inc., Turku, Finland).

Statistical analysis. One-way analysis of variance was used to compare means across groups. For each variable of CPM, a pairwise multiple comparison procedure (Tukey's test) was used to determine the significance of differences among means. Comparisons of the ALP activities between 2 groups were analyzed using a grouped *t*-test. For all analyses, a level of $p < 0.05$ was used to indicate statistical significance.

Results

Characteristics of the microcapsules

The microcapsules were generally spherical, smooth, and of a uniform size, with a diameter of about 250 μm (Fig. 1A). The X-gal staining of the encapsulated cells was still positive 28 days after encapsulation (Fig. 1B). This indicated that the cells were viable and could express the gene products in the microcapsules.

BMP-2 proteins released from the encapsulated cells and the biologic effects of gene products

The BMP-2 proteins in the supernatants from the encapsulated BMP-2 gene-transfected MSCs were accumulated in the supernatant continually (Fig. 2). Little BMP-2 protein was detected in the supernatant from the control cells. The ALP activity of 52.537 ± 1.774 units (mean ± SD) in the experimental group was higher than that of 42.336 ± 0.857 U (mean ± SD) in the control group, a statistically significant difference ($p < 0.01$). The results indicated that the gene products secreted from the microcapsules could induced the MSCs to differentiate into osteoblasts.

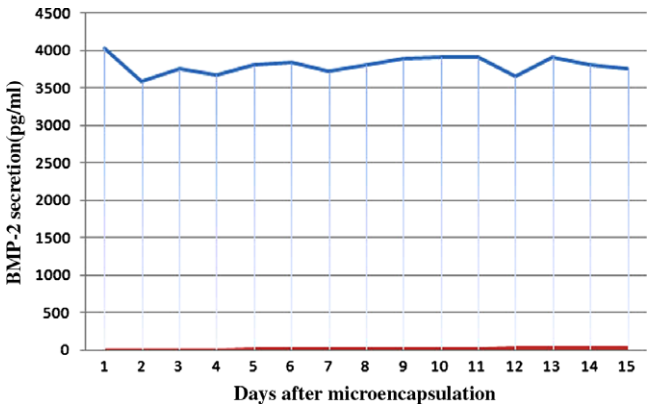

Fig. 2. Bone morphogenic protein-2 (BMP-2) released from the encapsulated cells and the data is presented as the mean of 5 aliquots.

Permeability of APA microcapsules

A photograph (Fig. 3) taken by confocal laser scanning microscopy indicated that the intact APA microcapsules prevented entry of the FITC-IgG but that the broken microcapsules did not.

Lymphocyte proliferation

The comparison of the CPM that indicated lymphocyte proliferation among different groups is presented in Fig. 4. There was significant difference between groups 3 and 1 ($p < 0.05$) but no significant difference between groups 3 and 6 ($p > 0.05$), which indicated that the APA microcapsules could prevent lymphocyte proliferation stimulated by allogenous Adv-BMP-2-infected MSCs. The difference between groups 1 and 2 is also statistically significant ($p < 0.05$), which may be related to the amounts of cells or adenovirus lysate. Although empty APA microcapsules or alginate/Ca microbeads could stimulate lymphocyte proliferation, there was no statistic significant difference among groups 3, 4, 5, and 6 ($p > 0.05$).

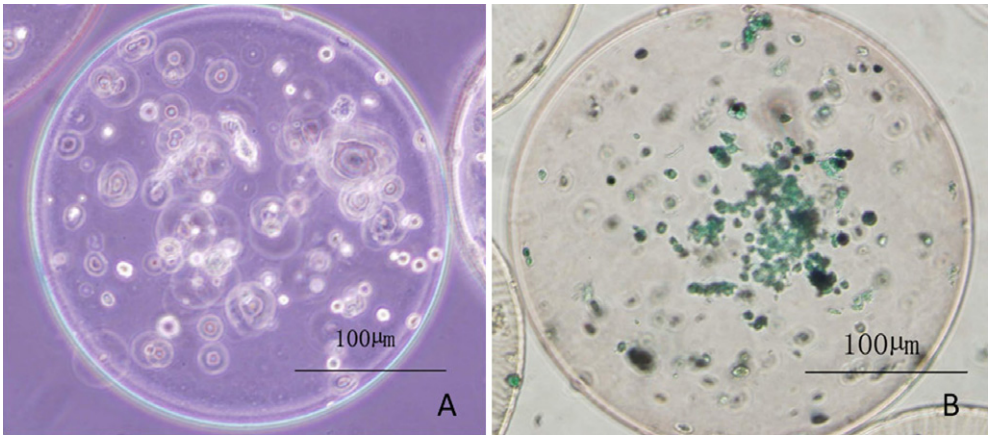

Fig. 1. (A) Microcapsules were spherical, smooth, and of uniform size. (B) X-gal staining of the encapsulated cells was still positive 28 days after encapsulation.

