Original Article

Basic Fibroblast Growth Factor as a Potential Stent Coating Material Inducing Endothelial Cell Proliferation

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***Aim*: The use of drug-eluting stents has reduced the incidence of in-stent restenosis following percutaneous coronary intervention; however, almost all drugs eluting from polymers on stents induce antiproliferative effects on vascular endothelial and vascular smooth muscle cells. Due to injury of the endothelium and delayed reendothelialization, the risk of thrombosis increases over time. Enhancing rapid reendothelialization after stent placement is important for solving these problems. Basic fibroblast growth factor (bFGF) is one of the most important growth factors involved in vascular lesion formation. In this study, we evaluated the potential of bFGF as a stent coating promoting endothelial cell proliferation.**

***Methods*: Human umbilical vein endothelial cells (HUVECs) and human aortic smooth muscle cells (HASMCs) were cultured with various doses of bFGF *in vitro*, and the effects of bFGF on the degree of cell proliferation and migration were monitored. We also investigated the effects of bFGF on the protein expression of endothelial nitric oxide synthase (eNOS) in HUVECs using Western blotting.**

***Results*: Cell proliferation and migration were promoted in HUVECs by bFGF in a dose- and time-dependent manner. On the other hand, bFGF stimulation had little effect on the HASMCs. Basic FGF increased the eNOS protein levels in the HUVECs, with a maximum at 10 ng/mL followed by a decline at 100 ng/mL.**

***Conclusions*: Basic FGF is a possible candidate stent coating and/or eluting drug material stimulating endothelial cell proliferation and early reendothelialization without excessive vascular smooth muscle cell proliferation.**

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Key words: Basic fibroblast growth factor, Stent, Reendothelialization, Human umbilical vein endothelial cells

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Introduction

Cardiovascular disease is one of the leading causes of morbidity and mortality worldwide. According to the World Health Organization, 7.3 million people (12.8%) died from cardiovascular diseases in 2008. Angioplasty with vascular stenting is a mini-

mally invasive percutaneous treatment for narrowing of the arteries, termed "stenosis," due to atherosclerosis.

A stent is a mesh "tube" inserted into a natural passage/conduit in the body to prevent or counteract a disease-induced, localized constriction in flow. Stents are widely used in patients with cardiovascular diseases. However, in-stent restenosis after stent placement remains a significant clinical problem. The primary causes of in-stent restenosis are acute/late thrombosis and neointimal hyperplasia[1]. Stent placement results in acute mechanical injury to the arterial wall and the activation of platelets, thereby inducing thrombosis accompanied by the recruitment of blood-borne monocytes, neutrophils and lymphocytes into the intimal area. These cells release cytokines that trigger the activation and proliferation of smooth muscle cells[2].

Although the introduction of drug-eluting stents has reduced the incidence of in-stent restenosis, recent clinical studies indicate that drug-eluting stents stimulate antiproliferative effects on endothelial and vascular smooth muscle cells[2]. Due to injury of the endothelium and delayed reendothelialization, late thrombosis induced by drug-eluting stents is a serious complication.

Therefore, enhancing rapid reendothelialization can stimulate prompt wound healing at denuded regions, consequently preventing late stent thrombosis and the cytokine-driven activation of vascular smooth muscle cells. Many attempts have been made to accelerate reendothelialization, including the use of endothelial cell preseeding[3], vascular endothelial growth factor (VEGF) transfection of human endothelial cells[4] and endothelial progenitor cell capturing methods[5].

Basic fibroblast growth factor (bFGF) is one of the most important growth factors involved in vascular lesion formation and is known to induce the migration and proliferation of endothelial cells, smooth muscle cells and fibroblasts and promote the production of type I collagen[6]. FGF, which was first discovered in extracts from the pituitary gland in 1973, is widely expressed in cells and tissues. Acidic FGF and bFGF were originally isolated from the brain and pituitary gland as growth factors for fibroblasts. Since then, at least 22 distinct FGFs have been identified or isolated. Moreover, bFGF is currently used as a treatment for skin ulcers[7]. However, to date, no studies have examined the effects of bFGF on endothelial cell proliferation/migration.

