Accelerated Bone Regeneration by Chitosan/Nanometer Hydroxyapatite/Collagen Composite Incorporating BMP-7 Mimetic Peptide

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Abstract: The purpose of the present study was to examine whether the chitosan/nanometer hydroxyapatite/collagen composite (chitosan/nHAC) incorporating BMP-7 mimetic peptide could accelerate bone regeneration. The chitosan/nHAC composite was prepared and the BMP-7 mimetic peptide introduced into the composite by vacuum adsorption. The released peptide content from the composite was detected using high performance liquid chromatography at different set times. 5 mm diameter cranial bone defects were created on both sides of the parietal bone in 24 adult Sprague-Dawley rats, which were randomized into two treatment groups; one receiving the chitosan/nHAC composite loaded with 1 mg BMP-7 mimetic peptide and the other one receiving the unload composite. Bone healing was evaluated with radiographic and histological analysis. The results showed that significantly improved and effective bone regeneration was achieved with the composite loaded with 1 mg BMP-7 mimetic peptide compared to the unloaded composite. In conclusion, the BMP-7 mimetic peptide in combination with the chitosan/nHAC composite could successfully accelerate the healing of rat’s cranial bone defects. The chitosan/nHAC/BMP-7 mimetic peptide composite is an ideal bone substitute material.

Key words: BMP-7 mimetic peptide, Bone regeneration, Bone substitute material, Cranial bone defect

Introduction

Repair of bone defects caused by trauma or pathology is always a big challenge in reconstructive surgery. The long history of orthopedic practice has confirmed the “golden standard” efficacy of autogenous bone grafting, but potential disadvantages such as morbidity of the donor site, infection, limited availability, and uncontrolled bone resorption have limited its usage\(^1-3\). Recently, bone tissue engineering has been developed as an alternative approach to autogenous bone grafting. The main principle of bone tissue engineering strategy is to implant an osteoconductive porous scaffold in combination with osteoinductive molecules or osteogenic cells\(^4,5\).

The bone morphogenic protein (BMP) family is assumed to be the only group of osteoinductive growth factors that can regulate each key steps of the sequential cascades in bone morphogenesis\(^6\) and induce bone formation in both ectopic and orthotopic sites\(^7,8\). Among them, BMP-7 and BMP-2 have been successfully used in the reconstruction of long bones, spine and the facial skeleton in clinical and preclinical studies\(^9,10\). Currently, BMP-7 and BMP-2 have mainly been produced by genetic engineering, but it is very expensive, owing to the complexity, low yields, expense and length of time necessary to prepare it. Therefore, to develop safer and cheaper cytokines, to enhance bone regeneration, may be of great practical significance. For this purpose, our group designed and synthesized BMP-7 mimetic peptide derived from residues of BMP-7. Our previous study demonstrated that the BMP-7 mimetic peptide could enhance the osteoblastic differentiation of bone marrow stromal cells\(^11\).

It is generally accepted that the therapeutic concentration of growth factor is difficult to be maintained at wound sites due to its rapid diffusion by body fluid and short half-life\(^12\). Moreover, periodic addition of growth factors often requires invasive procedures, such as injection or infusion, which may be clinically impractical, while excessive doses may have undesirable systemic side effects\(^13\). Therefore, the use of an appropriate carrier for delivery of growth factor is required. For bone regeneration, the choice of carrier material is based on biocompatibility, biodegradability, mechanical properties, interface properties and...
nonimmunogenicity. Recently, composite materials are playing a predominant role as scaffolds in bone tissue engineering. Our group has developed the chitosan/nHAC composite as a carrier for BMP-7 mimetic peptide.

In this paper, our group presents a growth-factor based tissue engineering strategy for bone regeneration using the BMP-7 mimetic peptide carried by a chitosan/nHAC composite material. The osteoinductive characteristics of chitosan/nHAC loaded with the BMP-7 mimetic peptide are evaluated by rat calvarial defect model. Our findings demonstrated that BMP-7 mimetic peptide loaded by chitosan/nHAC could efficiently promote bone regeneration.

Materials and Methods

Synthetic peptides

The purity of the BMP-7 mimetic peptide (KQLNAISVLYFDD, GL Biochem, Shanghai, China) was 97% determined by high performance liquid chromatography (HPLC).

