

Original

**Glucagon-like Peptide-1 Suppresses the Proliferation
and Migration of Vascular Smooth Muscle Cells :
Implications for Preventive Effects on Atherosclerosis**

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Abstract : Our group previously demonstrated the suppressive effect of glucagon-like peptide-1 (GLP-1) on macrophage-driven atherosclerosis in apolipoprotein E-deficient (apoE^{-/-}) mice. In the present study we investigated the suppressive effect of GLP-1 on the atherogenic phenotype of vascular smooth muscle cells (VSMCs) *in vivo* using apoE^{-/-} mice, and the proliferation and migration of human VSMCs *in vitro*. A 4-week infusion of GLP-1 in 17-week-old apoE^{-/-} mice significantly reduced the proliferative VSMC phenotype stained with SMemb. Platelet-derived growth factor (PDGF)-BB significantly stimulated the proliferation of human aortic VSMCs by three fold. Both 0.1 and 1 nmol/l GLP-1 significantly suppressed the PDGF-induced VSMC proliferation, and this suppressive effect was significantly abolished by the GLP-1 receptor antagonist exendin (9-39) (50 nmol/l). The GLP-1 receptor agonists liraglutide (100 nmol/l) and exendin-4 (100 nmol/l) mimicked GLP-1, significantly suppressing PDGF-induced VSMC proliferation. PDGF-BB significantly stimulated the migration of human aortic VSMCs by 1.7 -fold, and this effect was significantly suppressed by 1 nmol/l GLP-1. These findings suggest that GLP-1-related treatments may prevent the progression of atherosclerotic lesions by suppressing the proliferation and migration of VSMCs, which are characteristic features of atherosclerosis.

Key words : atherosclerosis, GLP-1, vascular smooth muscle cells

Introduction

The incretins glucagon-like peptide-1 (GLP-1) and glucose-dependent insulintropic polypeptide (GIP) are secreted postprandially from the L-cells of the lower gut and the K-cells of the intestines, respectively, to regulate glucose homeostasis¹⁻³⁾. GLP-1 and GLP-1 receptor (GLP-1R) agonists, such as exendin-4 and liraglutide, have been reported to have cardioprotective and vasodilatory actions⁴⁾ and to improve vascular endothelial dysfunction and inflammation^{5, 6)}. Exendin-4 stimulates endothelial cell proliferation⁷⁾ but suppresses monocyte adhesion to the

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vascular wall and vascular smooth muscle cell (VSMC) proliferation^{8, 9)}. Liraglutide stimulates nitric oxide production¹⁰⁾ and attenuates tumor necrosis factor- α -induced oxidative stress and inflammation, as well as the expression of vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and plasminogen activator inhibitor-1 in human umbilical vein endothelial cells^{11, 12)}. Chronic infusion of GLP-1 suppresses monocyte/macrophage infiltration into the aortic wall and the development of atherosclerotic lesions in apolipoprotein E-deficient (apoE^{-/-}) mice, an animal model of spontaneous atherosclerosis¹³⁾. GLP-1 works via GLP-1R to suppress oxidized low-density lipoprotein (oxLDL)-induced foam cell formation in exudate peritoneal macrophages, and this suppressive effect is followed by activation of cAMP¹³⁾.

In the present study we evaluated the degree to which GLP-1 inhibited the development of aortic atherosclerotic lesions as a result of reducing the area of atherosclerotic lesions infiltrated by proliferative VSMCs in apoE^{-/-} mice. We assessed the suppressive effects of GLP-1 and GLP-1R agonists on the proliferation and migration of human VSMCs, pivotal features of atherosclerosis.

Materials and Methods

Chemicals and Reagents

Human GLP-1 (7–36) amide, exendin (9–39), and exendin-4 were purchased from AnaSpec (San Jose, CA, USA). Liraglutide was obtained from Bachem (Torrance, CA, USA). The dipeptidyl peptidase-4 (DPP-4) inhibitor vildagliptin and its analog PKF275-055 were generous gifts of Novartis (Basel, Switzerland). Platelet-derived growth factor (PDGF)-BB was purchased from Wako (Osaka, Japan).

