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Enhanced bone formation using hydroxyapatite ceramic coated with fibroblast growth factor-2

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ABSTRACT

Our objective was to develop a bone substitute coated with fibroblast growth factor-2 (FGF-2) that subsequently releases FGF-2. We investigated the use of our system of bone substitutes to induce bone formation. Hydroxyapatite ceramic buttons (HAP-CBs) were coated with FGF-2 by precipitation in supersaturated calcium phosphate solution. HAP-CBs were coated with high or low doses of FGF-2, denoted as FGF-H and FGF-L. The release of FGF-2 from FGF-H and FGF-L was evaluated using its release profile and bioactivity. The efficacy of the subsequent bone formation was quantified using rats with round-shaped bone defects (5 mm in diameter) of the right parietal bone. Group 1 was treated only with HAP-CBs, group 2 with HAP-CBs and drops of FGF-2 solution, group 3 with FGF-L and group 4 with FGF-H. To detect the release of FGF-2 in vivo, the expression of bone morphogenic protein-2 (BMP-2) was measured in the defective bone tissue. FGF-2 was released in vitro from FGF-H and FGF-L, and maintained its bioactivity. Rats treated with FGF-L showed better bone formation than rats from the other groups. BMP-2 expression was detected in the defective bone tissues of group 3 at 14 days, which might indicate in vivo FGF-2 release during this period. A specific FGF-2 concentration may be needed for bone formation, and our system can release FGF-2 at adequate concentrations to induce bone formation.

1. Introduction

Recently, many studies have reported that certain proteins, such as fibroblast growth factor-2 (FGF-2) [1–3], transforming growth factor-β (TGF-β) [4] and bone morphogenetic proteins (BMPs, which belong to the TGF-β superfamily) [5–8], can stimulate bone formation. As clinical medicines, safety and quality are necessary conditions for their use. FGF-2 is one of the most promising and practical options because it has already been approved as a clinical pharmaceutical in Japan and is commercially available. When cytokines such as FGF-2 are administered in vivo, a large proportion is rendered inactive due to degradation or non-specific binding [5,9]. In general, the delivery system cannot maintain the in vivo effects of FGF-2 for very long. Therefore, a new delivery system is needed for the administration of FGF-2 to be useful [3].

The ability to hold and release various kinds of proteins, including FGF-2, has been studied previously [10,11]. In studies using cytochrome C (cyt C) as a harmless dummy protein, cyt C was effectively coated to a hydroxyapatite (HAP) ceramic, forming a cyt C/calcium phosphate composite layer in supersaturated calcium phosphate solutions prepared by mixing several types of infusion fluids [10]. Because the molecular weight and isoelectric focusing point of FGF-2 are analogous to those of cyt C, an FGF-2/calcium phosphate composite layer would be expected to form on HAP ceramic [11] and then be slowly released from it.

HAP has already been applied to various clinically approved bone substitutes, such as the burr-hole buttons that are used to repair cranial defects. HAP causes minimal foreign-body reactions and acts as an osteoconductive material, binding easily to bone [12,13]. Therefore, HAP may be a good material with which to repair bone defects, such as in cranioplasties, and may also be a suitable substrate for binding FGF-2.

In this study, an FGF-2/calcium phosphate composite layer was formed on a HAP ceramic button (HAP-CB) using clinically approved pharmaceutical solutions and FGF-2. The amount of FGF-2 released and the biological activity of the resulting material were studied to confirm its abilities both in vitro and in vivo.

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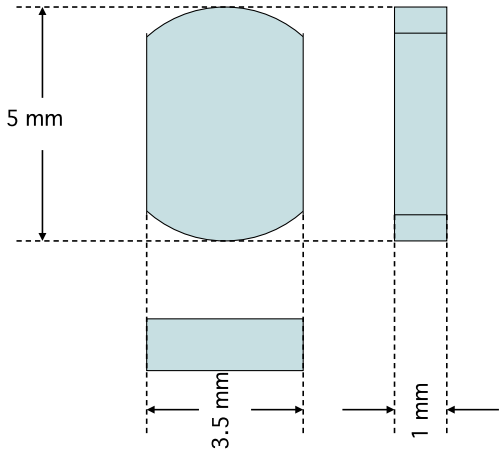

Fig. 1. Three-dimensional views of the HAP-CB, which was made for cranial repair (cranioplasty) in rats. Both sides of the HAP-CB were cut in order for bone formation to extend into the space around the bone defect.

2. Materials and methods

2.1. Preparation of HAP-CB

Pure, stoichiometric HAP powder was supplemented with 3 wt.% polyvinyl alcohol and 1 wt.% polyethylene glycol, sieved to select only particles under 75 μm in size and then formed into disks at 98 MPa and sintered at 1150 °C for 1 h. The resulting shape of HAP-CB is shown in Fig. 1, with a surface area of 15.94 mm^2 and a mean thickness of 1.00 mm per button. The HAP-CB was designed for a round cranial bone defect 5 mm in diameter, and its sides were cut bilaterally to permit bone formation into the space that was created by cutting.