Implant preparation

Type I collagen solution (Sigma, USA) was diluted in deionized water at a concentration of 2 mg/ml for 3 h. Solutions of CaCl₂ and H₃PO₄ (Ca/P=1.66) were then added separately by drops. The solution was gently stirred and titrated at room temperature with sodium hydroxide solution to pH 7.4. After 24 h, the nHAC deposition was harvested by centrifugation and freeze-dried. A chitosan (Sigma, USA) solution of 2 mg/ml was prepared by dissolving chitosan in 0.1 M lactic acid. Then the nHAC powder was added at a 2.5:1 nHAC: chitosan weight ratio. After homogenization, the solution was then ultrasonicated, poured into a mold, frozen at a temperature -20°C overnight, and then lyophilized to remove dioxane. The morphology and microstructure of the composite were examined by scanning electron microscopy (SEM).

The chitosan/nHAC composites were pre wetted in pure ethanol. Ethanol was removed with excess water and shaken continuously for 24 hours. The pre wetted composites were impregnated with 1 mg of the BMP-7 mimetic peptide in 100 µL water and then vacuum dried. The implants were sterilized with ethylene oxide gas.

In vitro BMP-7 mimetic peptide release

The chitosan/nHAC loaded with 1 mg BMP-7 mimetic peptide was incubated in 2 mL PBS at 37°C for 21 days (n=3). The elution of BMP-7 mimetic peptide was examined at 1, 2, 3, 5, 7, 10, 12, 14, 16, 18 and 21 days. At each time interval, the supernatant was completely removed and replaced with fresh buffer. The amounts of the BMP-7 mimetic peptide in the collected supernatants were measured by HPLC.

Rat cranial defect repair

Twenty-four male Sprague-Dawley rats (body weight 100-120 g, Xiangya Experimental Animals center, Changsha, China) were fed a standard laboratory diet, housed in plastic cages at ambient temperature. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Central South University. The rats were anesthetized by intravenous injection of pentobarbitol sodium (40 mg/kg). A calvarial defect, measuring 5 mm in diameter was created on both sides of the parietal bone of rats using a bone trephine bur. The defect on the right side was filled with BMP-7 mimetic peptide loaded chitosan/nHAC (1 mg per scaffold), while the defect on the left side was filled with unloaded chitosan/nHAC. After the scaffolds were implanted into the defect, the periosteum and scalp were closed.
in layers. All above procedures were approved by the committee on animal experimentation of Xiangya Hospital.

Animals were sacrificed after 6 or 12 weeks and the crania were harvested intact. All of the specimens underwent histological analysis and a multi-slice spiral computed tomography (CT) scan. The specimens retrieved from the 12-week implanted rats underwent Masson trichrome staining analysis. CT scan was performed with a 16-slice spiral CT scanner (Somatom Sensation 16; Siemens, Erlangen, Germany) with the following scan parameters: collimation 1.5 mm, table feet 5 mm, 280 mA, 120 kV, reconstructed slice thickness 5 mm and images were reconstructed using 3-dimensional (3D) image reconstruction software supplied by the CT scanner. The repair percentage, defined as the repaired (radiopaque) volume over the entire defect was obtained from the CT scans by auxiliary software after processed. For histological analysis, the decalcified calvarial explants were fixed in 4% neutral buffered formaldehyde solution for 2 days and dried through gradient ethanol baths and xylene, then embedded in paraffin and sectioned into 5 μm thick sections. The sections were stained with Haematoxylin and Eosin to visualize the morphology of the new bone. The sections from the specimens at the 12th week were stained with Masson trichrome methods to evaluate the synthesis of collagen fibrils. All histological sections were observed with an Olympus BX-51 light microscope.

Statistical analysis

All quantitative data was presented as mean±standard deviation. Student’s t-test was used to test for statistical significance, which was accepted when P<0.05 (two-tailed). The difference was regarded statistically significant when P<0.05. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Central South University.

Results

Characterization of the chitosan/nHAC composite

On scanning electron microscopy, the chitosan/nHAC scaffold had a uniformly distributed and interconnected pore structure, with pore size ranging from several 10 μm to about 100 μm (Fig. 1).

In vitro release kinetics of the BMP-7 mimetic peptide

The cumulative release of BMP-7 mimetic peptide from chitosan/nHAC scaffold was measured and shown in Fig. 2. Apparently, the release curve exhibited a sharp initial burst at the first day with about 30.57% of BMP-7 mimetic released from the scaffold. After an initial burst, the protein was released in a
sustained manner, and the rate decreased with time. At 21 days, 65.98% of total BMP-7 mimetic peptide was released.