Aim

We evaluated the potential of bFGF as a candidate stent coating material with the ability to promote endothelial cell proliferation. As a preliminary experimental study, primary human umbilical vein endothelial cells (HUVECs) and human aortic smooth muscle cells (HASMCs) were cultured *in vitro*, and the effects of bFGF on the degree of cell proliferation and migration were monitored. We also investigated the effects of bFGF on the protein expression of endothelial nitric oxide synthase (eNOS) in HUVECs.

Methods

Ethics Statement

This study was approved by the Ethics Committee of Toho University Sakura Medical Center. The HUVECs were isolated as previously described and used at passages 3-5.

Materials

HUVECs and HASMCs were purchased from LONZA Walkersville, Inc. In addition, HUVECs were isolated from single donors as previously described[8, 9]. The cells were grown in endothelial cell basal medium-2 (Clonetics) containing 2% fetal bovine serum (FBS) and endothelial cell growth supplements provided by the company. Both the HUVECs and HASMCs were used at passages 3-5 in culture. Human recombinant basic fibroblast growth factor (rh-bFGF) was obtained from Kaken Pharmaceutical Co., Ltd.

Cell Proliferation

HUVECs were maintained in EBM-2 containing EGM-2 (Clonetics) and grown in a humidified incubator at 37℃ in a 5% CO_2 atmosphere. For the bFGF stimulation assays, the cells were seeded in 12-well plates at a density of 2.0×10^4 cells/mL and 1 mL/well in complete HUVEC medium. The next day, the medium was changed. After three days, the cells were washed twice with phosphate-buffered saline (PBS) and then subjected to a serum-starved culture overnight in EBM-2 containing 0.2% FBS. The cells were activated by the addition of 0, 1, 10 and 100 ng/mL of bFGF and maintained for the indicated time periods at 37℃ in a 5% CO_2 atmosphere.

After 24, 48 and 72 hours of incubation, the number of viable cells was measured using a WST-8 assay. Briefly, 50 μL of CCK-8 (Dojindo) was added to each well, and the cells were incubated at 37℃ for two hours, and the absorbance was measured at 450

nm (reference wavelength: 660 nm).

HASMCs were maintained in SmBM-2 containing SmGM-2 (Clonetics) and grown in a humidified incubator at 37℃ in a 5% CO_2 atmosphere. For the bFGF stimulation assays, the cells were seeded in 96-well plates at a density of 2.0×10^4 cells/mL and 100 μL/well. The next day, the medium was changed. After three days, the cells were washed twice with PBS and then subjected to a serum-starved culture overnight in SmBM-2 containing 0.2% FBS. The cells were activated by the addition of 0, 1, 10 and 100 ng/mL of bFGF and maintained for the indicated time periods at 37℃ in a 5% CO_2 atmosphere.

After 24, 48 and 72 hours of incubation, the number of viable cells was measured using a WST-8 assay. Briefly, 10 μL of CCK-8 (Dojindo) was added to each well, and the cells were incubated at 37℃ for three hours, and the absorbance was measured at 450 nm (reference wavelength: 660 nm).

Cell Migration Assay

For the *in vitro* assay of HUVECs and HASMCs, BD BioCoat™ FluoroBlok™ Fibronectin 3.0-μm PET Membrane 24-well cell culture inserts were used to provide a platform for the real-time analysis of samples using fluorescence-based detection[10]. A bottom-reading fluorescence plate reader is required to monitor the degree of fluorescence in the chamber located below the insert.

