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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

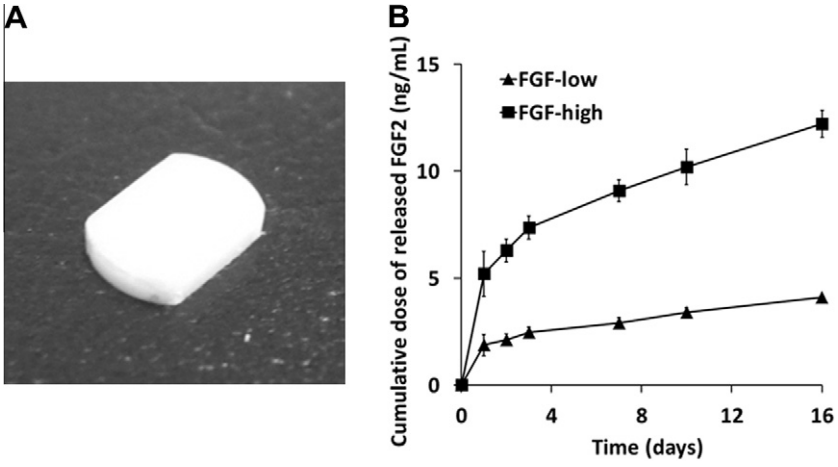

Fig. 1. The hydroxyapatite ceramic used in this study. (A) Photograph of the hydroxyapatite ceramic. (B) FGF2 release curves from the FGF-low (triangles) and FGF-high (squares) groups in DMEM. The results shown are the mean and standard deviations of three independent experiments.

Doppler probe was attached to the surface of the cranium for 30 s to stabilize CBF data [11]. The CBF was measured bilaterally on day 0 and day 14 after the procedure and was expressed as a percentage of the contralateral measurement.

2.4. Assessment of infarction area

On day 14 after the procedure, all rats were anesthetized with a lethal dose of intraperitoneal pentobarbital and perfused with phosphate-buffered saline through the left ventricle. The brains were carefully removed and sectioned into 2 mm coronal slices from the frontal pole. Every third section, 4–6 mm from the frontal pole, was transferred to 10% phosphate-buffered formalin for fixation. Brain sections were then embedded in paraffin and the fixed brain slices were sliced into 5 μm-thick coronal sections. The area of brain infarction in each animal was measured in a coronal section stained with hematoxylin & eosin (H&E). Infarction was defined as the area in which >90% of neurons were injured (necrosis or apoptosis) [11]. Each section was scanned microscopically (Biozero, BZ8000; Keyence, Osaka, Japan) and the area of the infarction was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and the ratio of the infarct area of the coronal section to the contralateral hemisphere (percentage of ipsilateral hemisphere) was calculated.

2.5. Immunohistochemical analysis of the brain infarction

For identification of neurons, brain sections were treated with microwave after deparaffinization and immunohistochemical analysis (IHC) was performed. For IHC, sections were incubated with mouse anti-human (rat) smooth muscle actin (SMA) antibody (M0851, 1:50 dilution; Dako Japan, Tokyo, Japan) in 1% bovine serum albumin (BSA) at 4 °C overnight. Sections were incubated using a LSAB 2 Kit (K0675; Dako Japan) for 10 min. 3-Amino-9-ethylcarbazole and hematoxylin were used as sensitive chromogens for light microscopy.

Fluorescence immunohistochemistry of brain sections was performed as follows. The sections were incubated with the following primary antibodies: a monoclonal mouse anti-human (rat) Factor VIII (M616, 1:50 dilution; Dako Japan) and a polyclonal rabbit anti-human (rat) VEGF (147) (SC-507, 1:100 dilution; SantaCruz, Santa Cruz, CA). After incubation, the sections were then incubated with the following secondary antibodies: Alexa Fluor 488 rabbit anti-mouse (1:1000 dilution; Molecular Probes) and Alexa Fluor 555 goat anti-mouse (1:1000 dilution; Molecular Probes). The sections were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Roche Diagnostics-Applied Science, Mannheim, Germany). Moreover, the section from the FGF-high group was incubated with the following primary antibodies: a monoclonal mouse anti-rat glial fibrillary acidic protein (GFAP) (2A5, 1:100 dilution; Abcam Japan, Tokyo, Japan), a polyclonal rabbit anti-human (rat) VEGF (A-20, 1:100 dilution; SantaCruz), a monoclonal mouse anti-rat ionized calcium-binding adaptor molecule 1 (Iba1) (1022-5, 1:50 dilution; Abcam Japan), and a polyclonal rabbit anti-human (rat) VEGF (A-20, 1:100 dilution; SantaCruz). After incubation, the sections were incubated with the following secondary antibodies: Alexa Fluor 488 goat anti-mouse (1:400 dilution; Molecular Probes) and Alexa Fluor 555 goat anti-rabbit (1:400 dilution; Molecular Probes). The sections were counterstained with DAPI.