Radiographic analysis

The healing of calvarial defect was examined using 3D-CT imaging. Representative 3D-CT images of bone defect sites at 6 weeks and 12 weeks post-implantation are shown in Fig. 3 and the quantitative analysis of radiopacity by 3D-CT images is shown in Fig. 4. At 6 weeks, the experimental group that was treated with BMP-7 mimetic peptide loaded chitosan/nHAC, shadows of uniform high density between the intermedial defect sites and the cranial bone defect edge were seen. In the control group treated with chitosan/nHAC alone, there were only nonuniform low-density shadows at the center of the defect sites. The repair percentages were 37.25% and 9.50%, respectively. At the 12th week, in the experimental group, massive calcified tissues grew...
from the host bones and nearly complete bony-union was observed in the defect sites, and the repair percentage was 89.09%. However, in the control group, only minimal calcified tissues were detected and 30.47% of the defect volume repaired. The overall statistical analysis was then carried out with the results confirming a significantly greater healing percentage in the BMP-7 mimetic peptide loaded chitosan/nHAC when compared with the unloaded scaffold.

**Histological observation**

The photomicrographs of the histological slices of implants are shown in Fig.5. At the 6th week, in the experimental group, a large amount of osteoid tissue and new bone was observed. There was simultaneous scaffold remodeling by osteocyte invasion and new bone formation. While the control group showed only minimal new bone formation at the defect margins. At the 12th week, in the experimental groups, the preexisting defect was filled with massive mature lamella bone and the bony-union between new bone and host bone was observed. Meanwhile, the composite was almost completely degraded. In the control group, there were still slight amounts of new bone, but this bone was more mature compared with the regenerated bone at the 6th week. The scaffolds were only partly degraded and the residual materials were surrounded by areas of new bone formation.

Representative Masson trichome staining photographs of regenerated bone at the 12th week are shown in Fig.6. Massive calcified bones stained red color were observed in the experimental group. However, in the control group there was only slight amounts of fresh-formed bone stained blue color (Fig.6).

**Discussion**

In the present study, our group investigated the osteoinductive potential of a synthetic BMP-7 mimetic peptide, in combination with the chitosan/nHAC composite as a local drug delivery system in vivo. For this purpose, firstly, our group analyzed the BMP-7 mimetic peptide release kinetics from the chitosan/nHAC composite. Secondly, our group evaluated the bone formation capacity of the BMP-7 mimetic peptide loaded chitosan/nHAC in a rat calvarial bone defect. The results of these studies demonstrated that the BMP-7 mimetic peptide loaded chitosan/nHAC significantly enhances bone formation. Thus, the present study suggests that the chitosan/nHAC/BMP-7 mimetic peptide composite is a kind of ideal bone substitute material.

The BMP-7 mimetic peptide utilized in this study is derived from the BMP-7 sequence. Our previous in vitro experiment with rat bone marrow stromal cells (BMSCs) showed that the BMP-7 mimetic peptide could induce osteoblastic differentiation of BMSCs. However, the mechanism involved in osteogenic differentiation of BMSCs induced by the BMP-7 mimetic peptide has not been clear and needs to be elucidated in further studies. Recently, some synthetic peptides with potential to treat bone defects have been reported, including TP508, P-15, 73-92 peptides, B2A2, F2A4-K-NS. TP508 is a thrombin derived peptide with wide specificity that causes angiogenesis, cytokine release, and appears to act through activation of an inflammatory response. P-15 is a collagen-derived high-affinity cell-binding peptide, which increases cell attachment and modulates a number of gene products. The 73-92 peptide corresponds to residues 73-92 of the knuckle epitope of BMP-2, and it can induce ectopic calcification when it is immobilized on a covalently cross-linked alginate gel. B2A2 consisting of a BMP receptor targeting sequence, a hydrophobic spacer, and a heparin-binding sequence, is a positive modulator of recombinant BMP-2. It can be used to reduce the effective dose of recombinant BMP-2 on or in a medical device. F2A4-K-NS, a peptidemimetic of FGF-2, can augment ectopic bone production following the subcutaneous implant of human demineralized bone matrix. These synthetic peptides represent a new kind of bioactive molecules that can accelerate the healing of bone defects. The BMP-7 mimetic peptide belongs to this kind of bioactive molecules. Compared with their homologous proteins, these peptides are less expensive to produce, easy to chemically modify for enhanced drug delivery and vastly more chemically stable during storage and delivery due to their linear structure.

