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Original

In vivo Evaluation of a Novel Chitosan/ HAp Composite Biomaterial as a Carrier of rhBMP-2

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Abstract: We developed a porous chitosan/ hydroxyapatite (HAp) composite, in which the HAp nanocrystals align along the chitosan molecules, and examined the biocompatibility, osteoinductive activity, and the ability to act as a carrier of recombinant human bone morphogenetic protein-2 (rhBMP-2) of this novel biomaterial. The composite was subcutaneously implanted into the backs of 11-week-old SD rats, with or without rhBMP-2 (5 μg). At 2 and 8 weeks after the implantation, the composite was explanted for morphohistological evaluation. In the presence of rhBMP-2, ectopic bone formation was found at 2 weeks and maturation of the newly formed bone around the composite at 8 weeks. Chitosan/HAp composite alone caused little inflammation, and new blood vessel growth and multinucleated giant cells were found around the composite, accompanied with roughening of the surface due to degradation at 2 weeks; however, neither cartilage nor bone formation was found around the composite. With rhBMP-2, the bioabsorption of the composite was accelerated as the rhBMP-2-induced bone matured. Histomorphometrical analysis showed that the mean value of the composite areas with rhBMP-2 was significantly smaller than that without rhBMT-2 at 2 and 8 weeks after the implantation. These results suggested that the novel chitosan/ HAp composite was an effective bioabsorbable material as a carrier of rhBMP-2.

Key Words: Chitosan, Hydroxyapatite (HAp), Osteoinduction, Bioabsorption, Recombinant human bone morphogenetic protein-2 (rhBMP-2)

Introduction

Hydroxyapatite (HAp: $Ca_{10}(PO_4)_6(OH)_2$) has been used as a biocompatible and osteoconductive substitute in the fields of orthopedic, craniofacial, and dental surgeries [1,2]. However, due to its hardness and brittleness, HAp ceramics are difficult to shape into the specific forms required for bone substitution. HAp powder, used for the treatment of bone defects, has also the problem that it easily migrates from the implanted sites. Novel composites of HAp and organic polymers are arousing great interest because of their potential to compensate for such mechanically weak properties of HAp [3,4].

Among the organ polymers, chitosan is an N-deacetylation product of chitin. It consists of glucosamine and N-acetylglucosamine units linked with 1-4 glycosidic bonds. In commercially available chitosan products, the acetyl content rates vary from 5 to 30 % due to imperfect N-deacetylation of chitin. The most important feature of chitosan is its biodegradability [5], while it has a good solubility in various organic acid solutions and sufficient resistance to alkali environments. Furthermore, chitosan is flexible and has high resistance upon heating due to the intramolecular hydrogen bonds formed between hydroxyl and amino groups[6-8]. The composite biomaterial of HAp and chitosan is therefore thought to exert increased osteoconductivity and biodegrading activity, with sufficient mechanical strength for orthopedic or dental use. To this day, various methods to develop HAp/chitosan composites have been reported, such as mixing HAp powder in a chitosan solution[9], or coating a chitosan sheet with HAp particles[10]. However, the composites prepared by those methods were not homogenous microscopically and often caused inflammation when implanted. By using the co-precipitation and porogen-leaching method, we recently developed a novel

