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Evaluation of the *in vitro* and *in vivo* angiogenic effects of exendin-4

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ABSTRACT

Exendin-4, an analog of glucagon-like peptide (GLP)-1, has beneficial effects on cardiovascular disease induced by diabetes mellitus (DM). Recently, exendin-4 was reported to induce the proliferation of endothelial cells. However, its angiogenic effect on endothelial cells has not been clearly evaluated. Therefore, we investigated the effects of exendin-4 on the angiogenic process with respect to migration, sprouting, and neovascularization using *in vitro* and *in vivo* assays. Treatment with exendin-4 increased the migration of human umbilical vein endothelial cells (HUVECs) in *in vitro* scratch wound assays, as well as the number of lumenized vessels sprouting from HUVECs in *in vitro* 3D bead assays. These responses were abolished by co-treatment with exendin (9–39), a GLP-1 receptor antagonist, which suggests that exendin-4 regulates endothelial cell migration and tube formation in a GLP-1 receptor-dependent manner. In an *ex vivo* assay, treatment of aortic rings with exendin-4 increased the sprouting of endothelial cells. Exendin-4 also significantly increased the number of new vessels and induced blood flow in Matrigel plugs in *in vivo* assays. Our results provide clear evidence for the angiogenic effect of exendin-4 in *in vitro* and *in vivo* assays and provide a mechanism underlying the cardioprotective effects of exendin-4.

1. Introduction

Angiogenesis is a complex process involving endothelial cells sprouting from preexisting vessels, migrating directionally toward angiogenic stimuli in the perivascular space, and then elongating new vessels [1]. Angiogenesis can be observed in various vascular diseases such as coronary artery disease (CAD) and peripheral artery disease (PAD) [2]. Diabetes mellitus (DM) is associated with an increased incidence of morbidity and mortality from atherosclerotic diseases, including CAD and PAD. Although there have been experimental attempts to rescue impaired angiogenesis by adding abundant amounts of angiogenic growth factors or cells, a clinically available and safe treatment is still required to treat the vascular complications of DM.

Glucagon-like peptide (GLP)-1 is an incretin hormone that plays an important role in the regulation of glucose homeostasis and has been used in clinical treatments [3,4]. Exendin-4 is a stable analog of GLP-1 that was originally isolated from the saliva of the Gila monster (*Heloderma suspectum*) [5,6]. Exendin-4 displays ligand-binding affinity for the GLP-1 receptor (GLP-1R) and resistance to degradation by dipeptidyl peptidase IV (DPP-IV) [7,8], and is approved in both Europe and the United States for clinical treatment of type 2 diabetes. Endothelial cells express GLP-1R and acute administration of GLP-1 improves endothelial dysfunction in type 2 diabetes patients with CAD [9], demonstrating an important role of GLP-1 in endothelial function. Interestingly, exendin-4 stimulates the proliferation of human coronary artery endothelial cells through cAMP-dependent protein kinase (PKA)- and phosphoinositide 3-kinase (PI3K)/Akt-dependent pathways [10]. Although some molecular mechanisms of the effect of exendin-4 on the proliferation of endothelial cells have been investigated, the effects of exendin-4 on angiogenic processes such as migration, sprouting, and tube formation by endothelial cells have not been elucidated. In the present study, we evaluated the angiogenic role of exendin-4 with scratch migration assays, fibrin gel bead assays, aorta ring sprouting assays, and Matrigel plug assays for neovascularization.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained from the Yale Vascular Biology and Therapeutics Core. Both cell types were cultured at 37 °C in a 5% CO_2 incubator. For HUVECs, the growth medium was EGM-2 (Lonza, Basel, Switzerland) containing 2% fetal bovine serum (FBS) (Lonza). For experimental treatments, HUVECs (passages 3–7) were grown to 80–90% confluence.

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2.2. Scratch migration assay

HUVECs were incubated on 6-well plates in EGM-2 medium overnight. After starvation for 6 h with EGM-2 medium, monolayers of HUVECs were scratched with a universal blue pipette tip and the widths of the scratches in four fields per well were captured using Zeiss microscope with a 10× objective. Cells were incubated for 9 h in starvation medium containing PBS (Lonza), 100 nM exendin-4 (Sigma, St. Louis, Missouri), or 100 nM exendin-4/200 nM exendin (9–39) (Sigma). The same fields were captured again after migration. Differences in the widths of scratches before and after migration were calculated. The width of one field is an average of the widths at three different places in the same field. The means and SEM of triplicate wells were calculated. All experiments were replicated at least three times, with similar results.