Each section was analyzed microscopically (Biozero, BZ8000). In the peri-infarction region of IHC, the number of morphologically intact capillaries was counted to determine the capillary density using ImageJ software. A total of five different fields were randomly selected and the number of capillaries was assessed as the mean number of capillaries per square millimeter.

2.6. Statistical analysis

All values are expressed as the mean ± standard deviation. All data were analyzed using Student's *t*-test and analysis of variance. Differences with a value of $p < 0.05$ were considered statistically significant.

3. Results

3.1. In vitro release of FGF2 from the FGF-low and FGF-high groups

FGF-low released approximately 4.1 ng/mL of FGF2 and FGF-high released 12.2 ng/mL of FGF2. FGF-low and FGF-high continued to release FGF2 over 16 days (Fig. 1B). The total amount of FGF2 released by FGF-low and FGF-high was 8.2 ng and 24.4 ng, respectively.

3.2. Physiologic parameters

There were no significant differences in physiologic parameters, such as mean arterial pressure, pH, PO_2, PCO_2, or rectal temperature, between the groups during the procedures. No rats in any group died and there were no significant differences in body weight during the observation period.

3.3. Assessment of CBF

There were no significant differences in the CBF ratio at day 0. However, the CBF ratio at day 14 was significantly higher in the FGF-low (0.76 ± 0.06) and the FGF-high (0.77 ± 0.11) groups than in the BHB (0.51 ± 0.14; $p < 0.01$ and $p < 0.01$, respectively) and FGF-drop (0.58 ± 0.12; $p < 0.05$ and $p < 0.01$, respectively) groups.

3.4. Assessment of infarction area and capillary density

The area of the infarction, as a percentage of the ipsilateral hemisphere, was significantly smaller in the FGF-high group (33.9 ± 6.4%) than in the BHB (44.5 ± 10.4%; $p < 0.05$) and FGF-drop (46.2 ± 9.3%; $p < 0.01$) groups (Fig. 2A). Infarction regions were present in the cortex, external capsule, and lateral side of the putamen. The infarction regions in the FGF-high group were reduced in the external capsule and lateral side of the putamen, compared to the BHB and FGF-drop groups. IHC with anti-SMA antibody was performed at the peri-infarction region to evaluate angiogenesis. The capillary density was significantly greater in the FGF-low (66.6 ± 17.1 vessels/mm^2) and FGF-high (75.1 ± 22.5 vessels/mm^2) groups compared to the BHB (36.6 ± 6.0 vessels/mm^2; $p < 0.01$ and $p < 0.01$, respectively) and FGF-drop (31.3 ± 8.7 vessels/mm^2; $p < 0.01$ and $p < 0.01$, respectively) groups (Fig. 2B). The angiogenesis was prominent only the peri-infarction region, not in other brain regions. The vessels of the FGF-low and FGF-high groups were 10–30 μm in size and thick-walled, while almost every vessel in the BHB and FGF-drop groups was <10 μm in size (Fig. 2C–F).

3.5. Fluorescence immunohistochemistry of the peri-infarction region

Fluorescence immunohistochemistry of the peri-infarction region was performed to assess the biological activity of released FGF2 in vivo. There was marked expression of VEGF in the FGF-low and FGF-high groups compared with the BHB and FGF-drop groups (Fig. 3A–D).

Fluorescence immunohistochemistry of FGF-high group showed the expression of VEGF on GFAP-positive cells and Iba1-positive cells in the peri-infarction region (Fig. 3E and F).