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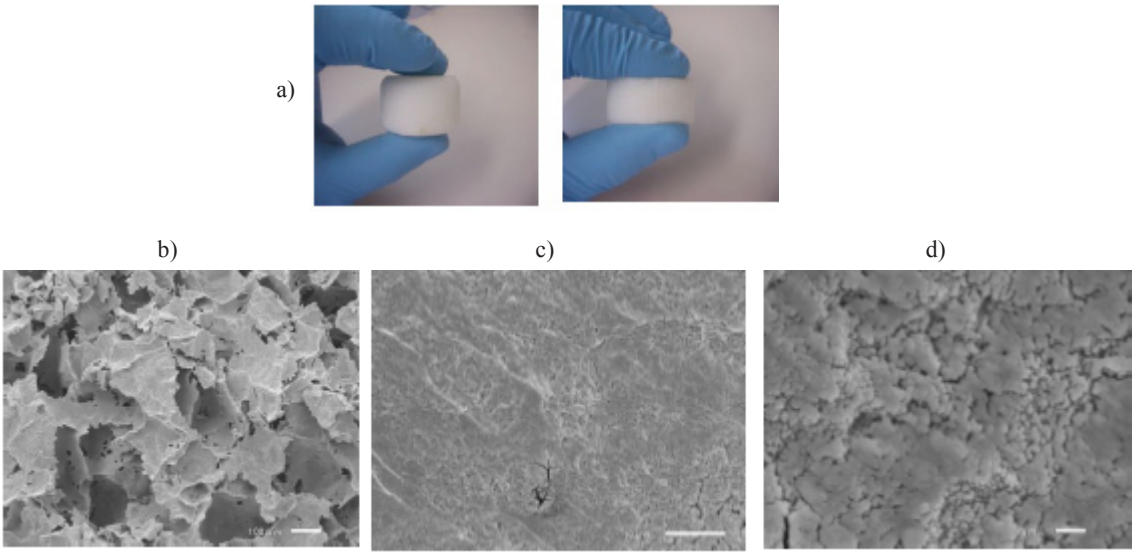

Figure 1 Macroappearance and scanning electron microscopic (SEM) photographs of the porous chitosan/HAp composite. (a) The spongy composite is mechanically flexible and could be easily formed into any desired shape; if deformed with force, it soon recovers the original form by itself.
(b) The interconnected porous structure is observed in cross-sectional SEM images. Porosity: 60–90%, pore diameter: 100–300 μm. (c)Higher magnification of (b). (d) Higher magnification of (c). Scale bar = 100 μm (in b) or 10 μm (in c) or 1 μm (in d).

homogeneous chitosan/HAp composite with various porosities, in which the HAp nanocrystals align along the chitosan molecules[11]. The spongy composite was found to be mechanically flexible and easily formed into any desired shape.

One of the important points in clinical use of biomaterials is that they must be not only biocompatible but also stable immediately after they are implanted. To facilitate bone formation and shorten the time for bone union, recombinant human bone morphogenetic proteins (rhBMPs) are available, which are known to induce ectopic bone formation in skeletal and nonskeletal sites[12-14]. To introduce rhBMPs, biocompatible carriers are indispensable, and many carriers have already been reported[15,16]. For example, β-tricalcium phosphate (β -TCP) [17], biphasic calcium phosphate (BCP) [18], and insoluble bone matrix (IBM) [19] have good osteoconductive activities, but their mechanical properties are far inferior to cortical bone tissue; ceramics[1,2] shows a superior osteoconductive activity and mechanical property, but it is not bioabsorbable and thus clinical problems may occur after its implantation. For these reasons, to develop a new biocompatible carrier to introduce rhBMPs is required.

The present study was designed to evaluate the osteoinductive efficacy of a newly developed porous chitosan/ HAp composite as a bioabsorbable carrier of rhBMP-2 in the subcutaneous tissue of rats.

Materials and Methods

Synthesis of the porous chitosan/ HAp composite

Chitosan powder was obtained from Hokkaido Soda Co. (Tomakomai, Japan). The degree of deacetylation was estimated to be 96.0 % from measurements of ^{1}H-NMR and the weight-averaged molecular weight being 9.4×10^4 Da. measured with the use of gel permeation chromatography.

The chitosan/HAp composite was synthesized by our previous method[20]. A chitosan aqueous solution of 3 wt % was prepared by dissolving chitosan powder into distilled water containing 1 wt % acetic acid. Then the solution was added into an 8.5 wt % H_3PO_4 solution. The ratio of chitosan to H_3PO_4 was adjusted for the final chitosan/HAp composition to 70/30 because of suitable mechanical properties for clinical use. The obtained chitosan/ H_3PO_4 solution was then added drop by drop into 0.5M $Ca(OH)_2$ suspension with vigorous stirring until pH 9 ± 0.2; then chitosan/ HAp composite was coprecipitated. The reaction temperature was kept at 25°C, and the dropping speed was controlled at 3.2 mL/ min with the use of a tube pump. The resulting slurry was aged for 24 h upon continuous stirring. Then, the precipitate was filtered and washed with distilled water.