Human VSMC Proliferation Assay

Human aortic VSMCs were seeded on six-well plates (3×10^4 cells/ml per well) and cultured in smooth muscle cell basal medium (SmBM) supplemented with 5% fetal calf serum (FCS) for 24 h before being transferred to SmBM without FCS to synchronize the cell cycle. VSMCs were incubated for 48 h with PDGF-BB (20 ng/ml) and GLP-1, liraglutide, or exendin-4 at concentrations indicated, together with 2 μ mol/l PKF-275 to prevent the incretin from degrading. Cells were exposed to 10 μ mol/l 5'-bromo-2'-deoxyuridine (BrdU) for the last 20 h of culture¹⁴⁾. BrdU-positive cells were visualized by immunostaining and counted under a microscope.

Human VSMC Migration Assay

Cell migration was measured using modified Boyden chambers (Neuro Probe, Gaithersburg, MD, USA). Each chamber housed a polycarbonate filter with 8- μ m pores that had been incubated with 5.3 mg/ml collagen (Centrix, Santa Clara, CA, USA) in phosphate-buffered saline (PBS) for 30 min before the migration assay¹⁴⁾. Human aortic VSMCs ($3\text{--}5 \times 10^3$ cells/100 μ l per well) were plated on the top chamber of a Transwell plate (Transwell-Costar, Cambridge, MA, USA) and 20 ng/ml PDGF-BB was dispensed into the lower chamber. The cells were then incubated for 20 h with the indicated concentration of GLP-1 together with the vildagliptin

analog PKF-275 ($2\ \mu\text{mol/l}$). After incubation, the non-migrating cells were removed from the upper face of the Transwell membrane with a cotton swab and the migrating cells were fixed and stained with crystal violet in 3.7% paraformaldehyde. Migration was quantified by counting the number of positively stained cells.

Animal Experiments

Animal experiments were performed in accordance with National Institutes of Health (NIH) guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Showa University. Sixteen male apoE^{-/-} mice were purchased from Sankyo Labo Service (Tokyo, Japan) at 8 weeks of age and were maintained on a normal diet until they reached 17 weeks of age. At this time, apoE^{-/-} mice were divided into two groups and infused with either GLP-1 ($2.2\ \text{nmol/kg}$ per day) or saline (vehicle) for 4 weeks via osmotic mini-pumps (Alzet Model 1007D; Durect, Cupertino, CA, USA)^{13, 15}. The osmotic mini-pumps had been implanted subcutaneously on the back of the mice. Mice were simultaneously fed an atherogenic diet containing 30% fat, 20% sucrose, 8% NaCl, and 0.15% cholesterol (Oriental Yeast, Tokyo, Japan) for 4 weeks¹⁶. Then, after 4 weeks infusion, systolic blood pressure (SBP) was measured using indirect tail-cuff equipment (MK-2000; Muromachi Kikai, Tokyo). Blood samples were collected from the inferior vena cava after mice had been fasted for 12 h. All surgery was performed in mice anesthetized with diethyl ether. Plasma concentrations of glucose were measured by enzymatic methods using an autoanalyzer (Hitachi 7020; Hitachi, Tokyo, Japan).

Assessment of Atherosclerotic Lesions

After the 4-week infusion period, the 21-week-old apoE^{-/-} mice were anesthetized with diethyl ether, drained of blood via the descending vena cava, and subjected to whole-body perfusion with PBS via a left ventricular cannula^{13, 16-19}. The whole aorta was fixed by perfusion with PBS containing 4% paraformaldehyde, excised from the root to the abdominal area, and carefully stripped of connective and adipose tissues. Next, cross-sections of the aortic root were stained with anti-human SM2 antibody (Yamasa, Tokyo, Japan) and anti-SMem antibody (Yamasa) to assess the contractive and proliferative VSMC phenotypes, respectively. VSMC-positive areas are expressed as a percentage of the area of atheromatous plaques.

Statistical Analysis

Data are expressed as the mean \pm SEM. Parameters were compared between the two groups by unpaired Student's *t*-test. The significance of differences was determined using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). Differences were considered significant at $P < 0.05$.