2.3. Fibrin gel bead assay

The fibrin gel bead assay was performed as described by Nakatsu et al. [11]. HUVECs were mixed with Cytodex 3 microcarriers (Amersham Pharmacia Biotech, Piscataway, New Jersey) at a concentration of 400 HUVECs per bead in 1 mL EGM-2 (Clonetics, Walkersville, Maryland). Beads with cells were shaken gently every 20 min for 4 h at 37 °C in 5% CO_2. After incubation, the beads with cells were transferred to a 25 cm^2 tissue culture flask (Falcon, Bedford, Massachusetts) and left for 12–16 h in 5 mL EGM-2 at 37 °C in 5% CO_2. The following day, these were washed three times with 1 mL EGM-2 and were resuspended at a concentration of 200 cell-coated beads/mL in 2.5 mg/mL fibrinogen (Sigma) containing 0.15 U/mL aprotinin (Sigma). A total of 500 μL fibrinogen/bead solution was added to 0.625 U of thrombin (Sigma) in one well of a 24-well tissue culture plate. Fibrinogen/bead solution was allowed to clot for 5 min at room temperature and then at 37 °C in 5% CO_2 for 15 min. One milliliter of EGM-2 containing PBS, 100 nM exendin-4, or 100 nM exendin-4/200 nM exendin (9–39) was added to one well. Fibroblasts were layered on top of the clot at a concentration of 20,000 cells/well. The medium was changed every other day and exendin-4 and exendin (9–39) were added every day.

2.4. Quantification of vessels in vitro

At day 5, high-resolution images of beads were captured using an IX70 Olympus microscope with a 10× objective. Fifteen fields were captured per well. Images were analyzed using ImageJ software (National Institutes of Health). For quantification of sprouting, only sprouts whose lengths were greater than or equal to the diameter of the bead were counted. Lumen formation was quantified by measuring the lengths of sprouts that had formed lumens. The mean and SEM of triplicate wells were calculated. All experiments were replicated at least three times, with similar results.

2.5. Animals

Six-week-old male Sprague–Dawley rats and seven-week-old male C57BL/6 mice (DAEHAN BIOLINK, Chungbuk, Korea) were used in animal experiments. All animals were housed in groups in temperature-controlled (20 ± 2 °C) housing with access to food and water (12 h light/dark cycle). All experiments were carried out in accordance with the animal care guidelines of the National Institutes of Health and the Korean Academy of Medical Sciences.

2.6. Aortic ring assay

Aortae were excised from 6-week-old male Sprague–Dawley rats and fibroadipose tissue surrounding the aortae was removed. The aortae were sectioned into 1 mm slices, and then aortic rings were placed in wells coated with Matrigel (BD Biosciences, San Diego, CA), covered with additional Matrigel, and allowed to gel for 30 min at 37 °C. Four days after treatment with exendin-4 (Sigma–Aldrich, St. Louis, MO) images of sprout outgrowth were captured under a phase contrast microscope.

2.7. Matrigel plug assay

C57BL/6 mice (7 weeks old) were injected subcutaneously with 250 μL Matrigel containing bFGF (100 ng/mL) and heparin (50 U/500 μL) with or without exendin-4 (1 μg/kg) ($n = 5$ per group). After 7 days, the skin of the mice was pulled back to expose the Matrigel plug, which remained intact. The Matrigel plugs were photographed and fixed in 4% paraformaldehyde overnight. Then they were embedded in OCT, sectioned, and stained with hematoxylin and eosin (H&E). Vessels were identified by immunostaining with an anti-mouse CD31 antibody (BD Biosciences).

2.8. Image analysis and statistics

Assays were performed in duplicate, and three independent experiments were performed unless otherwise stated. ImageJ was used to analyze cell counts or stained areas. Statistical significance was analyzed using Student's *t*-test or one-way analysis of variance (ANOVA) using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA). A p value of <0.05 was taken to be significant.

3. Results

3.1. Exendin-4 induces the migration of HUVECs in the wound migration assay

To exclude any possible interference by HUVEC proliferation, the effects of exendin-4 on HUVEC migration were observed for 9 h, less than the doubling time of HUVECs (~24 h). In response to wounding, HUVECs migrated to the denuded area in a manner that mimicked the pattern of endothelial cell migration *in vivo*. Untreated HUVECs hardly migrated into the denuded area, while treatment with exendin-4 increased migration by 25%. Co-treatment with exendin (9–39) completely abrogated the effect of exendin-4 on HUVEC migration (Fig. 1A and B).