The porogen-leaching method was used to produce porous chitosan/ HAp composite for tissue engineering applications. The 70/30 chitosan/ HAp composite and NaCl particles (particle size range: 100-300 μm) were homogenously mixed at weight ratios of 1:1, 1:5, 1:10, and 1:20. The slurry obtained was mixed with the NaCl particles and stirred. The mixture was subsequently compressed into a cylindrical form under a uni-axial pressure of 20 MPa for 24 h, followed by dehydration. After pressing, NaCl particles were subsequently removed from the scaffolds by extraction in water (approx. 20°C), followed by extraction in warm

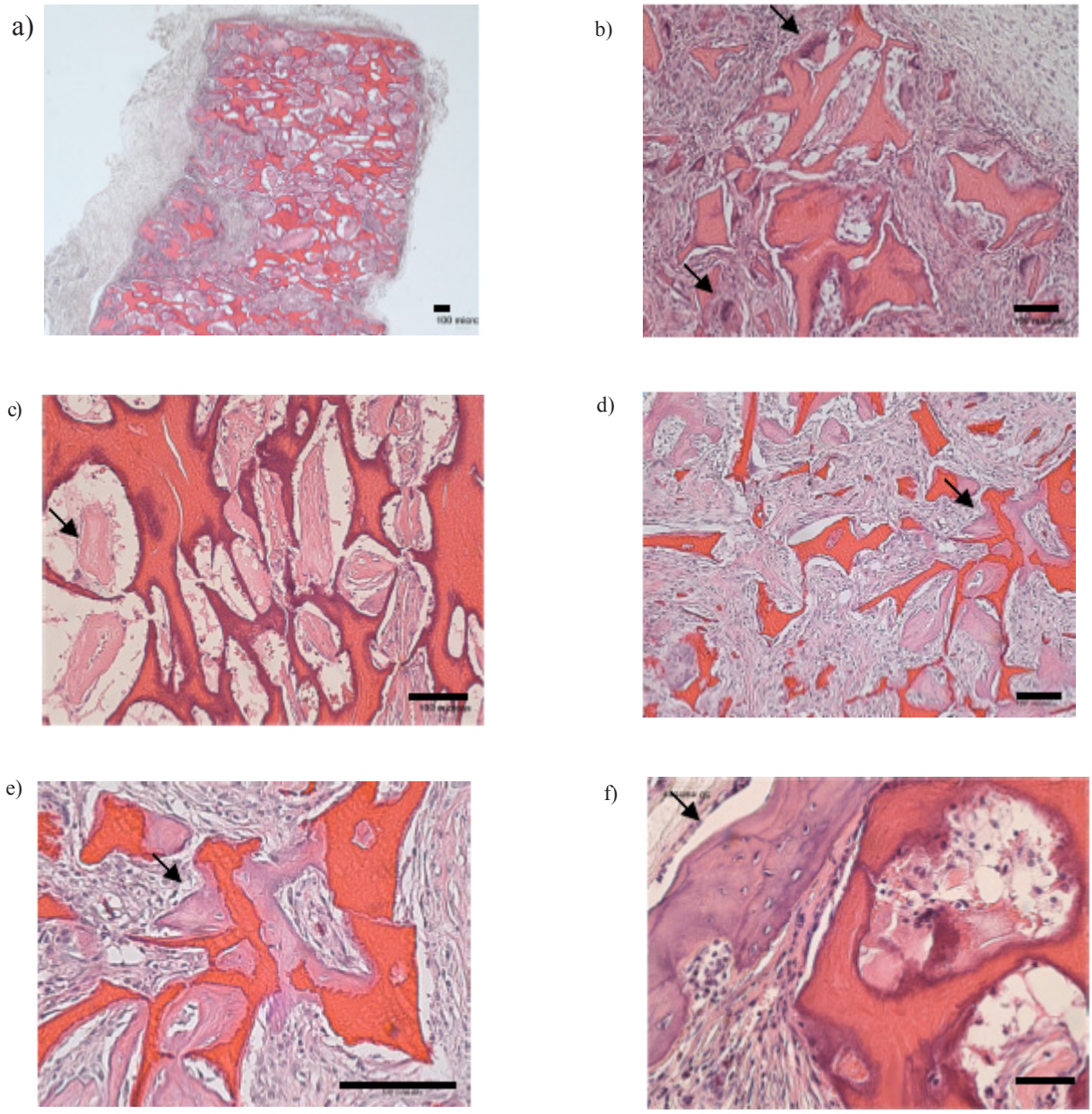

Figure 2 HE-stained histological sections of the composites subcutaneously implanted into the backs of SD rats.
(a) chitosan/HAp alone at 2 weeks. The composite alone caused little inflammation, and (b) new blood vessel growth and multinucleated giant cells (arrow) were found around the composite accompanied with roughening of the surface due to degradation. (c) chitosan/HAp alone at 8 weeks. Degradation of the composite in the surface and the depth proceeded, and the total size of the composite decreased remarkably at 8 weeks. Dense connective tissues were seen (arrow). Neither bone nor cartilage was seen in any of composites without rhBMP-2.
(d) chitosan/HAp with rhBMP-2 at 2 weeks. New bone formation (arrow) occurred on the surface of the composite. (e) Higher magnification of (d). (f) chitosan/HAp with rhBMP-2 at 8 weeks. Maturation of the newly formed bone (arrow) covering the composite was seen through a trabeculae structure development. The bioabsorption of the composite was accelerated as BMP-2-induced bone matured. Scale bar = 100 μm (in a, b, c, d, e) or 50 μm (in f).

water (approx. 50°C) for about 12 hours each. Figure 1 shows a macroappearance and scanning electron microscopic (SEM) photographs of the composite. The spongy composite was found to be mechanically flexible and could be easily formed into any desired shape; it soon recovered the original form by itself, if deformed with forces. The interconnected porous structure was observed in SEM images (porosity: 60–90%, pore diameter: 100–300μm) [11]. For all the composites, comparatively homogeneous

Table 1 The changes in percentage proportion of bone tissue, chitosan/HAp comosite, and other connective tissues to total volume of subcutaneous implant

	2 weeks			8 weeks		
	BT	composite	CT	BT	composite	CT
chitosan/HAp	0	66.7 ± 2.1	33.3 ± 2.5	0	53.3 ± 5.0	46.7 ± 2.5