To facilitate retention of growth factors at the treatment site and reduce the effective dose, delivery systems that retain growth factors and release it slowly, as well as serving as scaffolding for new bone formation, are essential. An ideal delivery system also must be biocompatible and biodegradable; possess mechanical properties, large porosity and high pore interconnectivity; permit the biologic activity of osteoinductive growth factors; lack immunogenicity, toxicity, and carcinogenicity; be easily handled; be sterilizable; and be inexpensive to produce commercially. A large number of materials have been considered as osteoinductive growth factors delivery systems and tested in animals, including inorganic hydroxyapatite, natural biopolymers such as, collagen and alginate, and synthetic polymers such as, poly(lactic-co-glycolic acid) (PLGA), poly(lactic acid) (PLA), and poly(glycolic acid) (PGA). These delivery systems enhance bone repair and accelerate fracture healing. However, the optimal retention/release rates for growth factors and their carriers have not been established yet. In this study, our group developed the chitosan/nHAC composite as a carrier for BMP-7 mimetic peptide. Fig 2 showed that the chitosan/nHAC scaffold has a uniformly distributed and interconnected pore structure. Such a well-interconnected porous structure is of importance for an adequate supply of the body fluids with nutrients throughout the implant. In our in vitro release study, a burst release from the porous scaffold during the first day in which more than 30.57% of the BMP-7 mimetic peptide was released. After an initial burst, the protein was released in a...
sustained manner. At 21 days, 65.98% of total BMP-7 mimetic peptide was released. This result suggested that the porous chitosan/nHAC scaffold may be a promising carrier for BMP-7 mimetic peptide. Although the exact mechanism is not completely clear, the HA has high affinity to drugs and proteins \(^{27}\). The chitosan/nHAC scaffold contains a great quantity of HA. Therefore, the in vitro release kinetics of the BMP-7 mimetic peptide could be explained by the binding affinity of the BMP-7 mimetic peptide for the chitosan/nHAC scaffold. Firstly, the initial burst effect may be due to diffusion of loosely bound or unbound BMP-7 mimetic peptide from the scaffold. Secondly, after the initial burst, the following sustained release might be caused by the diffusion of the BMP-7 mimetic peptide adhered to HA due to the slow degradation of the scaffold.

To further evaluate the osteogenic effect of the chitosan/nHAC incorporating BMP-7 mimetic peptide, our group implanted the BMP-7 mimetic peptide loaded chitosan/nHAC into rat cranial defects and evaluated healing over a 12-week time-course. In this study, the calvarial defect was selected as the model for bone defect repair because this model has been used as a standard protocol for the testing of both scaffold materials and delivery of osteoinductive factors in bone sites \(^{28,29}\). Our group examined these defects using radiographic and histological analysis. CT scans revealed high levels of mineralization and nearly-complete closure in defects implanted with the BMP-7 mimetic peptide loaded chitosan/nHAC at 12 weeks postoperatively (Fig. 3), whereas the defects filled with scaffolds alone had about 30.47% of the defect volume repaired. We consider the fact that the unloaded chitosan/nHAC scaffolds supported a fair amount of bone formation in the defects is due to the osteoconductive qualities of the scaffold. Histological analysis of the specimens revealed that defects treated with the BMP-7 mimetic peptide loaded chitosan/nHAC were filled with dense trabecular bone with no evidence of scaffold remnants at 12 weeks postoperatively. (Fig. 5) In contrast, the defects treated with the chitosan/nHAC alone were filled with a small quantity of woven bone and the maturity of the regenerated bone was significantly lower than that the BMP-7 mimetic peptide loaded chitosan/nHAC. The Masson trichrome staining coincided with the CT scans and histological analysis findings (Fig 6). There were massive calcified bones stained red color in the experimental group. However, in the control group there were only slight remnants at 12 weeks postoperatively. The diffusion of the BMP-7 mimetic peptide in the chitosan/nHAC scaffolds supported a fair amount of bone formation and accelerating the healing of calvarial bone defects.

Conlusion

In conclusion, the current study demonstrates that the chitosan/nHAC loaded with the BMP-7 mimetic peptide could successfully accelerate bone regeneration and healing of rats calvarial bone defect, indicating that the BMP-7 mimetic peptide may represent a new class of bioactive molecules that can instigate osteogenesis following incorporation into a local drug delivery system and it may be useful in clinical repair of large bone defects and other orthopaedic indications.

References