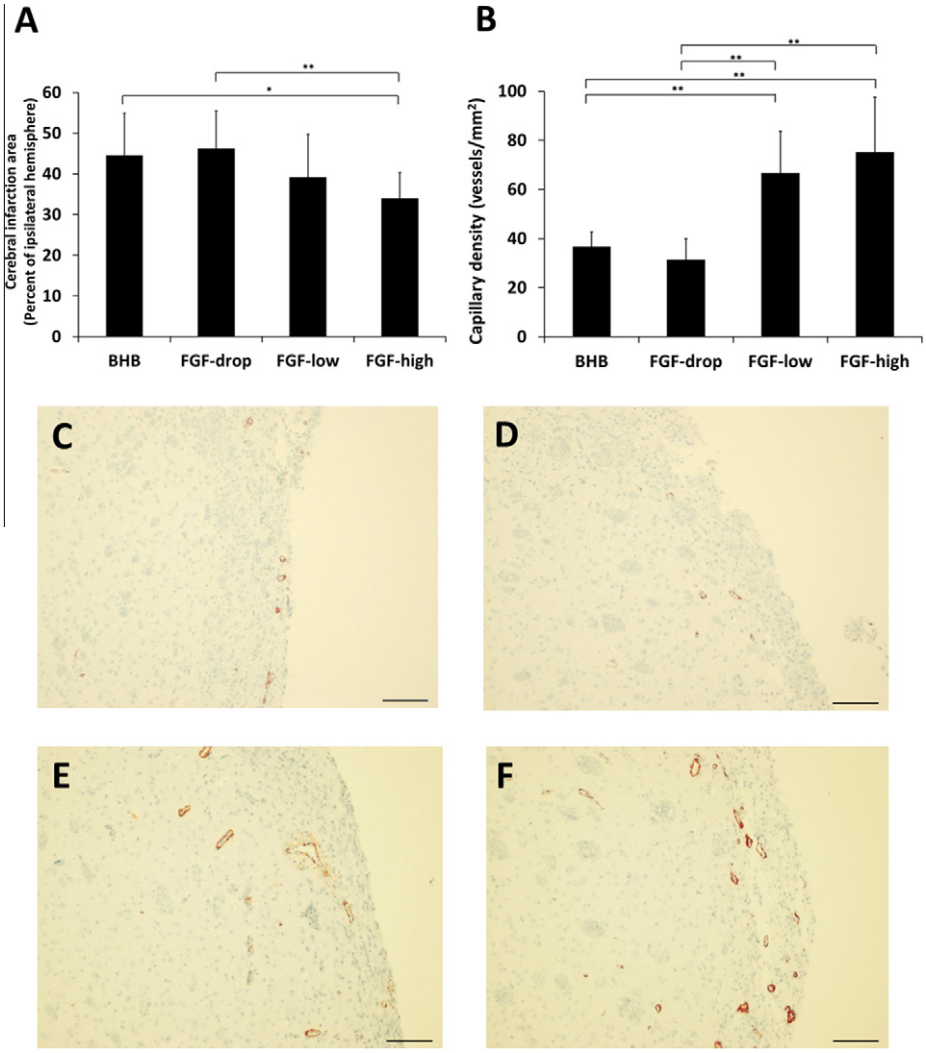

Fig. 2. The infarction area and capillary density on day 14 after infarction. (A) The infarction area was significantly smaller in the FGF-high groups ($^{*}p < 0.05$, $^{**}p < 0.01$). (B) Capillary density was significantly greater in the FGF-low and FGF-high groups than in the BHB and FGF-drop groups ($^{**}p < 0.01$). (C–F) The vessels in the FGF-low (E) and FGF-high (F) groups were larger than those in the BHB (C) and FGF-drop (D) groups. Scale bar = 200 μm.