chitosan/HAp+rhBMP-2	5.3 ± 2.1*	54.0 ± 3.0*	40.7 ± 1.4*	11.3 ± 2.5*	26.7 ± 4.0*	62.0 ± 3.0*

The total volume is designed as 100%, BT: bone tissue, CT: other connective tissues. Values are mean ± standard deviation.
* $P<0.05$ relative to chitosan/HAp alone. N = 3.

distribution of Ca and P was confirmed from the Ca or P-X-ray images by EPMA (Electron Probe Micro Analyzer) analysis.

The composite was cut into pieces of 3 x 3 x3 mm in size and sterilized with saturated steam at 120°C for 20 min using an autoclave (Pasolina IST-150, Iuchi, Japan) and soaked in a rhBMP-2 solution (0 and 5μg of rhBMP-2 in 20 μl of phosphate-buffered saline solution; PEPROTECH Co., London) in a sterilized culture dish.

Subcutaneous implantation

Eleven-week-old Sprangue Dawler (SD) male rats were used for animal experiments. The surgery and animal care conformed to the Guideline for the Care and Use of Animals of Hokkaido University. The rats were operated on under general anesthesia (Nembutal, 40 mg kg^{-1} body weight, Dainippon Sumitomo Pharma Co.,Ltd., Osaka, Japan). After their backs were shaved and disinfected with 70% ethyl alcohol, shallow incisions were made in the skin of their backs, and pockets were created. The composite with or without rhBMP-2 (5μg) was subcutaneously inserted in the left pocket (below the subcutaneous muscle layers) and the skin was closed with cotton sutures. Three rats were sacrificed respectively at 2 and 8 weeks after the implantation.

Histological observations

The implanted pieces of the composite were removed and fixed in 10% neutral buffered formalin, dehydrated and cleared with graded alcohols and xylol, and embedded in paraffin wax. Sectioned at 5 μm thickness with a microtome, the paraffin wax-embedded specimens were stained with hematoxylin and eosin (H & E). Histological observation and recording were carried out with an optical microscope (Olympus BX-51, Tokyo, Japan). For morphometric analysis, tissues of the implant areas at 2 and 8 weeks were respectively divided into three parts: bone tissue that included newly formed bone and marrow, the composite residues that had little or no cellular invasion, and connective tissue that contained a certain number of mesenchymal cells. Those three parts were measured respectively by using Weibel's method[21,22] at three separate points 100 μm apart, the midpoint being at the center of the implant. The average values of the three points respectively at 2 and 8 weeks after the implantation were compared. The statistical significance of the data was evaluated by Mann-Whitney U-test.