In detail, HUVECs were seeded at 1.0×10^6 cells/mL in EBM-2 containing 0.2% FBS at 250 μL/well in the top chambers of BD BioCoat™ FluoroBlok™ Fibronectin-coated 24-well insert plates, after which 750 μL of EBM-2 with 0.2% FBS containing 0, 1, 10 and 100 ng/mL of bFGF was immediately added to the bottom chambers. The resultant mixture was incubated for 24 hours at 37℃ in a humidified atmosphere of 5% CO_2. The medium was then removed from the upper chambers, and the insert chambers were transferred to additional 24-well plates containing 500 μL/well of 4 μg/mL of Calcein-AM in PBS, and the resultant mixture was incubated for 90 minutes at 37℃ in 5% CO_2. Images were subsequently obtained using a fluorescence microscope (Olympus). The number of migrated cells was measured according to the ratio of the area of fluorescence in four randomly chosen fields (×100 per field). The number of both HASMCs and HUVECs was measured using 0.2% FBS SmBM-2.

Western Blotting

HUVECs were maintained in EBM-2 with EGM-2 and grown in a humidified incubator at 37℃ in a 5% CO_2 atmosphere. For the bFGF stimulation assays, the cells were seeded in 6-well plates at a density of 2.0×10^5 cells/ml and 2 mL/well in complete HUVEC medium. When the cells reached subconfluence, the medium was aspirated, and the cells were washed twice with PBS and subjected to a serum-starved culture for 24 hours in 0.2% FBS medium. The cells were activated by the addition of 0, 1, 10 and 100 ng/mL of bFGF and maintained for 24 hours at 37℃. The process was terminated by washing the cells once with ice-cold PBS, and the cells were scraped from the surface of the plate. The cells were then placed on ice, and 1 mL of cold lysis buffer containing 1U benzonaze nuclease and a 1% (v/v) protease inhibitor mixture was immediately applied. The cell homogenate was incubated for one hour on ice and then centrifuged at 1,500 rpm for 10 minutes at 4℃. The supernatant was aliquoted and stored at −80℃ until further use. The protein concentrations were determined using a bicinchoninic acid assay (BCA; Sigma), with bovine serum albumin (BSA; Sigma) as the protein standard. The protein concentrations of the HUVEC samples were calculated from the linear region of the standard curve, freshly prepared for each experiment.

Western blotting was performed to examine the effects of bFGF on the protein expression of eNOS in the HUVECs using 10% SDS-PAGE mini and an STC-888 electrophoresis system (TEFCO). Individual protein bands were electrotransferred to an activated PVDF membrane with 0.45-μm pores (Hybond-P, GE Healthcare) over two hours at a constant voltage of 30 V at 4℃ in transfer buffer (25 mM Tris, pH 8.3, 192 mM Glycine, 20% v/v methanol). The membranes were washed with 0.05% (v/v) Tween 20/PBS (−), blocked overnight with blockace (DS pharma biomedical) and then probed with the appropriate primary antibody in 0.05% (v/v) Tween 20/PBS (−) for one hour at room temperature. The membranes were then washed and probed with the appropriate secondary antibody for one hour at room temperature. After the final washing step, protein bands were visualized using the ECL Plus Western Blotting Detection System (GE Healthcare). The following antibodies were used: rabbit anti-eNOS polyclonal (PA1-037; Thermo Scientific), mouse anti-actin (sc-8432; Santa Cruz Biotechnology, Inc.) and Horseradish Peroxidase Conjugated Second Antibodies (GE Healthcare).

Statistical Analysis

The data are presented as the mean ± SEM. Statistical comparisons were made using a one-way ANOVA followed by either Student's *t*-test or Dunnett's multi-