Results

The body weight of saline- and GLP-1-infused apoE^{-/-} mice was comparable (30 ± 1 vs $29 \pm$

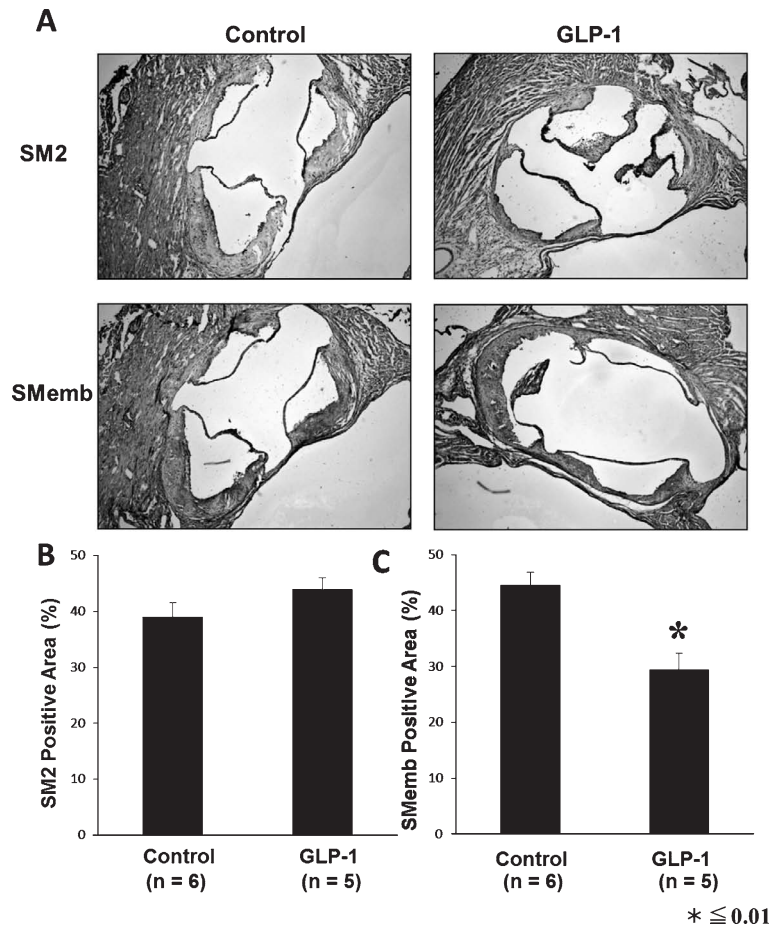


Fig. 1. Suppressive effects of chronic glucagon-like peptide-1 (GLP-1) infusion against the proliferative or contractile vascular smooth muscle cell (VSMC) phenotype in aortic atherosclerotic lesions in apolipoprotein E-deficient ($\text{apoE}^{-/-}$) mice. Sixteen $\text{apoE}^{-/-}$ mice were infused with GLP-1 (2.2 nmol/kg per day) or saline (control) by osmotic mini-pump for 4 weeks from 17 weeks of age. (A) Cross-sections of the aortic root were stained with anti-SM2 antibody or anti-SMemb antibody to identify contractile and proliferative phenotypes, respectively. (B, C) Quantitative analysis of areas positive for anti-SM2 antibody (B) and anti-SMemb antibody (C) relative to atherosclerotic lesions in percentage terms. Data are the mean \pm SEM. * $P < 0.01$ compared with control.

1 g, respectively; $P = 0.56$), as was systolic blood pressure (99 ± 3 vs 102 ± 3 mmHg, respectively; $P = 0.61$), and serum glucose (131 ± 7 vs 141 ± 10 mg/dl, respectively; $P = 0.48$).

Effects of GLP-1 on Proliferative VSMCs in Atherosclerotic Lesions in $\text{apoE}^{-/-}$ Mice

Similar to results reported previously¹³⁾, $\text{apoE}^{-/-}$ mice infused with saline vehicle in the present study exhibited marked atherosclerotic lesions in the proximal portion of the aorta at 21 weeks of age, whereas 4-week infusion of GLP-1 significantly suppressed the size of the atherosclerotic lesions with infiltrating monocytes/macrophages in the aortic root. Staining of cross-sections of the aortic root with anti-SM2 antibody or anti-SMemb antibody (Fig. 1A) revealed that GLP-1 infusion significantly decreased SMemb (i.e. the proliferative VSMC phenotype) by 33.9% compared with staining in the saline-treated group ($P < 0.01$; Fig. 1A, C). However, GLP-1

