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Angiogenesis therapy for brain infarction using a slow-releasing drug delivery system for fibroblast growth factor 2

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ABSTRACT

Although fibroblast growth factor 2 (FGF2) is a promising agent for treating brain infarction, current methods of FGF2 administration are associated with a short circulating half-life. An FGF2 apatite coating was developed as a slow-releasing drug delivery system (DDS) by forming an FGF2/calcium phosphate composite layer. Hydroxyapatite was coated with high or low doses of FGF2, denoted as FGF-high and FGF-low. This study investigated the efficacy of the coating as angiogenesis therapy for brain infarction. Rats were subjected to permanent occlusion of the middle cerebral artery, an FGF2 apatite-coated implant was inserted, and the rat brains were removed 2 weeks after implantation. Rats in groups treated with FGF-high had significantly smaller areas of brain infarction, particularly in the external capsule and the lateral side of the putamen, and better capillary density than rats in groups treated with non-FGF2 apatite-coated implants. Histologic analysis indicated that the new vessels were larger and had thicker walls in the FGF2 apatite-coated groups than in the non-FGF2 groups. Fluorescence immunohistochemistry of the peri-infarction region showed that FGF2 released from FGF2 apatite-coated implants might have biological activity. Moreover, fluorescence immunohistochemistry showed that released FGF2 influenced microglia cells. This new FGF2 DDS involving an FGF2 apatite coating can prevent infarction of the penumbra through the multipotential effects of FGF2.

1. Introduction

Fibroblast growth factor 2 (FGF2) is involved in the regulation of neuronal cell differentiation and survival, as well as maintenance and proliferation of glia, fibroblasts, endothelial cells, and other cell types [1,2]. Intrinsic FGF2 levels in the brain are known to increase following focal brain infarction and the level of serum FGF2 could be useful in creating estimates of infarction volume and clinical prognosis [3]. Animal experiments have shown that FGF2 administered intravenously within hours after stroke onset reduces the volume of an infarction, presumably due to direct protection of cells at the penumbra of the infarction area [1]. In contrast, delayed administration of FGF2 may play a role in functional recovery, probably due to the effects on axonal sprouting and new synapse formation in intact brain tissue rather than a reduction in infarction volume [1,4]. Furthermore, the effects of FGF2 vary according to the timing of administration and, importantly, the FGF2 effect is long lasting. However, FGF2, when administered intravenously, has a short circulating half-life and is sequestered rapidly in several organs, including the kidney, liver, and spleen [5,6]. To date, an effective system for the delivery of FGF2 to the brain to limit infarction volume has not been developed.

Previous studies have described a slow-release drug delivery system (DDS) for FGF2. Using this method, FGF2 was coated onto a hydroxyapatite ceramic, forming an FGF2/calcium phosphate composite layer in supersaturated calcium phosphate solution [7], which would then slowly release FGF2 in vivo [8]. The FGF2/calcium phosphate composite layer was developed on a hydroxyapatite ceramic using clinically approved pharmaceutical solutions and FGF2 [9]. This coating system releases FGF2 at precise concentrations adequate for inducing bone formation in vivo [9].

The purpose of this study was to evaluate the therapeutic effects and safety of FGF2 apatite coating using models of permanent brain infarction.

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2. Materials and methods

2.1. Preparation of the FGF2 apatite coating

The procedure used to create the FGF2 apatite coating has been described previously [9]. Particles were pure sieved using 3 wt% polyvinyl alcohol and 1 wt% polyethylene glycol, to select particles < 75 μm in size. Particles were then formed into disks at 98 MPa and sintered at 1150 °C for 1 h. An FGF2 solution was prepared by dissolving FGF2 (Fiblast®; Kaken Pharmaceutical Co., Ltd., Tokyo, Japan) into a sterilized physiologic salt solution. A calcium-containing solution, a phosphate-containing solution, and an alkalinizer were prepared by dissolving reagent-grade KCl, CH_3COONa–$3H_2O$, $NaHCO_3$, KH_2PO_4, Xylitol (Wako Pure Chemical Industries, Ltd., Osaka, Japan), NaCl, $CaCl_2$–$2H_2O$, K_2HPO_4–$3H_2O$, and $MgCl_2$–$6H_2O$ (Nacalai Tesque, Inc., Kyoto, Japan) in ultra-pure water. These solutions are equivalent in their chemical compositions to infusion fluids available clinically. The above-mentioned solutions were mixed to prepare supersaturated calcium phosphate solutions that included the FGF2 solution. The hydroxyapatite ceramics were designed for a round cranial bone defect 5 mm in diameter with two opposing sides cut to form flat edges (Fig. 1A). The hydroxyapatite ceramics were immersed in 2 mL of the supersaturated calcium phosphate solution at 25 °C for 24 h. Hydroxyapatite ceramics were coated with either high-dose (10%) FGF2 (FGF-high) or low-dose (4%) FGF2 (FGF-low). As an additional control condition, a burr-hole button (BHB) was created using a hydroxyapatite ceramic treated under FGF-high conditions, but without FGF2 (Table 1).