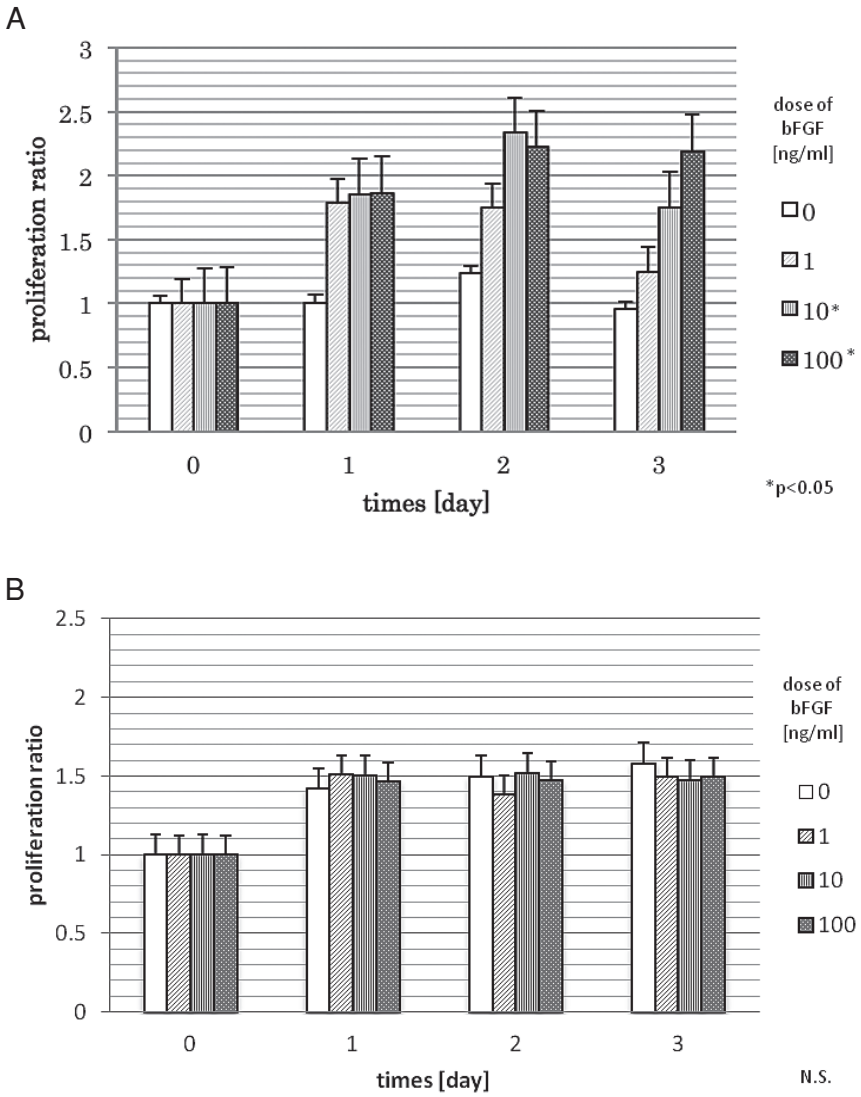

Fig. 1. Proliferation rates of HUVECs (Fig. 1A) and HASMCs (Fig. 1B) stimulated by bFGF.

ple-comparison test. A value of $p < 0.05$ was considered to indicate statistical significance.

Results

Effects of bFGF on the Proliferation of HUVECs and HASMCs

Fig. 1A shows the rates of proliferation of HUVECs in all samples stimulated by bFGF compared with that observed in the unstimulated control (rate = 1.0). Cell proliferation was promoted in HUVECs by bFGF in a dose- and time-dependent manner (**Fig. 1A**). Basic FGF (bFGF) stimulated cell proliferation, with a 70% increase over the control value at 1 ng/mL for 24 hours, reaching a maximum effect (~230%) at 10 ng/mL for 48 hours, then declining slightly (~210%) for 74 hours. On the other hand, bFGF stimulation had little effect on the proliferation of HASMCs, although the degree of proliferation increased by 50% compared with that observed in the unstimulated control (**Fig. 1B**). Each group contained a sample size of $n = 3$.