Results

Histological findings

The chitosan/HAp composite alone caused little inflammation(Figure 2a), and new blood vessel growth and multinucleated giant cells were found around the composite, accompanied with roughening of the surface due to degradation of the composite at 2 weeks (Figure 2b). Degradation of the composite in the surface and the depth proceeded, and the total size of the composite decreased remarkably at 8 weeks (Figure 2c). However, neither bone nor cartilage was seen in any of composites without rhBMP-2.

In contrast, on the surface of the composite with rhBMP-2, ectopic bone formation occurred at 2 weeks (Figure 2d, e). Absorption of the composite proceeded in the surface and depth. Blood vessel and connective tissue invaded in the pores. At 8 weeks, maturation of the newly formed bone prevailed over the composite through a trabeculae structure development (Figure 2f). The bioabsorption of the composite was accelerated as rhBMP-2-induced bone matured.

Histomorphometrical analysis

The morphometric results are shown in Table I. The chitosan/HAp composite with rhBMP-2 implant showed 5.3 % at 2 weeks and 11.3 % at 8 weeks of the total volume of bone and marrow, while the composite implant alone showed 0 %. The mean percentages of the newly formed bone in the composite implanted with rhBMP-2 were 5.3 % at 2weeks and 11.3 % at 8 weeks after the implantation whereas those in the composite without rhBMP-2 were 0 % at the post-implantation points. The mean value of the composite areas with rhBMP-2 was significantly smaller than that without rhBMP-2 at 2 and 8 weeks after the implantation ($p < 0.05$).

Discussion

Many reports show that HAp ceramics may be used as substitutes for autogenous bone grafts in bone defects[23-26]. The disadvantages of HAp ceramics are, however, (a) fragility under

mechanical stress, especially to a torsional force, and (b) inability of rigid osteosynthesis at the implanted site. Although HAp shows bone conductive activity and is able to unite with bone in the recipient, the clinical value of HAp remains to be improved, as new bone formation can not be obtained within a short period of time after its implantation, and as it is not finally replaced with bone. To facilitate the completion of rigid bone union, and to prevent a possible implant failure after implantation, rhBMPs may be used. However, rhBMPs must be implanted with an appropriate carrier; otherwise these proteins are not able to induce bone formation because their molecules are immediately dispersed. Namely, biocompatible carriers are indispensable to effectively introduce rhBMPs; porous HAp has osteoconductivity and is able to act as a carrier of rhBMPs, but it is poorly resorbed and problems as above are likely to arise.

We developed a novel chitosan/HAp composite with various porosities, using the co-precipitation and porogen-leaching method[11]. The spongy composite was found to be mechanically flexible and could be easily formed into any desired shape; it does not easily break either with tensile or compressive force, and if deformed with force, it soon recovers the original form by itself. The mechanical properties of the composite have been improved by heat treatment with saturating steam, which is ascribed to the formation of hydrogen bonds among chitosan molecules[11,20]. The elasticity and flexibility will contribute to easy handling in surgical operations. SEM observations confirmed the interconnected porous structure (porosity: 60–90%, pore diameter: 100–300 μm). A previous research suggested that human osteoblasts can penetrate pores >20 μm in size[27], and the porosity needs to be >30% to achieve interconnection[28]. The porosity of the composite scaffolds we developed was sufficient for good interconnection and supply of nutrition. Also, the pore size of the scaffolds could support the growth of cells. To evaluate the efficacy of the newly developed composite as a carrier of rhBMP-2, we subcutaneously implanted it into the backs of SD rats, with or without rhBMP-2 (5 μg). The composite with rhBMP-2 implant resulted in ectopic bone formation and the accelerated bioabsorption of the composite harmonized with bone remodeling. These results suggested that the novel chitosan/ HAp composite was an effective bioabsorbable material for the delivery of rhBMP-2 and bone engineering.

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