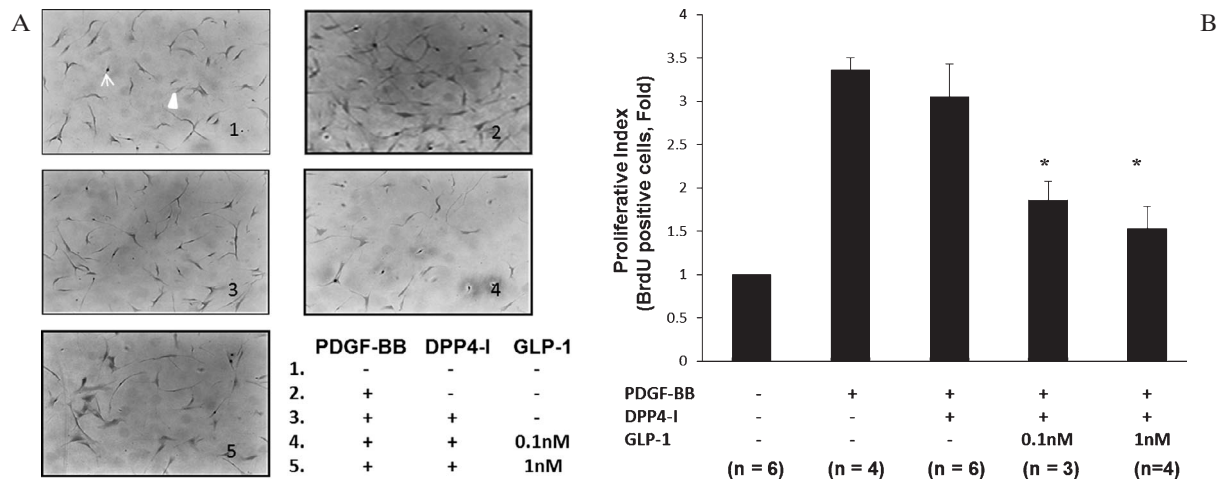


Fig. 2. Suppressive effects of glucagon-like peptide-1 (GLP-1) on the platelet-derived growth factor (PDGF)-BB-induced proliferation of human vascular smooth muscle cells (VSMCs). Human aortic VSMCs were seeded onto six-well plates (3×10^4 cells/ml per well), cultured in smooth muscle cell basal medium (SmBM) supplemented with 5% fetal calf serum (FCS) for 24 h, and then transferred to SmBM without FCS. VSMCs were incubated for 48 h with PDGF-BB (20 ng/ml) and the indicated concentrations of GLP-1, together with a vildagliptin analog (2 μ mol/l). Cells were exposed to 10 μ mol/l bromodeoxyuridine (BrdU) for the last 20 h of culture. (A, B) BrdU-positive cells (arrows in 2A) were visualized by immunostaining (A) and counted under a microscope (B). Data are the mean \pm SEM. * $P < 0.01$ compared with column 3.

infusion had no significant effect on SM2 staining (i.e. the contractile phenotype) within aortic atherosclerotic lesions in apoE^{-/-} mice (Fig. 1A, B).

Effects of GLP-1 on the Proliferation of Human VSMCs

PDGF-BB significantly stimulated the proliferation of human aortic VSMCs by three fold (Fig. 2A, B). Alone, PKF-275, a DPP-4 inhibitor, did not suppress PDGF-BB-induced VSMC proliferation. Incubation of cells with 0.1 and 1 nmol/l GLP-1 significantly suppressed PDGF-BB-induced VSMC proliferation by 39.1% and 49.9%, respectively ($P < 0.001$ for both; Fig. 2A, B). In another series of experiments, 1 nmol/l GLP-1 significantly suppressed PDGF-BB-induced VSMC proliferation ($P < 0.03$; Fig. 3A) and this effect was significantly ameliorated by the GLP-1R antagonist exendin (9–39) (50 nmol/l; $P < 0.05$; Fig. 3A). The GLP-1R agonist liraglutide (100 nmol/l) mimicked GLP-1, significantly suppressing PDGF-BB-induced VSMC proliferation by 42.7% ($P < 0.03$; Fig. 3B), as did the other GLP-1R agonist exendin-4 (100 nmol/l), which suppressed PDGF-BB-induced VSMC proliferation by 48.6% ($P < 0.03$; Fig. 3B).