Effects of bFGF on the Migration of HUVECs and HASMCs

The cell migration assay was conducted using area-based measurements of the fluorescently stained cytoplasm, as described above. The sum of all areas of stained cytoplasm in each image was determined. The

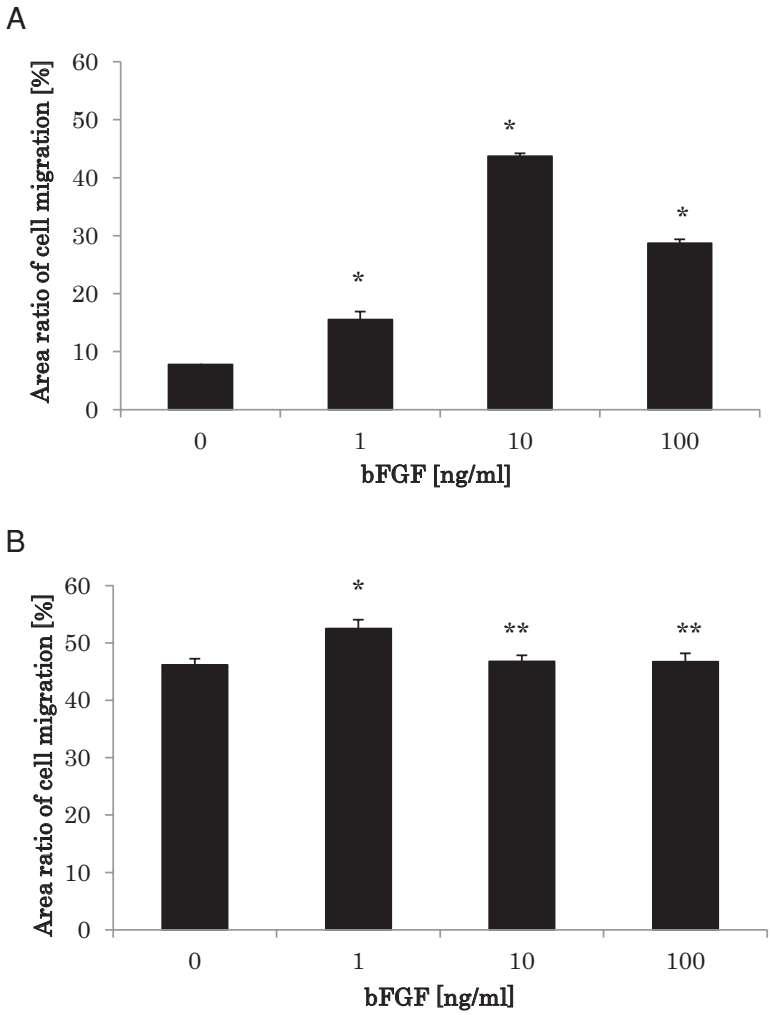

Fig. 2. Cell migration of HUVECs (Fig. 2A) and HASMCs (Fig. 2B).
*$p < 0.05$ **N.S.

equation editor was used to generate the ratio of the area of migration cell cytoplasm to the total area of the microscopic field.

Cell migration was increased among the HUVECs by bFGF in a dose-dependent manner. A maximal response was obtained at approximately 10 ng/mL (**Fig. 2A**). On the other hand, no significant differences were noted in the degree of HASMC migration between the different doses of bFGF (**Fig. 2B**). There were no dose-dependent effects of bFGF on HASMC migration. Each group contained a sample size of $n = 3$. Representative images of the migration assay are shown in **Figs. 3A** and **3B**.

Effects of bFGF on the Protein Expression of eNOS in the HUVECs

Nitric oxide synthase (NOS), a cell-type specific enzyme, catalyzes the synthesis of nitric oxide (NO). NO is a short-lived radical that transmits signals involved in vasorelaxation, neurotransmission and cytotoxicity. Endothelial NOS (eNOS) contain recognition sites for NADPH (nicotinamide adenine dinucleotide phosphate), FAD (flavin adenine dinucleotide), FMN (flavin mononucleotide) and calmodulin. The human forms exhibit 52% amino acid identity. Endothelial NOS (eNOS) proteins have a unique N-myristylation consensus sequence that may explain their membrane localization. The Western blot analysis showed a major signal band at 140 kDA for eNOS, which corresponded to the respective band of the positive control. Basic FGF (bFGF) induced a dose-dependent increase in the eNOS protein level (**Fig. 4**). Basic FGF (bFGF) increased the eNOS protein levels in the HUVECs, with a maximum at 10 ng/mL fol-