4. Discussion

The FGF-high group demonstrated smaller infarction areas than the non-FGF2 apatite coating groups (the BHB and the FGF-drop groups). The infarction region (the external capsule and the lateral side of the putamen) forms the watershed regions of the MCA, ACA, and the perforators of the MCA. The peri-infarction region in this permanent MCA occlusion model forms the penumbra [10]. The penumbra in the FGF-high group was preserved from infarction. Angiogenesis was detected in all specimens in the peri-infarction region. Not only the FGF-high group but also the FGF-low group showed significantly increased angiogenesis, vessels of larger size, and endothelial cells with increased thickness. It is possible that the FGF2 apatite coating induced this effect on angiogenesis. Increased synthesis of angiogenic growth factors, such as FGF2 and VEGF and their receptors, was seen in the brain after brain infarction [3,12–15]. Intrinsic FGF2 can control angiogenesis after brain infarction by influencing other intrinsic growth factors, such as VEGF and chemokines [16–18]. Fluorescence immunohistochemistry showed that expression of VEGF was increased in the FGF-low and FGF-high groups. This result indicated that FGF2 released from FGF2 apatite-coated implants might have biological activity in vivo. Moreover, this result may indicate that continuous stimulation by released FGF2 promotes the release of intrinsic angiogenic growth factors, leading to an increase in angiogenesis in the peri-infarction region and a reduction in the area of the infarction. Immunohistochemical analysis of VEGF/GFAP and VEGF/Iba1 was used to investigate which type(s) of brain cells were influenced by released FGF2. Due to the expression of VEGF demonstrated in GFAP-positive cells and Iba1-positive cells, it is likely that glial cells and microglia are influenced by released FGF2. Released FGF2 is thought to effect brain infarction via two mechanisms: (1) FGF2 is absorbed into the vessel via arachnoid granulations and penetrates the area of infarction through the impaired blood brain barrier and (2) FGF2 is directly delivered to the brain parenchyma with inflammatory factors after the infarction [19].

These results indicate that FGF-high had more potent effects than FGF-low. A previous study suggested that strict control of the released FGF2 dose might be needed for bone formation, as osteoblast cells were stimulated when they were cultured with FGF2 concentrations of 3–10 ng/mL and osteoblast stimulation decreased at concentrations >30 ng/mL [9]. In contrast, microvessel formation from mouse embryonic aorta was increased at FGF2 concentrations of 10–50 ng/mL [20]. A maximum of 2.72 μg/cm^2 of FGF2 was immobilized in the composite layer formed on the