Effects of GLP-1 on the Migration of Human VSMCs

PDGF-BB significantly stimulated the migration of human aortic VSMCs by 1.8 -fold. Alone, the DPP-4 inhibitor PKF-275 had no significant effect on PDGF-BB-induced VSMC migration. Incubation of cells with 1 nmol/L GLP-1 significantly suppressed PDGF-induced VSMC migration by 19.3% ($P < 0.01$; Fig. 4A, B). Although incubation of cells with 0.1 nmol/l GLP-

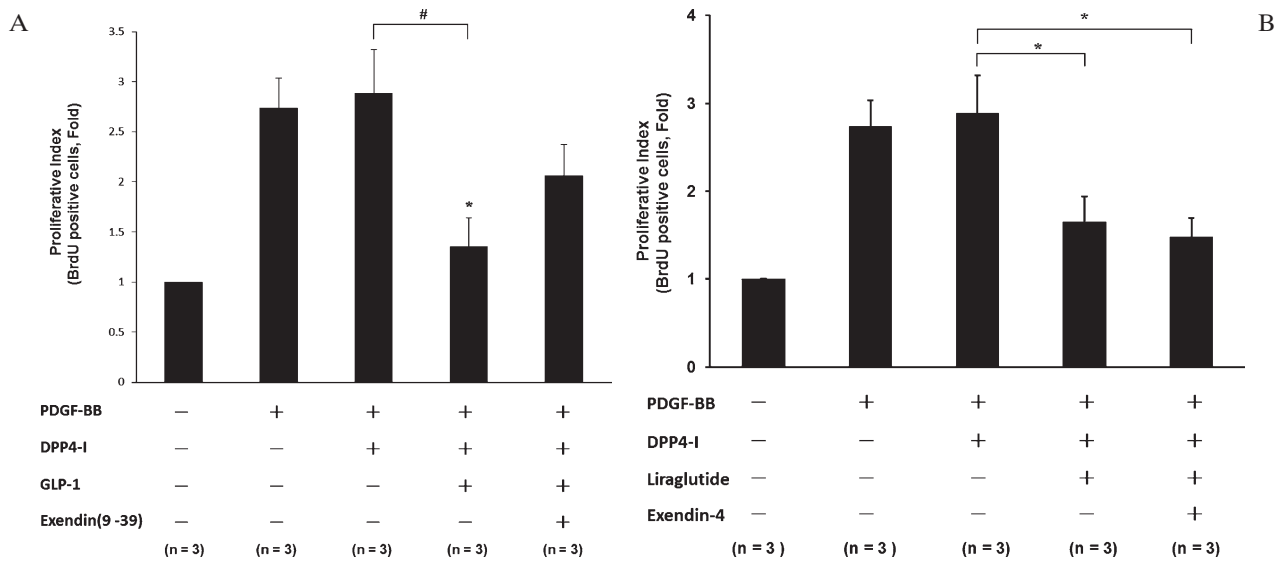


Fig. 3. Effects of a glucagon-like peptide-1 (GLP-1) receptor (GLP-1R) antagonist and agonists on human vascular smooth muscle cell (VSMC) proliferation. Human aortic VSMCs were seeded onto six-well plates (3×10^4 cells/ml per well), cultured in smooth muscle cell basal medium (SmBM) supplemented with 5% fetal calf serum (FCS) for 24 h, and then transferred to SmBM without FCS. (A) VSMCs were incubated for 48 h with platelet-derived growth factor (PDGF)-BB (20 ng/ml) and GLP-1 (1 nmol/l), together with a vildagliptin analog (2 μ mol/l), in the presence or absence of 50 nmol/l exendin (9-39). (B) VSMCs were incubated for 48 h with 20 ng/ml PDGF-BB and 100 nmol/l liraglutide or 100 nmol/l exendin-4, together with a vildagliptin analog (2 μ mol/l). Cells were exposed to 10 μ mol/l bromodeoxyuridine (BrdU) for the last 20 h of culture. BrdU-positive cells were visualized by immunostaining and counted under a microscope. Data are the mean \pm SEM. * $P < 0.03$; # $P < 0.05$.

