The Behavior of \(rhBMP2\) Gene Transfecting Cells on Nanometer Hydroxyapatite/Chitosan Scaffold

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Abstract

The purpose of this study was to prepare nanometer hydroxyapatite/chitosan composite (nHA/CS) and evaluate its biocompatibility with \(rhBMP2\) gene transfecting cells. The cell line expressing \(rhBMP-2\) gene was grown, and the nanometer hydroxyapatite powder was fabricated with the sol-gel method and then dispersed completely in 2% nHA/CS and dense HA. It was shown in X-ray diffraction that characteristically diffracted peaks of precipitated HA nanocrystals were identical with those of stoichiometric HA with reference to JCPDS. HA powder in composites was shown to be nanoparticles by transmission electron microscope. After being co-cultured for 3 days, 5 days and 7 days, the proliferation of attached cells on the scaffold materials was more than that on dense HA. It was believed that compared with dense HA, the novel scaffold material, nHA/CS, had significant ability for cell adherence and considerable improvement in the biocompatibility of hydroxyapatite. Adherence and proliferation of \(rhBMP2\) gene transfecting cells on the surface of nanoHA/CS were better than those on dense HA/CS composite. And the Alkaline phosphatase (ALP) activity from nanoHA/CS was approximately the same as that of dense HA/CS. From these results, it is suggested that the porous nano-HA/chitosan composite scaffolds are useful materials in tissue engineering research.

Keywords: Hydroxyapatite (HA), Chitosan (CS), Bone morphogenetic protein (BMP), Biocompatibility

1. Introduction

Recently, many materials, including calcium phosphate, hydroxyapatite (HA), extracellular matrix components and other synthetic materials, have been applied in bone regeneration of humans [1-4]. Hydroxyapatite, \(Ca_{10}(PO_4)_6(OH)_2\), is one of the major constituents of the inorganic materials in human and animal bone and tooth, and it makes up more than 95 percent of dental enamel [5]. Artificial HA is similar to the apatite crystal of natural bone. Because of its good bioactivity and good biocompatibility, it can form a compact bond with surrounding tissues after implantation. It can also supply good physiological scaffold for deposition and regeneration of new bone. As an attractive bone substitution material, HA has been widely used in medical applications such as dental material, bone substitute and hard tissue paste [6,7]. Recently, HA prepared at nanoscale has played a significant role in various biomedical applications owing to its unique functional properties including high surface area, volume effect and so on [8]. Moreover, it has been determined that it could be absorbed or degraded by periodontal fibroblasts in vitro. However, ideal biomaterial should be compatible with organic as well as inorganic contents because all natural hard tissues contain small but very significant amounts of organic contents [1,9]. So hybrid composite has been believed to be a main direction for artificial scaffold materials. Chitosan (CS) is a nature polygone with significant alkalinity, which is obtained from chitin through its deacetylation. Its final metabolites are N-acetylgluco-samine and glucidamin, which are natural metabolites [10]. Chitosan was suggested as an alternative polymer for use in orthopedic applications to provide temporary mechanical support due to its good biocompatibility, non-toxicity, biodegradability, and inherent wound healing characteristics [6]. It can be utilized in combination with other bioactive inorganic ceramics, especially HA, to further enhance tissues’ regenerative efficacy and osteoconductivity. Bone morphogenetic proteins (BMPs), which belong to the transforming growth factor beta superfamily, were discovered as a factor present within bone matrix. It has been believed that BMPs are powerful regulators of cartilage and bone formation during embryonic development and regeneration in post-natal life, which induce new cartilage and bone formation when implanted ectopically into an animal. This osteoinductive
ability has led to use of BMPs as therapeutic agents for creation of new bone in treatment of periodontal disease. In our previous papers [1,11], secreted BMP and its biologic activity were studied through gene-transfecting skills and nanometer hydroxyapatite, and its significance on scaffold were reported. In this study, the cell line with osteoinductive gene, which is used in bone tissue engineering, was seeded on a composite scaffold. Incorporation of HA with chitosan was fabricated in order to improve the bioactivity and the cell bonding ability.