3.2. Exendin-4 induces tube formation by HUVECs in the fibrin gel bead assay

To investigate in more detail the effect of exendin-4 on sprouting and tube formation by HUVECs, we performed *in vitro* fibrin gel bead assays with HUVECs. Treatment with exendin-4 increased the number of sprouts formed by HUVECs twofold (Fig. 1C). Co-treatment with exendin (9–39) dramatically decreased the number of sprouts formed by HUVECs (Fig. 1C and D). Interestingly, we found many lumenized sprouts in the exendin-4-treated HUVECs (Fig. 1C, lower panels: arrow and asterisks). Treatment with exendin-4 further increased the average length of lumenized sprouts of HUVECs by 15%. Co-treatment with exendin (9–39) dramatically decreased the average length of lumenized sprouts formed by HUVECs (Fig. 1C and E).

3.3. Exendin-4 induces sprouting in the aortic ring assay

To study the angiogenic effects of exendin-4 *ex vivo*, we performed an aortic ring assay. Incubation with 1 μM exendin-4 for 4 days increased sprout outgrowth in rat aortic rings (Fig. 1F and G).

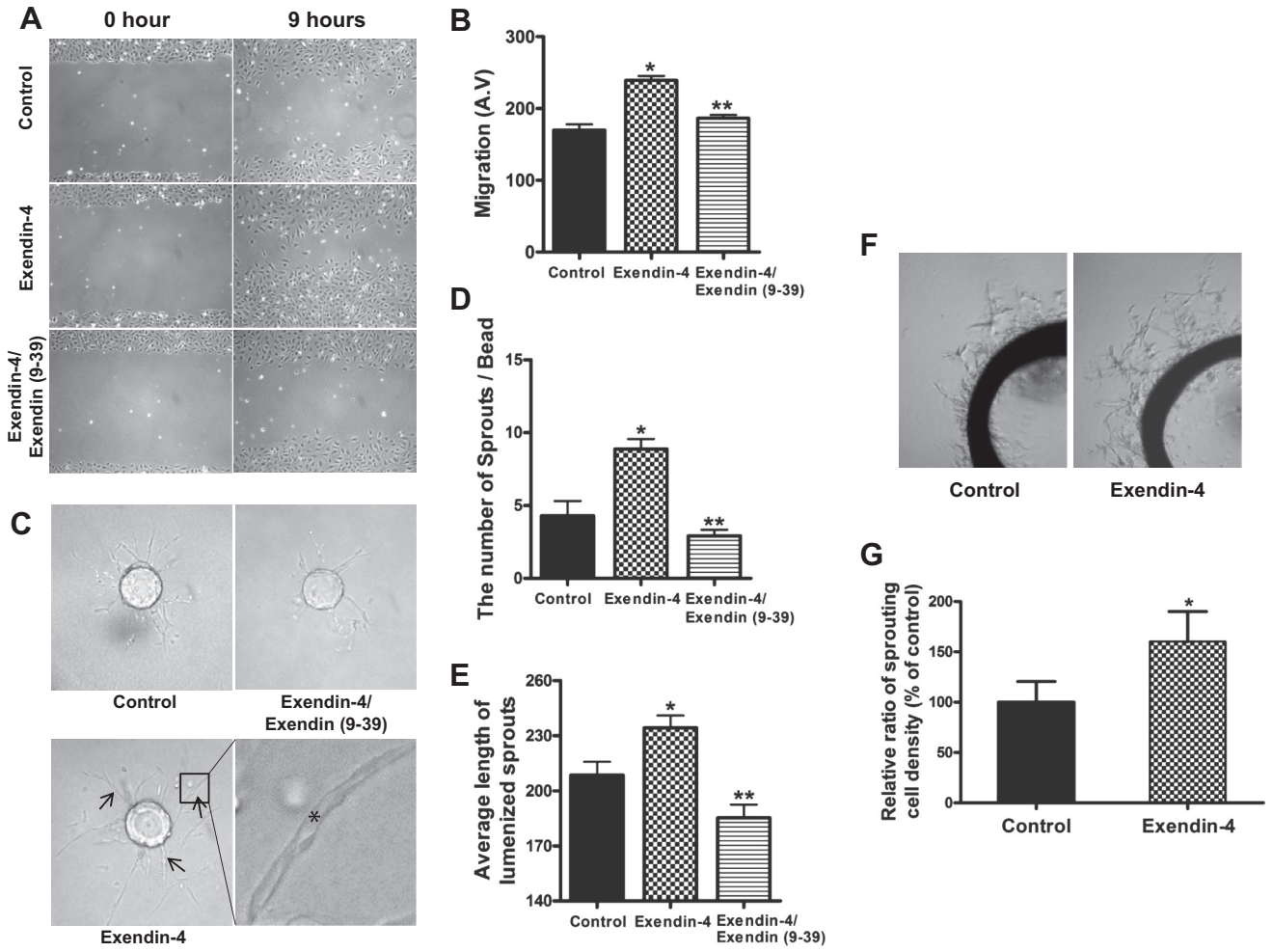

Fig. 1. Exendin-4 induces EC migration and tube formation in *in vitro* and *ex vivo* assays. (A) An *in vitro* scratch migration assay was performed using HUVECs in the presence of exendin-4 or exendin-4 plus exendin (9–39). Exendin-4 increased the migration of HUVECs. Co-treatment with exendin (9–39) reduced the migration of HUVECs. (B) Quantification of the number of migrated cells after 9 h. Values are expressed as the mean ± SEM. $^{*}p < 0.0001$ *vs.* the control group; $^{**}p < 0.0001$ *vs.* the exendin-4 group. (C) An *in vitro* fibrin gel bead assay was performed using HUVECs in the presence of exendin-4 or exendin-4 plus exendin (9–39). Exendin-4 increased the number of sprouts, and lumenized sprouts (black arrow) were mainly detected in exendin-4-treated samples. The asterisk shows a lumen formed by HUVECs. (D) Quantification of the number of sprouts. (E) Quantification of the average length of lumenized sprouts. Values are expressed as the mean ± SEM. $^{*}p < 0.0001$ *vs.* the control group; $^{**}p < 0.0001$ *vs.* the exendin-4 group. (F) An *ex vivo* aortic ring assay was performed using HUVECs in the presence of exendin-4. Exendin-4 increased the sprouting of aortic rings. (G) Quantification of relative sprouting cell density. Values are expressed as the mean ± SEM. $^{*}p < 0.0001$ *vs.* the control group; $^{*}p < 0.0001$ *vs.* the exendin-4 group.