2.2. In vitro FGF2 release assay

FGF-low and FGF-high were immersed in 2 mL Dulbecco's modified Eagle's medium (DMEM; Life Technologies/Gibco-BRL, Grand Island, NY, USA) without fetal bovine serum (FBS) and allowed to stand at 37 °C for up to 16 days. At each measured time point, a 0.15-mL sample was collected from each well and 0.15 mL of DMEM was added after sample collection. The FGF2 concentration in these samples was measured using an ELISA (Human Fibroblast Growth Factor 2 ELISA kit; Calbiochem, EMD Biosciences, San Diego, CA, USA) and the amounts of FGF2 released were calculated.

2.3. Animal experiments

All experiments were approved by the Institutional Animal Care and Use Committee. The animals were housed and handled in accordance with the guidelines of the National Institutes of Health. Eight-week-old male Sprague–Dawley rats (Charles River, Wilmington, MA, USA) were maintained on a 12 h light/dark cycle and allowed free access to food and water. Rats were anesthetized with isoflurane (4% induction, 2% maintenance) using an anesthesia machine 400 (Medicare Co., Ltd., Kanagawa, Japan). Body temperature was maintained at 37 °C with the use of a heating pad. Focal brain infarction was produced by occluding first the left common carotid artery (CCA) and then the left middle cerebral artery (MCA) just inferior to the rhinal fissure [10]. The left CCA was dissected free of connective tissue through a midline cervical incision and the vessel was permanently occluded with a 4–0 silk ligature. A skin incision was made between the left eye and the left ear hole. The temporal muscle was dissected to expose the left zygoma. The left MCA was exposed through a burr hole drilled (Leutor Mini Gold; Natsume Seisakusho, Co., Ltd., Tokyo, Japan) 2–3 mm rostral to the fusion of the zygomatic arch with the squamosal bone. The MCA was then electrocauterized and disconnected distal to crossing the olfactory tract. The above-described hydroxyapatite ceramic was then inserted into the craniotomy area. The skin incision was sutured to close. Animals were then returned to their cages and allowed free access to food and water. A 24-gauge cannula was introduced into the right femoral artery to monitor physiologic parameters (e.g., mean arterial blood pressure) (UB-103U; Unique Medical, Co., Ltd., Tokyo, Japan) and arterial blood was analyzed using iSTAT (Fuso Pharmaceutical Industries, Osaka, Japan).

Rats were randomly assigned to four groups. (1) BHB group ($n = 13$), in which a BHB was inserted; (2) an FGF-drop group ($n = 13$), in which 500 ng of FGF2 was dropped into the craniotomy area, then covered with a BHB; (3) an FGF-low group ($n = 13$); and (4) an FGF-high group ($n = 13$), in which the FGF-low and FGF-high implants were inserted, respectively. The cerebral blood flow (CBF) was measured at a point 2 mm rostral and 4 mm lateral to the bregma using laser Doppler (Advance, Tokyo, Japan), after the laser

Table 1
Preparation of the solutions and reagents.

	BHB (mL)	FGF-low (mL)	FGF-high (mL)
Calcium-containing solution	1.423	1.518	1.423
Phosphate-containing solution	0.213	0.228	0.213
Alkalinizer	0.164	0.174	0.164
FGF2 solution (100 μg/mL)	0	0.080	0.200
Physiological solution	0.200	0	0
Total	2.000	2.000	2.000

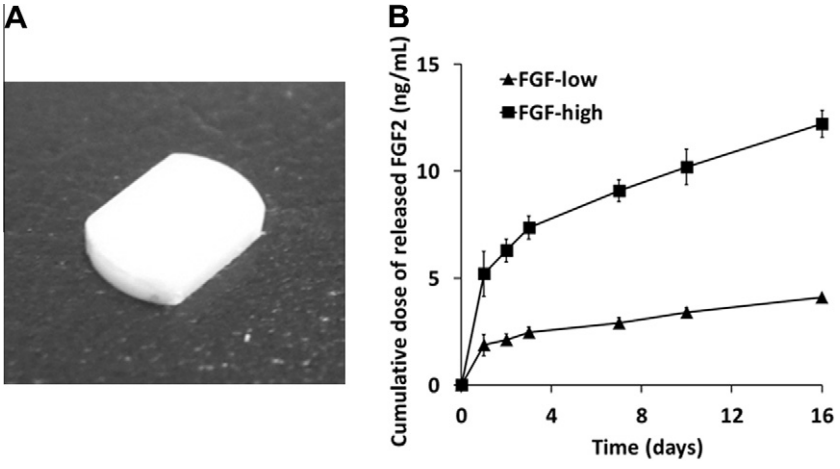

Fig. 1. The hydroxyapatite ceramic used in this study. (A) Photograph of the hydroxyapatite ceramic. (B) FGF2 release curves from the FGF-low (triangles) and FGF-high (squares) groups in DMEM. The results shown are the mean and standard deviations of three independent experiments.

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