2. Materials and methods

2.1 Establishment and identification of cell line transfected with rhBMP-2 gene

The cell line expressing rhBMP-2 gene was grown as previously described [11]. Mainly, pcDNA3.1-BMP2 was transfected into NIH3T3 with Sofast™ positive compound transfection agent (Xiamen Summa Biotechnology Co., Ltd., China). After 48 hours, primary medium was removed and then cultured at 37°C and 5% CO2. Then, 2 to 3 weeks later, the majority of original cells died under selective circumstance and the positive cell clones were selected to further grow and named B2/3T3. The empty vector pcDNA3.1 was transfected into NIH3T3 cells as control, which was named T/3T3. The cell sections were fixed and then incubated with polyclonal rabbit anti-human BMP2 (Boster Co., Ltd., China) for 2 hours at 37°C and washed three times with PBS (pH 7.3). Goat biotinylated anti-rabbit secondary antibody (Boster Co., Ltd., China) was incubated for 30 minutes at room temperature and washed three times with PBS (pH 7.3). Streptavidin biotin complex (Boster Co., Ltd., China) was incubated for 20 minutes at room temperature and washed five times with PBS (pH 7.3). DAB staining was performed by DAB substrate (Boster Co., Ltd., China).

2.2 Fabrication of nanophase and dense hydroxyapatite powder

Nanophase hydroxyapatite powders (nHA) were fabricated using a sol-gel method [1]. Aqueous solution of calcium nitrate tetrahydrate [Ca(NO3)2·4H2O] and alcohol solution of trimethyl phosphate (CH3O)PO were used as precursors. A small amount of citric acid (about 5%), used as a chelating reagent, was added to the aqueous solution of calcium nitrate tetrahydrate. The solutions were mixed at room temperature with Ca/P in molar ratio of 5:3 and were kept at pH 7.5 by ammonium hydroxide. After 48 h reaction, the gel thus obtained was dried at 190°C for 2 hours, calcined at 600°C for 1 h and ground into fine powders. Dense hydroxyapatite powders (HA) were prepared with the same method as mentioned above except for citric acid added. Both nanometer and dense hydroxyapatite were analyzed and observed with X-ray diffraction (XRD), Fourier transformed infrared (FTIR) spectroscopy, scanning electron microscope (SEM) and transmission electron microscope (TEM).

2.3 Fabrication of nanometer hydroxyapatite/chitosan (nHA/CS) composite

A chitosan aqueous solution of 2% was prepared by dissolving chitosan powder into distilled water containing 0.2 mol/L acetic acid [7]. Then, under agitation, 0.1 mol/L Ca(NO3)2 aqueous solution was slowly added into the chitosan solution. Subsequently, 0.06 mol/L (NH4)2HPO4 was added drop by drop. Nanometer hydroxyapatite was dissolved in the chitosan solution, and stirred for 6 hours till deposition appeared. The mixed solution was blended thoroughly and added to the model which was 13 mm in diameter and 10 mm in height. All of the models were frozen at -20°C and the frozen composites were lyophilized. The dry composite scaffolds were soaked in 10% NaOH solution for 10 h. and then rinsed in distilled water to neutrality and lyophilized again. The composite discs were immersed in media for 2 h before use. The dense hydroxyapatite/chitosan (HA/CS) discs were prepared and analyzed as the controls.

2.4 Morphological observation of cells

All discs were exposed to UV light for 1 hour before being immersed in media for 30 min. After seeding 3.0 × 104 B2-3T3 cells on the scaffold and culturing for 3, 5 and/or 7 days, the discs were washed twice with PBS and all unattached cells were removed. The discs were fixed with 2.5% glutaraldehyde for 24 h before being postfixed for 1 h with 1% osmium tetroxide. Then the samples were dehydrated with series of graded alcohol solutions, critical-point-dried and sputter-coated with gold. The mounted slabs were observed with scanning electron microscopy and affiliated energy-dispersive X-ray analysis (Siron Field Emission SEM, FEI, USA). T-3T3 cells were also seeded as the controls.