3.4. Exendin-4 induces angiogenesis in the Matrigel plug assay

The angiogenic potential of exendin-4 was subsequently validated using the *in vivo* Matrigel plug model. In the absence of exendin-4, Matrigel plugs appeared pale, whereas the Matrigel plugs in mice treated daily with exendin-4 appeared bright red, indicating that exendin-4 activated mouse endothelial cells to develop functional neo-vessels in the plug (Fig. 2A, left panels). H&E staining of sections of Matrigel showed that exendin-4 induced tube formation and the flow of blood *in vivo*. We found that erythrocytes were recruited to the Matrigel through newly formed microvessels (Fig. 2A, right panels). The number of erythrocytes was significantly increased in Matrigel plugs treated with exendin-4 (Fig. 2B). To identify endothelial cells in the Matrigel plugs, we performed immunostaining for CD31. We mainly found newly formed capillaries in the Matrigel plugs from the exendin-4-treated group (Fig. 2C: arrows). In addition, the stained areas were significantly increased in the Matrigel plugs from the exendin-4-treated group (Fig. 2D).

4. Discussion

We evaluated the effect of exendin-4 on angiogenesis using *in vitro* and *in vivo* assays. Exendin-4 increased migration, sprouting, and tube formation by HUVECs in *in vitro* assays. In addition, it increased sprout outgrowth in aortic rings and induced the formation of new vessels in Matrigel in *in vivo* assays. Our data represent the first morphological evidence that exendin-4 may regulate endothelial cell migration, tube network formation, and neovascularization.

Exendin-4, a stable analog of GLP-1, is currently used to treat type 2 diabetes. In a large retrospective analysis, patients prescribed the GLP-1 analog exenatide showed a 20% reduction in cardiovascular disease events compared to patients on other glucose-lowering agents [12]. However, it is unclear whether exendin-4 has effects on the whole angiogenesis process. In the present study, we demonstrated that exendin-4 promotes the formation of blood vessels. Our data suggest that treatment of diabetic patients with exendin-4 may increase angiogenesis and reduce the possibility of complications of diabetes.