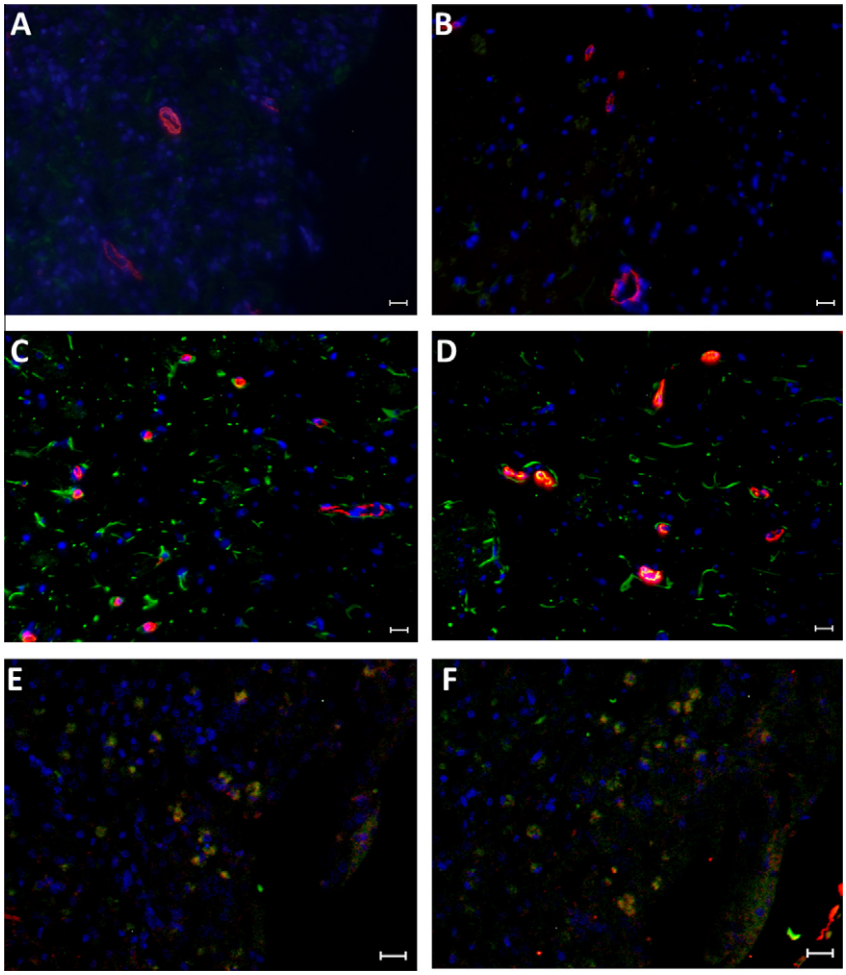

Fig. 3. (A–D) Fluorescence immunohistochemistry for Factor VIII (red), VEGF (green), and DAPI (blue). Each merged image was taken in the peri-infarction region. The expression of VEGF in BHB (A) and FGF-drop (B) groups was not increased. Marked expression of VEGF in FGF-low (C) and FGF-high (D) groups was noted not only around the vessels but also in brain tissue. (E) Fluorescence immunohistochemistry of the FGF-high group for VEGF (red), GFAP (green), and DAPI (blue). (F) Fluorescence immunohistochemistry of the FGF-high group for VEGF (red), Iba1 (green), and DAPI (blue). The expression of VEGF was shown on the GFAP and Iba1 positive cells in the peri-infarction region (E and F). Scale bars = 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

hydroxyapatite ceramic and approximately 1 μg/cm^2 of FGF2 was released from FGF2 apatite coating in a physiologic salt solution [7,8]. On the basis of calculations of the surface area of hydroxyapatite ceramic, the FGF-high and FGF-low conditions should have led to the release of approximately 150 ng and 60 ng of FGF2, respectively. Only a portion of the FGF2 was released in vitro because some of the released FGF2 might have been reabsorbed into the FGF2 apatite coating. Therefore, based on the current FGF2 release assay, it is difficult to accurately determine how much FGF2 is released from the FGF2 apatite coating. Nevertheless, the FGF-high condition reached an effective concentration for angiogenesis, while the FGF-low condition did not.

Preexisting arteriolar connections can be recruited to bypass the occluded region after brain infarction. This process involves the rapid proliferation of preexisting collateral arteries. These vessels are originally microvascular, thin-walled conduits that are composed of an endothelial lining, an internal elastic lamina, and 1 or 2 layers of smooth muscle cells [21]. The current findings suggest that it is impossible to form new large-diameter arteries with thick endothelial cells immediately after brain infarction. Profound angiogenesis has been observed in the brains of patients who have survived several days to weeks after brain infarction [13]. Moreover, it has been suggested that the vessels that stain for anti-SMA are mature vessels [22]. The anti-SMA-stained vessels observed in this study may represent profound angiogenesis induced from preexisting collateral arteries by released FGF2 and intrinsic angiogenic growth factors after infarction.

The advantage of an FGF2 apatite coating is that FGF2 and other fluids are approved for use as medicines by the Japanese government, meaning that their safety is guaranteed. Furthermore, the hydroxyapatite ceramic has already been applied to various clinically approved bone substitutes, such as the burr-hole buttons used to repair cranial defects. Intravenous injection of FGF2 has been reported to lower blood pressure in a dose-dependent fashion [23]. In this study, blood pressure was closely monitored and did not change significantly, nor did body weight increase significantly. Moreover, angiogenesis was only demonstrated in the peri-infarction region, although FGF2 released from an FGF2 apatite coating is expected to penetrate entirely into the cerebrospinal fluid through the subarachnoid space. This finding suggests that as intravenous FGF2 leaks into ischemic tissue and onto surroundings vessels through the damaged blood–brain barrier, the released FGF2 stimulates intrinsic angiogenic factors in the ischemic tissue [24]. Therefore, the observed adverse effects in this study were minor both with respect to the intracranial environment and the entire body. An FGF2 apatite coating can easily be removed in the event of a problem. The safety of other FGF2 slow-releasing DDSs have not been guaranteed and previous reports have indi-

cated that the safety of a FGF2 slow-releasing DDS using hydrogel and gene transfer cannot be guaranteed [22,25]. The cross-linker that is used to make the gels may be toxic to the nervous system in the FGF2-releasing system that uses hydrogel [26]. In addition, transferred genes cannot be removed if/when adverse effects occur in FGF2 gene transfer using viral vectors.

An FGF2 apatite coating, which is an FGF2 slow-releasing DDS, was created using FGF2 coated on hydroxyapatite ceramic. This system can promote intrinsic angiogenic factors due to slow release of FGF2 and can prevent infarction of the penumbra. An FGF2 apatite coating may be clinically effective for moyamoya disease and chronic brain ischemia.

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