2.5 Cell attachment assay

After the cells were seeded on nHA/CS discs in 12-well tissue culture plates at a density of 3.0 × 103 cells/well and incubated for 3, 5 or 7 d, allowing attachment to the scaffold plate, each well was rinsed with phosphate-buffered saline (pH 7.2, PBS) to remove unattached cells and stained with toluidine blue to distinguish the attached cells from the extra-cellular matrix. Three pictures of each sample were taken by stereoscope loaded digital camera and the number of cells attached to the plate was counted directly with Image J. The number of cells determined to be attached was normalized against the area available for the attachment (cells/mm²). Six specimens for each material were tested for each incubation time.

2.6 Alkaline phosphatase activity measurement and statistics

At 3rd, 5th and/or 7th d, the cells on the discs were washed twice with PBS and then trysined. 100 µl of mixture (10 mmol/L Tris-HCl, 5 mmol/L MgSO4, 0.1%, TritonX-100, 0.1% NaNO) were added then frozen for three times. Alkaline phosphatase (ALP) activity was then assayed using a commercial kit (Alkaline Phosphate Substrate Kit, Bio-Rad Laboratories, Hercules, CA). Eighty µl of substrate was added...
to 20 μl of each sample, and the mixture was incubated for 30 min at 37°C. The reaction was terminated by the addition of 100 μl of 0.4 M NaOH. The optical density at 405 nm was measured and compared with the value of a series of p-nitrophenol standards. Six specimens for each material were tested for each incubation time.

The statistical significance of differences among the results was analyzed with SPSS 13.0 using Student’s t test at a level of 0.05.

3. Results

3.1 XRD

In comparison with JCPDS Card No. 9-432, crystal structure of HA and nHA was verified as the shape of the crystal structure of HA (Fig. 1), which seemed not to result in any change in the composite of chitosan.

![Figure 1. XRD spectrum of nHA/CS.](Image)

3.2 TEM

TEM micrographs of nHA/CS composite are shown in Fig. 2. Here, nanometer HA particles with some nanometers to some tenth nanometers in diameter were dispersed well in chitosan matrix homogeneously. The intersurface distance of crystal, d value, was approximately equal to that of hydroxyapatite in JSDPS Card 9-432 with high-difference TEM (Fig. 3). It means that the crystals in the composite express the feature of hydroxyapatite. The insert figure shows multiple cycles of electron diffraction picture, in which the nHA seems to be multi-crystals.

![Figure 2. TEM graph of nHA, which shows some particles of tenth nanometer in dimension.](Image)

3.3 FTIR analysis

In FTIR spectroscopy (Fig. 4), HA and nHA showed presence of hydroxide and phosphate groups, i.e. two FTIR bands at 3573 and 634 cm⁻¹ of the -OH group or 1252 cm⁻¹ of the phosphate group. The other absorbance in the region 1252 cm⁻¹ was -HN₂, the main functional group of chitosan. The two absorbencies in 1400–1500 cm⁻¹ and 875 cm⁻¹ were the groups of -CO₃ from the structure of HA in which the -PO₄ groups were replaced by -CO₂ when the material was thermally treated. There was large amount of organic element in the gel made from sol adding citric acid. When thermally treated, the organic element was burnt into CO₂ and H₂O. As high viscosity of CO₂ was found, the group of CO₂ could enter HA crystal after citric acid was added. From the above results, the compound contained nHA as well as chitosan. And there was not new absorbance but only push-down of two absorbencies, and no chemical reaction happened.