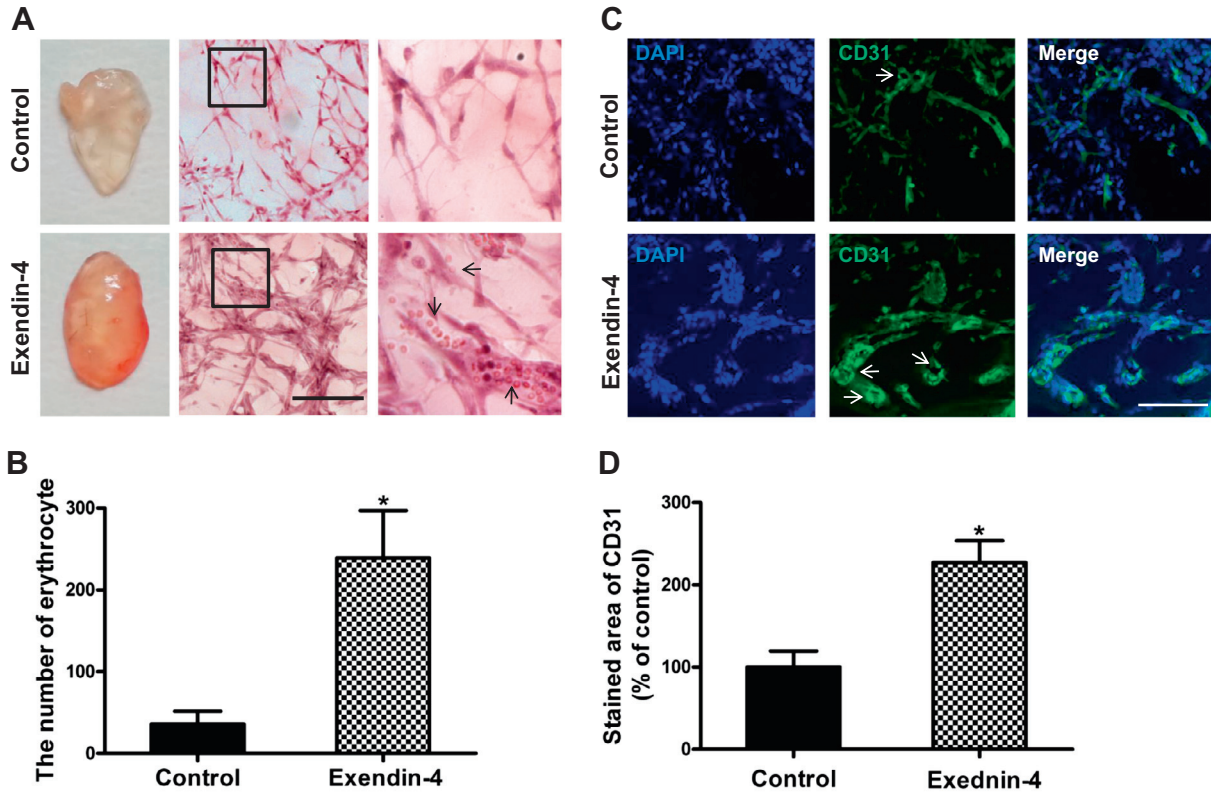

Fig. 2. Exendin-4 induces angiogenesis in *in vivo* assays. (A) An *in vivo* Matrigel plug assay was performed with daily treatment with exendin-4. Matrigel plugs of the exendin-4-treated group changed to a reddish color (left panels). Exendin-4 induced in-growth of capillaries into subcutaneous Matrigel plugs (right panels show higher-magnification images of the highlighted regions of the middle panels). H&E staining shows donut-shaped pinkish erythrocytes crammed in the endothelial capillary (arrows). (B) Quantification of the number of erythrocytes. Values are expressed as the mean ± SEM. $^{*}p < 0.001$ *vs.* the control group. (C) Immunostaining for CD31 shows the formation of capillaries in the Matrigel (white arrows). (D) Quantification of the CD31-stained area in Matrigel. Values are expressed as the mean ± SEM. $^{*}p < 0.001$ vs. the control group. Scale bars = 100 μm.

Several studies have shown that certain molecules are involved in the proliferation or differentiation of endothelial cells in response to GLP-1. VEGF secretion is increased by GLP-1 in endothelial progenitor cells and mediates the effects of GLP-1 on endothelial progenitor cells [13]. Recently, GLP-1 was reported to regulate the proliferation of human coronary artery endothelial cells through activation of PKA-PI3K/Akt-eNOS pathways by a GLP-1R-dependent mechanism [10]. Moreover, inhibition of DPP-4, which may have similar effects as analogs of GLP-1, has been reported to enhance endothelial cell growth [14]. These previous studies provide the molecular mechanism for our data showing that exendin-4 regulates the whole process of HUVEC angiogenesis in a GLP-1R-dependent manner.

In conclusion, the present study strongly suggests that exendin-4 directly regulates migration, spouting, and tube formation by endothelial cells, and also induces endothelial cell proliferation and neovascularization. Our results suggest that exendin-4 may be of potential use for the treatment of vascular complications of diabetes, as well as diabetes itself.

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