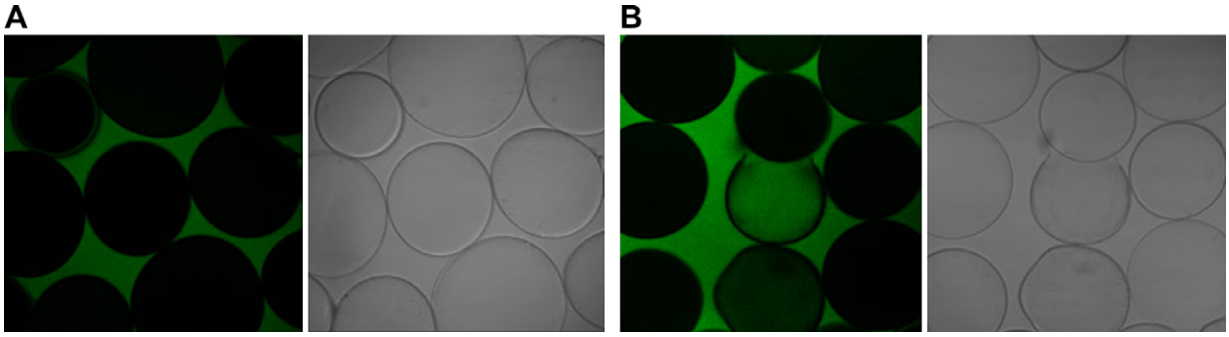

Fig. 3. Permeability of alginate-poly-L-lysine (APA) microcapsules as shown by confocal laser scanning microscopy. (A) The intact APA microcapsules prevented the entry of the fluorescein isothiocyanate-immunoglobulin G; (B) the broken microcapsules did not.

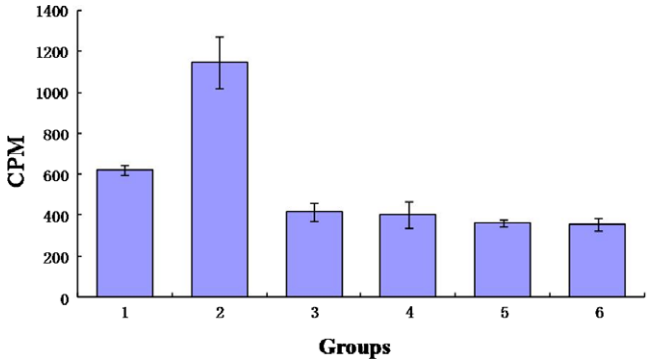

Fig. 4. Lymphocyte proliferation stimulated by cells or agents. The stimulators were Adv-BMP-2-infected mesenchymal stem cells ([MSCs] 1×10^5/mL) in group 1, Adv-BMP-2-infected MSCs (5×10^5/mL) in group 2, encapsulated Adv-BMP-2-infected MSCs (1×10^5/mL) in group 3, empty APA microcapsules without cells in group 4, alginate/Ca microbeads without cells in group 5, and 200 μL RPMI-1640 in group 6. Data are presented as the mean ± SD ($n = 5$). The differences between groups 1 and 6, groups 2 and 6, groups 1 and 3, groups 2 and 3, and between groups 1 and 2 were statistically significant ($p < 0.05$). The differences among groups 3, 4, 5, and 6 were not statistically significant ($p > 0.05$). Adv-BMP-2, recombinant adenovirus carrying the human *BMP-2* gene; CPM, counts per minute.

Discussion

Uniformly-shaped MSC-loaded capsules with a diameter of 250 μm were prepared at a concentration of 3×10^6 cells/mL of alginate in this experiment. Small, uniform microcapsules have been reported to have many advantages, including a higher degree of biocompatibility and better cell-product kinetics [24]. To obtain a sufficient therapeutic level of BMP-2 secretion, we tried to encapsulate the cells at as high a concentration as possible. In an earlier study, we encapsulated the cells at different concentrations of 10^6 cells/mL of alginate: 2×10^6 cells/mL of alginate, 3×10^6 cells/mL of alginate, and 4×10^6 cells/mL of alginate. We found that capsules prepared with the highest concentration of cells exhibited some irregular shapes and broken membranes, whereas capsules prepared at a concentration of 3×10^6 cells/mL of alginate were uniformly-sized intact spheres with a diameter of about 200–300 μm [25].

Cell survival and long-term expression of transgenes is important for the success of somatic gene therapy with cell encapsulation. We found that the reporter gene of β-galactosidase could survive and express transgenes 4 weeks after encapsulation. ELISA test results indicated that the BMP-2 proteins could be secreted stably from the microcapsules for 1 month. Moreover, ALP activity showed that the gene products from the microcapsules have the activity level required to induce MSCs to become osteoblasts. Further animal experiment is needed to test whether delivery of BMP-2 proteins by encapsulation is enough to promote bone repair.

The main advantage of cell microcapsules is their protective effect on immunity, which could permit the implantation of non-autologous recombinant cells. MSCs have been shown to suppress the activity of a broad range of immune cells, but new studies have further shown that MSCs interact with natural killer cells and act as antigen-presenting cells with interferon-γ stimulation [26–30]. Adenovirus vectors, which have been used in most gene-therapy strategies, are reported to induce immune response [9,10]. Our data also showed that lymphocyte proliferation stimulated by Adv-BMP-2-infected MSCs at different concentrations (1×10^5/mL or 5×10^5/mL) was significantly higher than that stimulated by RPMI-1640. The data further indicated that APA microcapsules could prevent stimulation of the cellular immune system by Adv-BMP-2-infected MSCs, thereby facilitating immunologic acceptance of grafts.

Recently, more attention has been paid to the use of xenogeneic cells for therapy [31]. We have found that intact APA microcapsules can inhibit permeation of FITC-IgG. Because the rejection of xenograft is mediated by the humoral immune system and because this response is initiated through the binding of IgM and IgG antibodies, these results indicate that APA microcapsules could be used to encapsulate xenogeneic cells.

Conclusions

Our results demonstrate that gene-modified stem cells can survive in APA microcapsules and prevent immune

response. Encapsulated *BMP-2* gene-transfected cells are capable of constitutive synthesis and delivery of biologically active BMP-2 proteins for at least 30 days and thus are of potential utility for enhancement of bone repair and bone regeneration.

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