![Figure 3. HTEM graph of nHA/CS, d = 0.2863 nm, insert: electron diffraction picture.](Image)

3.4 Scanning electron microscopy (SEM)

Under scanning electron microscopy, both nHA/CS and dense HA/CS scaffolds showed homogeneous porosity (Fig. 5). And the crystals distributed uniformly in the scaffold and spread well as aggregation. The gene transfecting cells were well adhered to nanometer HA/CS scaffolds (Fig. 6), which did not show any difference on dense HA/CS material. Both the
nanometer and dense HA chitosan composite showed the porous structure on which the dimensions of holes were about 15–40 µm. After cultured for 5 days, the cells attached and extended well on the scaffold surface with either dense or nanometer crystals.

Figure 5. SEM observation on nHA/CS (a) and dense HA/CS (b) scaffolds. No dense HA particle could be seen because of precipitation of HA in 2% chitosan solution.

Figure 6. Transflecting cells on nHA/CS after being cultured 5 days.

3.5 Cell proliferation

In the presence of nanoparticles of HA, the results on the 3rd, 5th and 7th days indicated that cell proliferations were significantly higher than those of the controls (Table 1).

Table 1. t test of cell adhesion quantity for two groups.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>HA/CS</th>
<th>nHA/CS</th>
<th>t value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>3rd day</td>
<td>6</td>
<td>75.173 ± 0.491</td>
<td>79.138 ± 0.053</td>
<td>12.790</td>
<td>0.049</td>
</tr>
<tr>
<td>5th day</td>
<td>6</td>
<td>92.961 ± 1.257</td>
<td>101.787 ± 0.476</td>
<td>15.973</td>
<td>0.040</td>
</tr>
<tr>
<td>7th day</td>
<td>6</td>
<td>130.500 ± 0.846</td>
<td>157.875 ± 0.247</td>
<td>17.353</td>
<td>0.036</td>
</tr>
</tbody>
</table>

3.6 ALP activity

In ALP activity (Table 2), there was no significant difference between nHA and dense HA compound. The result showed that the osseous activity in the gene transflecting cells was not affected in the materials.

Table 2. Unpaired t test of ALP activity values between two groups at each time.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>HA/CS</th>
<th>nHA/CS</th>
<th>t value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>3rd day</td>
<td>6</td>
<td>0.022 ± 0.002</td>
<td>0.031 ± 0.007</td>
<td>1.044</td>
<td>0.178</td>
</tr>
<tr>
<td>5th day</td>
<td>6</td>
<td>0.049 ± 0.006</td>
<td>0.059 ± 0.005</td>
<td>1.212</td>
<td>0.146</td>
</tr>
<tr>
<td>7th day</td>
<td>6</td>
<td>0.079 ± 0.012</td>
<td>0.062 ± 0.006</td>
<td>1.224</td>
<td>0.144</td>
</tr>
</tbody>
</table>

4. Discussion

In this study, nanometer hydroxyapatite/chitosan composite was used for rh-BMP2 gene transflecting cells in vitro to investigate the adhesion effects of the scaffold. Previous work suggested that the rh-BMP2 gene transflecting cells could transfer to osseous cells, which expressed the high activity of ALP [1]. In this study, the gene transflecting cells were fed onto the composite scaffold to evaluate qualities of adhesion and proliferation, and the number and morphology of attached cells on the surface were determined. Several studies have identified surface roughness as one of the major contributors to the development of cell-material interface [12,13]. In this paper, nHA/CS exhibited a rougher surface morphology even after immersion in the culture medium for 1 day. From previous experiment, it was shown that nanometer HA could be absorbable in vitro [1]. Therefore, in the early stages of culturing, the loss of the substrate surface and inhibition of anchorage-dependent adhesion of the cells is thought to occur through the dissolution of soluble components. This finding is in agreement with a previous report, which showed that a rough osteoceramic surface produced greater cell attachment efficiency than a smooth surface [14]. The assessment of cell morphology by SEM demonstrated that cell growth was not impaired on either nanoHA/CS or dense HA/CS, indicating that absorbable components of nanoHA/CS did not influence the growth of cells.