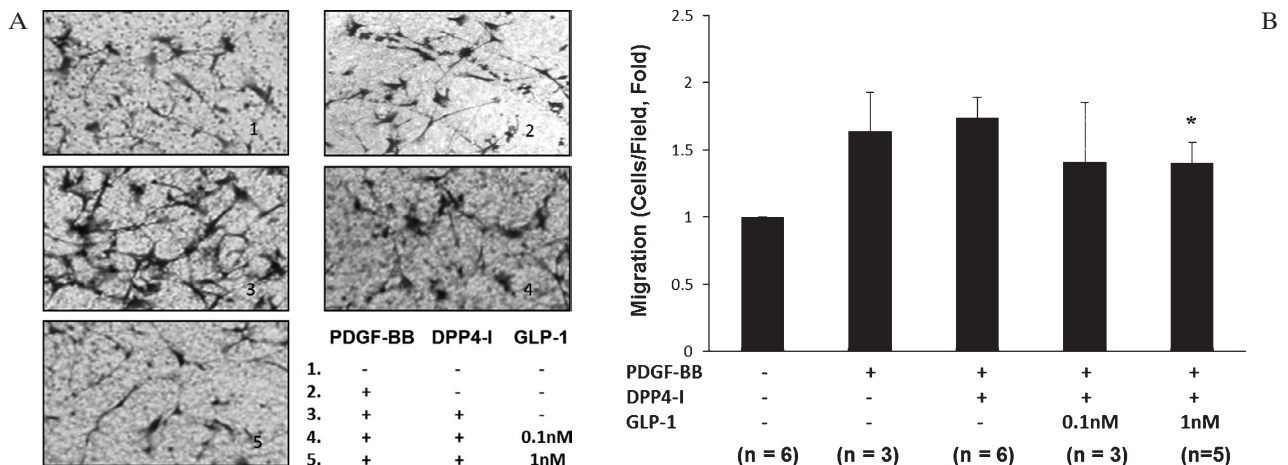


Fig. 4. Suppressive effects of glucagon-like peptide-1 (GLP-1) against the platelet-derived growth factor (PDGF)-BB-induced migration of human vascular smooth muscle cells (VSMCs). Human aortic VSMCs ($3-5 \times 10^3$ cells/100 μ l per well) were plated on the Transwell plates of the top chamber of a Boyden chamber assay kit, and PDGF-BB (20 ng/ml) was dispensed into the lower chamber. After 20 h incubation with the indicated concentrations of GLP-1 together with a vildagliptin analog (2 μ mol/l), all non-migratory cells were removed from the upper face of the Transwell membrane with a cotton swab and the migratory cells were fixed and stained with crystal violet in 3.7% paraformaldehyde (A). Migration was quantified by counting the number of stained cells (B). Data are the mean \pm SEM. * $P < 0.01$ compared with column 3.

1 suppressed PDGF-induced VSMC migration by 18.8%, the difference failed to reach statistical significance (Fig. 4A, B).

Discussion

The results of the present study suggest that GLP-1 treatments may prevent the progression of atherosclerotic lesions by suppressing the proliferation and migration of VSMCs, which are characteristic features of atherosclerosis. The present study is the first to show the suppressive effects of GLP-1 and GLP-1R agonists on PDGF-BB-induced proliferation and migration of human aortic VSMCs. The present study also showed that GLP-1 suppressed SMemb-positive VSMCs, a proliferative phenotype that acquires macrophage-like phagocytosis²⁰. In previous studies we showed that chronic infusion of GLP-1 significantly prevented the infiltration of monocytes/macrophages into the arterial wall, the development of atherosclerotic lesions, and oxLDL-induced macrophage foam cell formation in apoE^{-/-} mice¹³. Suppression of the proliferation and migration of VSMCs by GLP-1 may explain, in part, prevention of the atherogenic process in these mice. The anti-atherogenic effects exhibited by GLP-1 and GLP-1R agonists *in vitro* in human VSMCs and *in vivo* in apoE^{-/-} mice may have important implications for future research. Gaspari *et al* have shown that liraglutide infusion into apoE^{-/-} mice inhibits both endothelial dysfunction and expression of vascular adhesion molecule²¹. Goto *et al* reported that exendin-4 suppresses murine VSMC proliferation and reduces intimal thickening after vascular injury⁹. These findings are similar to those of the present study and suggest that incretin-based therapy may be effective for the prevention of atherosclerotic vascular diseases.