2.2. FGF-2 solution

An FGF-2 solution was prepared by dissolving FGF-2 (Fiblast®, Kaken Pharmaceuticals, Japan) in a sterilized physiological salt solution at the final concentration of 100 μg ml^{-1}. Fiblast is a pharmaceutical human recombinant FGF-2 containing an undisclosed amount of sucrose, ethylene diamine tetraacetic acid, and a pH adjustment agent.

2.3. Preparation of the other solutions

A calcium-containing solution, a phosphate-containing solution and an alkalinizer (Table 1) were prepared by dissolving reagent-grade KCl, $CH_3COONa{\cdot}3H_2O$, $NaHCO_3$, KH_2PO_4, Xylitol (Wako Pure Chemical Industry Ltd., Japan), NaCl, $CaCl_2{\cdot}2H_2O$, $K_2HPO_4{\cdot}3H_2O$ and $MgCl_2{\cdot}6H_2O$ (Nacalai Tesque Co., Japan) in ultrapure water. These solutions are equivalent in their chemical composition to the clinically available infusion fluids. The calcium-containing solution corresponds to Ringer's solution (Otsuka Pharmaceutical Co., Ltd., Japan), including 147 mM Na^+, 4.00 mM K^+, 2.25 mM Ca^{2+}, and 157 mM Cl^- at pH 6.43. The phosphate-containing solution corresponds to Klinisalz® B (I'rom Pharmaceutical Co., Ltd., Japan) and contains 45.0 mM Na^+, 25.0 mM K^+, 2.50 mM Mg^{2+}, 45.0 mM Cl^-, 10.0 mM $H_2PO_4^-$, 20.0 mM CH_3COO^- and 333 mM xylitol at pH 5.87. The alkalinizer is an $NaHCO_3$ solution corresponding to Bifil® (AJINOMOTO PHARMA Co., Ltd., Japan) and contains 166 mM Na^+ and 166 mM HCO_3^- at pH 8.28.

Table 1
Preparation of the solutions and agents used in this study, all of which have been approved for clinical usage in Japan.

	FGF-L (ml)	FGF-H (ml)	HAP-N (ml)
Calcium-containing solution (Ringer's solution)	1.518	1.423	1.423
Phosphate-containing solution (Klinisalz® B)	0.228	0.213	0.213
Alkalinizer (Bifil®)	0.174	0.164	0.164
FGF-2 solution (prepared from Fiblast®)	0.080	0.200	0
Physiological salt solution	0	0	0.200
Total	2.000	2.000	2.000

2.4. Formation of a calcium phosphate composite

The above-mentioned solutions were mixed to prepare supersaturated calcium phosphate solutions that included the FGF-2 solution. The supersaturated calcium phosphate solution with FGF-2 was filtered using a membrane with a pore size of 0.22 μm. HAP-CBs were immersed in 2 ml of the supersaturated calcium phosphate solution for 24 h at 25 °C. Two conditions were chosen for the FGF-2 coating with precipitation: a high-dose FGF-2 coating, which was named "FGF-H", and a low dose FGF-2 coating, which was named "FGF-L". An HAP-CB was also prepared under FGF-H conditions but without FGF-2, and this condition was named "HAP-N". All of the HAP-CBs were used immediately after washing with 2 ml of phosphate-buffered saline (PBS). The surfaces of the HAP-CB, FGF-L, FGF-H and HAP-N were observed using a scanning electron microscope (SEM; JSM-5500LV, JEOL Ltd., Japan).

2.5. In vitro FGF-2 release assay

FGF-L and FGF-H buttons were immersed in 2 ml of Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY) without fetal bovine serum (FBS) and allowed to sit at 37 °C for up to 16 days. DMEM was used to simulate the in vivo setting because it contains various minerals at concentrations close to those found in the body fluid. At each measured time point, 0.15 ml of DMEM was added after collecting 0.15 ml of sample from each well. The FGF- 2 concentration in these samples was measured with an ELISA (human Fibroblast Growth Factor-2 ELISA kit, Calbiochem, EMD bioscience, CA), and the amounts of the released FGF-2 were calculated.

2.6. In vitro biological activity induced by FGF-2

BHK-21 cells (RIKEN BioResouce Center, Tsukuba, Japan), which are fibroblastic cells derived from hamster and can respond to FGF-2, were used to evaluate the biological activity of FGF-2. MG-63 cells (RIKEN BioResouce Center), which are osteoblastic cells derived from humans, were used to evaluate the FGF-2 concentrations for bone formation. The BHK-21 cells were maintained in DMEM containing 10% FBS, and the MG-63 cells in modified Eagle's medium (MEM; Gibco-BRL) with 10% FBS. Cells (1×10^4 $well^{-1}$) were seeded on 24-well culture plates. In some wells, FGF-2 was added to the medium at each concentration, and FGF-H or FGF-L buttons were placed on the bottom of the other wells. The cells were cultured for 3 days at 37 °C in a humidified CO_2 incubator with 1 ml of medium without FBS. After this step, the cells were detached via a trypsin treatment and counted with a counting chamber slide. The wells grown with 10% FBS were used as a positive control. In some wells, bone morphogenetic protein-2 (BMP-2) expression was evaluated by the Mini Opticon real-time PCR system (Bio-Rad Laboratories Inc.).

2.7. Animal experiments

During all of the experiments that were approved by the Institutional Animal Care and Use Committee, the animals were housed