In 2004, Hu [6] reported that his groups had prepared homogenous, transparent and high/strength CS/HA nanocomposite, in which the matrix chitosan was precipitated and the filler of HA synthesized simultaneously by in situ hybridization. The results showed that the initial mechanical properties of bending strength and bending modulus of the composite were 86 MPa and 3.4 GPa, respectively, which was 2–3 times stronger than that of PMMA and bone ceramics. Nanometer HA particles identified by TEM and XRD were dispersed well in CS/HA composite. So it was suggested that nanophase HA/chitosan rod had potential property for internal fixation of bone fracture. Actually, it has been reported widely that chitosan, a basic polysaccharose, has been used in alternative polymer for use in orthopedic applications to provide temporary mechanical support to the regeneration of bone cells due to its good biocompatibility, non/toxicity, biodegradability, and inherent wound/healing characteristics. Chitosan had been used in various forms such as zero-dimensional microsphere, two-dimensional membrane and three-dimensional pin or rod. On the other hand,
hydroxyapatite (HA), \( \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 \), was used for various biomedical applications such as dental material, bone substitute and hard tissue paste. It was shown that HA could accelerate the formation of bone-like apatite on the surface of implant and periodontal restoration. In the present study, a novel material, nanophase hydroxyapatite and chitosan scaffold, was used for gene transfecting cell attachment, which is an available model of tissue engineering for bone replacement and repair. The results showed that the compound had good dissolvability and mechanic strength compared with simplex chitosan due to mixture with hydroxyapatite, and good feasibility compared with powder due to chitosan network.

Cell adhesion is the primary property of biocompatibility for scaffold materials. Some significant results were shown through the experiments. First, as shown in our previous papers [1,11], nanometer apatite could be fabricated by adding citric acid (about 5%) as a chelating agent through sol-gel method and rhBMP2 gene could be transferred into fibroblasts. Second, the gene transfecting cells could adhere to and grow normally on the surface of nanophase apatite and chitosan compound. Also, no chemical changes were found in the novel compound mixing chitosan with hydroxyapatite because essential content of two materials were shown by FTIR. Furthermore, under SEM and EDAX, \( \text{Ca}^{2+} \) decreased from 10.27% to 1.09% at the interface of cells and material. It is believed that organic contents increase and the inorganic decrease on the surface covered by cells. This result is similar with those of other investigators [15-17]. Moreover, the proliferation of the gene-transfeting cells on the chitosan scaffold with nanometer HA showed significant higher than that of dense HA material. The result is the same as our previous observation and those in other published reports [1]. In a study on the effect of nanophase HA on periodontal ligament cells in vitro, the cell “swallowing” of nanometer particles was seen; it was believed that the high proliferation of cells was due to higher phase activity of the nanometer material.

From the SEM images of this study shown in Fig. 5, the pore diameter was smaller than expected dimension of about 20–60 \( \mu \)m. Previous research suggested that human osteoblasts can penetrate pores > 20 m in size [7], and that the porosity needs to be > 30% to achieve interconnection [18]. The porosity of the composite scaffolds prepared in this study appeared sufficient for good interconnection and exchange of nutrition, in which the pore size of the scaffolds could support the growth of rhBMP2 gene transfecting NIH3T3 cells that were seeded on the scaffolds. They could grow into the scaffolds and distribute uniformly in the scaffolds. After 7 days’ culture, the cells proliferated and appeared as many clusters of cells and distributed uniformly and spread well. However, the method and significance regarding adjustment of the dimension of the porosity is worth studying in future.

5. Conclusion

In this study, novel composite scaffolds of nano-HA and chitosan were prepared and seeded on the surface with rhBMP2 gene transfecting cells. The porous structure of the composite scaffolds was made by lyophilization. Adherence and proliferation of rhBMP2 gene transfecting cells appeared better on the surface of nanoHA/CS than dense HA/CS composite. ALP activity from nanoHA/CS was approximately the same as that of dense HA/CS. The results suggest that the porous nano-HA/chitosan composite scaffolds are useful materials for tissue engineering research.

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References