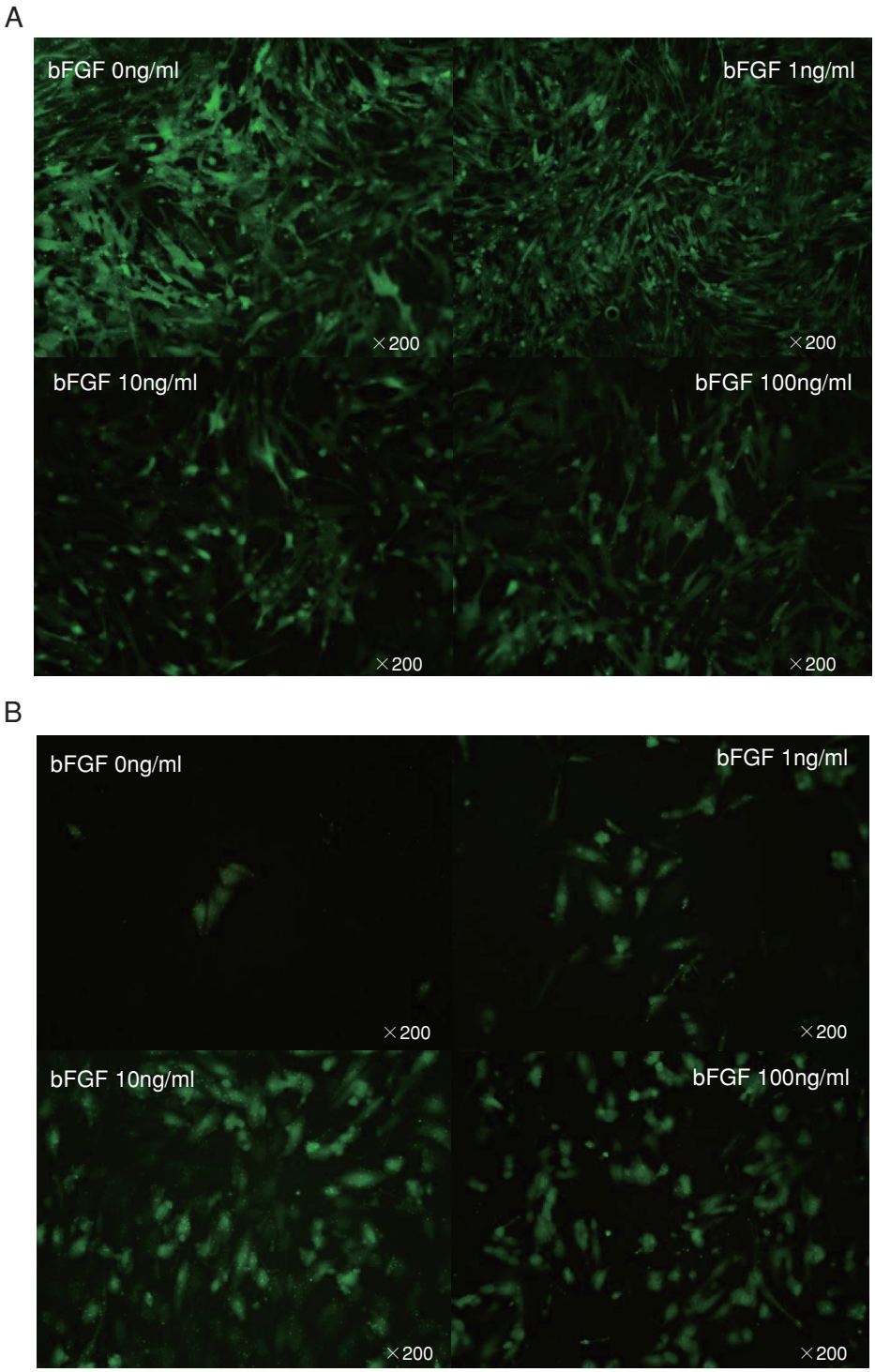

Fig. 3. Representative images of the migration assay. Fluorescence images of HUVEC migration (Fig. 3A) and HASMC migration (Fig. 3B) (×200).