DPP-4 acts on human VSMCs and monocytes/macrophages, and is present in human serum contained in culture medium^{22, 23}. In preliminary studies, GLP-1 had far weaker suppressive effects against human macrophage foam cell formation and human VSMC migration and proliferation when the culture media were prepared without DPP-4 inhibitor. Therefore, in the present study, we used DPP-4 inhibitors to prevent degradation of GLP-1 in all ensuing *in vitro* experiments investigating the suppressive effects of this incretin on human vascular cells.

The concentrations of GLP-1 used *in vitro* (0.1–1 nmol/l) in studies of human VSMC proliferation and migration are higher than concentrations found *in vivo* in plasma from non-fasted patients with diabetes (2–40 pmol/l)^{24, 25}. It has been reported that GLP-1 administered via buccal tablets (119 nmol) or via continuous infusion (2.16 nmol/kg per day for 2 days) increases plasma GLP-1 concentrations in humans to a peak of 100–120 pmol/l^{26, 27}. Therefore, the combined use of DPP-4 inhibitors and GLP-1 could conceivably lead to higher plasma concentrations of GLP-1. In humans infused with liraglutide once daily, plasma concentrations have been reported to reach 10 nmol/l liraglutide²⁸. Thus, the concentrations of GLP-1 used in the present study do not far exceed the plasma concentrations reached when a GLP-1 analog is administered.

The mechanisms underlying the suppressive effects of incretins on the proliferation and migration of VSMCs remain to be elucidated. Goto *et al*⁹ investigated the molecular mechanisms underlying exendin-4-mediated suppression of VSMCs, however their results did

not enable them to reach any definitive conclusions. Because GLP-1 has suppressive effects on VSMCs, it is likely that these effects are mediated by cAMP generation following activation of GLP-1R. However, Goto *et al*⁹⁾ did not find increased cAMP levels in exendin-4-treated VSMCs. Hattori *et al*¹⁰⁾ found that activation of GLP-1R induces phosphorylation of AMP-activated protein kinase (AMPK) independent of adenylate cyclase activity in endothelial cells. AMPK activation by the GLP-1 signal transduction pathway may be involved in the inhibition of VSMC proliferation. Further studies are required to elucidate the molecular mechanisms underlying the suppression of the proliferation and migration of VSMCs by incretins, and whether these mechanisms are dependent or independent of cAMP.

There are several obstacles in translating the findings of the present study to humans. First, unlike humans, in whom the etiology of atherosclerosis is multifactorial, the atherosclerosis in our animal model is due simply to hypercholesterolemia without any other risk factors. Furthermore, in the present study we started the intervention in young (17-week-old) mice and evaluated atherosclerotic lesions 4 weeks later. Therefore, this study evaluated the effects of intervention at a young age on the development of atherosclerosis, which differs from the design of intervention studies in humans, in which cardiovascular events or mortality are considered as end-points at older ages.

The Examine SAVOR (Saxagliptin and cardiovascular outcomes in patients with type 2 diabetes mellitus) study in humans failed to demonstrate a suppressive effect of a DPP-4 inhibitor on cardiovascular events²⁹⁾. Therefore, other evidence is needed to determine whether incretin-based treatment will really suppress atherosclerosis in humans. Based on the findings of Examin SAVOR, it is difficult at present to extend our findings to suppression of atherosclerosis in humans. However, if it could be demonstrated that incretin-based treatment suppressed atherosclerosis in humans, our results would contribute to an understanding of the mechanisms involved and perhaps the development of appropriate treatment.

In conclusion, the results of the present study indicate that GLP-1 and GLP-1R agonists suppress VSMC migration and proliferation, and are thereby capable of preventing the progression of atherosclerotic lesions. GLP-1-related treatments are expected to emerge as a new line of therapy against atherosclerotic vascular diseases in diabetics.

Disclosure

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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