lowed by a decline at 100 ng/mL. These results support the findings of the cell proliferation and migration assays.

Discussion

In this study, we explored the possibility of using

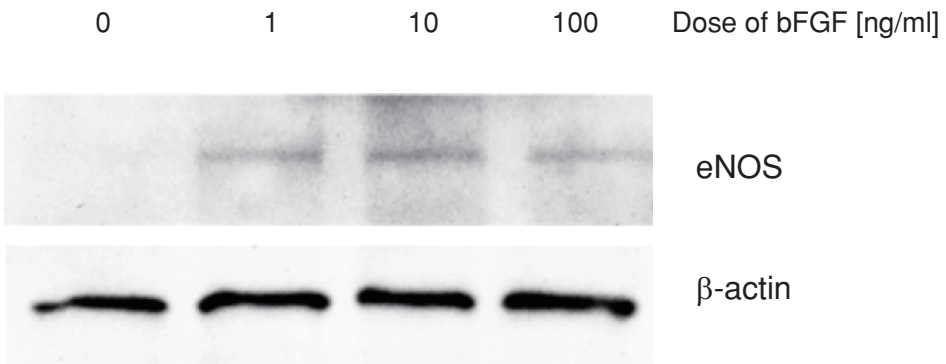

Fig. 4. A Western blot analysis of eNOS proteinss.

bFGF as a stent coating material. Basic FGF (bFGF) is known to be a potent mitogen and chemoattractant of various cells, including smooth muscle cells and endothelial cells. Specifically, bFGF induces the promotion of endothelial cell proliferation and the physical organization of endothelial cells into tube-like structures.

The primary cause of in-stent restenosis is neointimal hyperplasia, which involves the proliferation and migration of smooth muscle cells from the media into the intima. In order to prevent in-stent restenosis, using a stent coating is an important method for improving both the mechanical and physical properties of stents in direct contact with blood. There has been substantial interest in developing procedures to modify the surface of stents in order to increase biocompatibility. Stent coatings can be classified as active or passive[11]. Active coatings that release antiproliferative drugs directly interfere with the proliferation and migration of smooth muscle cells, thereby minimizing neointimal proliferation. Passive coatings provide a biologically inert barrier between the stent surface and bloodstream, with good biocompatibility[12].

The introduction of drug-eluting stents has revolutionized the field of interventional cardiology. Active coatings are loaded with drugs, such as paclitaxel, sirolimus, everolimus and biolimus A9. The benefits of drug-eluting stents include a remarkable reduction in the rates of both restenosis and target lesion revascularization. Although the use of drug-eluting stents has reduced the incidence of in-stent restenosis after percutaneous coronary intervention, almost all drugs eluting from polymers on stents induce antiproliferative effects on vascular endothelial cells. Due to delayed reendothelialization, the risk of thrombosis increases over time. Recently, concerns have been raised regarding the increased risk of late in-stent thrombosis (IST) in patients treated with drug-eluting stents, based on clinical experience. After approximately one year of treatment with an indwelling drug-eluting stent, almost all drugs are evacuated and the unprotected polymer surface becomes directly exposed to blood, possibly inducing thrombogenicity. In addition, delayed arterial healing processes, such as incomplete endothelial cell coverage and persistent fibrin deposition, have been identified after drug-eluting stent implantation and may play important roles in the development of IST.

Passive coatings provide a biologically inert barrier between the stent surface and surrounding tissue/blood. These coatings attempt to reduce inflammatory and thrombotic reactions and thereby prevent neointimal hyperplasia. A variety of different passive coatings have been evaluated, including gold, heparin, phosphocholine, carbon, silicon carbide and titanium-nitride-oxide. However, the results of several clinical trials are controversial.

The ideal agent for a stent coating would be one that inhibits thrombosis, inflammatory responses and neointimal formation while enhancing reendothelialization. Because both active and passive coatings fail to completely inhibit thrombosis and neointimal hyperplasia, we focused on coatings that actively promote reendothelialization. For example, endothelial cell preseeding, VEGF transfection of human endothelial cells and endothelial progenitor cell capturing methods demonstrate early reendothelialization.

We hypothesized that bFGF promotes endothelial cell proliferation and verified the potential use of bFGF as a stent coating material. However, we must further examine whether bFGF enhances smooth muscle cell proliferation, as excessive proliferation of smooth muscle cells results in neointimal hyperplasia. Our results showed that, with the addition of bFGF, the proliferation of HUVECs approximately doubled on day 1. The degree of HUVEC proliferation reached

a peak at a bFGF concentration of 10 ng/mL on day 2. On the other hand, bFGF exhibited little time- or concentration-dependent effects on the proliferation or migration of HASMCs. Our results are consistent with the findings of previous research showing that bFGF increased HASMC proliferation by only 24% 48 hours after seeding, as reported by DeLong S.A. *et al.*[13]. Our results and the results of DeLong S.A. *et al.* imply that bFGF does not trigger excessive proliferation or migration of HASMCs associated with in-stent restenosis. In addition to preventing the excessive proliferation and migration of HASMCs, bFGF directly promotes these processes among HUVECs, thereby achieving early reendothelialization. The observations from the *in vitro* experiments suggest that bFGF is a possible candidate stent coating material promoting endothelial cell proliferation and early reendothelialization.

Among the three concentrations of bFGF, 10 ng/mL of bFGF induced maximum proliferation and migration. When the bFGF concentration was increased to 100 ng/mL, the effects on proliferation and migration in the HUVECs decreased. This finding may be due to the saturated conditions of bFGF receptors on the cell surface.

In addition, the expression of NO, a factor for vasodilatation, was examined. The results showed that bFGF is useful not so much for inhibiting stenosis as for stimulating vasodilatation. NO has various functions, including enhancing vascular dilation, protecting the endothelium from platelet activation and leukocyte adhesion and inhibiting the proliferation of SMCs[14]. Previous reports have indicated that, in cultured bovine endothelial cells, bFGF enhances the mRNA and protein expression and activity of eNOS[15]. Other studies have demonstrated that NO promotes the migration and proliferation of endothelial cells while inhibiting the migration and proliferation of vascular smooth muscle cells both *in vivo* and *in vitro*[16-18]. The bFGF-induced eNOS expression plays an important role in early reendothelialization and the inhibition of neointimal hyperplasia after stenting.

Based on these results, we will further investigate whether bFGF-coated stents actually promote endothelialization *in vitro*. Furthermore, in addition to developing other drugs or extended-release methods, it is necessary to create stents that promote early endothelialization with as little neointimal proliferation as possible.

Conclusions

In this study, we clearly demonstrated that cell proliferation and migration are promoted by bFGF in HUVECs in a dose- and time-dependent manner; however, bFGF stimulation has little effect on these processes in HASMCs. The protein expression of eNOS detected using Western blotting strengthened the results of the cell proliferation and migration assays of the HUVECs and HASMCs treated with bFGF. Therefore, bFGF is a possible candidate stent coating or eluting drug material promoting endothelial cell proliferation and early reendothelialization. Further studies are needed to confirm the positive effects of bFGF on endothelialization without excessive smooth muscle cell proliferation using drug-eluting systems *in vivo*.

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Conflict of Interest (COI) Statement

There is no COI status to disclose.

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