수의학석사 학위논문

RELM-α reduces diabetic atherosclerosis in LDLR Knock-out mice

Ldlr 유전자가 제거된 마우스 모델에서 RELM-α 의한 당뇨성 동맥경화 감소효과

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Abstract

RELM-α reduces diabetic atherosclerosis in *LDLR* knockout mice

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Resistin-like molecule (RELM)-α belongs to a family of secreted mammalian proteins that have putative immunomodulatory functions. Recent studies have identified a role of RELM-α in the pathogenesis of hyperlipidemia-induced atherosclerosis. However, whether RELM-α regulates diabetic atherosclerosis is unknown. Here we report that RELM-α has anti-atherogenic effects and protects against diabetic atherosclerosis in low-density lipoprotein receptor-deficient mice (LDLR $^{-/-}$). Severity of the induced diabetic state was confirmed by monitoring of blood glucose levels and body weight. RELM-α overexpression appears to have a

cholesterol-lowering effect. In particular, there was significant difference in cholesterol levels of diabetic group. After 8 weeks on a High-fat diet (HFD), total en face aortic lesion area was reduced in RELM-α overexpressing (RELM-α Tg) mice compared with control mice in both non-diabetic and diabetic group. Plaque area in the aortic arch was also decreased in RELM-α Tg of both groups. We show RELM-α overexpression has a higher anti-atherogenic effect with decrease of cholesterol in diabetic atherosclerosis compared with non-diabetic group. These findings define RELM-α as a novel therapeutic target for treating diabetic atherosclerosis.

Keywords : RELM-α, Diabetic atherosclerosis, Low-density lipoprotein receptor-deficient mice, Aortic lesion

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1. Introduction

1.1. Diabetic atherosclerosis

Diabetes mellitus causes accelerated atherosclerosis, with greater inflammatory infiltrate (macrophages and T lymphocytes), larger necrotic core size, and more diffuse atherosclerosis in the coronary arteries (Shah & Brownlee, 2016). In the United States, 77% of diabetes related hospital admissions are for cardiovascular complications. High levels of glucose, which result in diabetes increases the production of reactive oxygen species (ROS) by mitochondrial dysfunction. Consequently, it promotes atherosclerotic lesion formation by upregulation of protein kinase C (PKC), activation of the hexosamine and polyol pathways, and accumulation of advanced glycation end-products (AGE) with upregulation of receptors for advanced glycosylation end-products (RAGE) (Siracuse & Chaikof, 2012; Yan, Ramasamy, & Schmidt, 2008).

1.2. Mechanisms of Streptozotocin-induced diabetes

Mice models commonly are used to study immunologic mechanisms and metabolic function in diabetes. Diabetes is induced in mice by

using streptozotocin (STZ), a glucosamine-nitrosourea compound derived from *Streptomyces achromogenes* that is used clinically as a chemotherapeutic agent in the treatment of pancreatic β cell carcinoma (Damasceno et al., 2014). STZ inhibits insulin secretion and causes a state of insulin-dependent diabetes mellitus, resulting in hypoinsulinemia and hyperglycemia which can be attributed to specific chemical properties, namely its alkylating potency (Lenzen, 2008). STZ is selectively accumulated in pancreatic beta cells via the low-affinity GLUT2 glucose transporter in the plasma membrane. Chemical structural similarity with glucose allows STZ to bind to this receptor (Graham, Janecek, Kittredge, Hering, & Schuurman, 2011). Thus, insulin-producing cells that do not express this glucose transporter are resistant to STZ (Lenzen, 2008). At high dose, typically given singly, STZ targets β cells by its alkylating property and at low doses, given in multiple exposures, STZ elicits an immune and inflammatory reaction, presumably related with the release of glutamic acid decarboxylase autoantigens(Paik, Fleischer, & Shin, 1980). Under this condition, the destruction of β cells and induction of the hyperglycemic state is associated with inflammatory infiltrates including lymphocytes in the pancreatic islets (Lenzen, 2008).

Although STZ is an alkylating agent, it is also useful in

genotoxicity studies with suggesting that STZ can irreversibly damage β cell DNA (Damasceno et al., 2014). Mossman et al. performed an *in vitro* study showing that STZ induces single-strand DNA breaks in rodent cells, and these lesions are repaired 24 hours after STZ exposition (Mossman, Ireland, Filipak, LeDoux, & Wilso, 1986). Studying the same cell lineage, Pettepher et al. demonstrated that STZ also induces alkali-labile site breaks in mitochondrial DNA, and even though the formation of this lesion is dose-dependent, it can be repaired as well (Pettepher, LeDoux, Bohr, & Wilson, 1991). After 8 hours of STZ exposition, 55% of the mitochondrial DNA lesions were repaired, rising to 70% in 24 hours. These data confirm that STZ by itself is not responsible for the high levels of DNA damage (Damasceno et al., 2014; Pettepher et al., 1991).

The effects of STZ on glucose and insulin homeostasis reflect the toxin-induced abnormalities in β cell function. Initially, insulin biosynthesis, glucose-induced insulin secretion and glucose metabolism are all affected (Lenzen, 2008). On the other hand, STZ has no immediate, direct inhibitory effect upon glucose transport (Elsner, Guldbakke, Tiedge, Munday, & Lenzen, 2000; Lenzen, 2008) or upon glucose phosphorylation by glucokinase (Lenzen, 2008; Lenzen, Tiedge, & Panten, 1987). However, at later stages of

functional beta cell impairment, deficiencies in terms of gene expression and protein production lead to the deterioration of both glucose transport and metabolism (Wang & Gleichmann, 1998).

1.3. Resistin-like molecules (RELM) family

Resistin was initially identified as an adipocyte-secreted factor that causes insulin resistance. Subsequent studies have suggested its association with type 2 diabetes mellitus, congestive heart failure, and coronary artery disease (Kushiyama et al., 2013). The family of resistin-like molecules (RELM) are also known as found in inflammatory zone (FIZZ) which consists of four members in mouse (RELM-α/FIZZ1/HIMF, RELM-β/FIZZ2, Resistin/FIZZ3, and RELM-ɣ/FIZZ4) and two members in human (Resistin and RELM-β) (Holcomb et al., 2000; Lee et al., 2014). These resistin family proteins share a cysteine-rich domain at their C terminus (Cx11Cx8CxCx2Cx10CxCxCx9CC) and other signature features (Steppan et al., 2001) have importance in many aspects of physiology and pathophysiology, especially inflammatory processes. The role of RELM-α, has been linked to various inflammatory conditions such as asthma and helminth infections (Dong et al., 2008; Holcomb et al., 2000). RELM-β, which is abundantly

expressed in the foam cells of atherosclerotic lesions, functions in both autocrine and paracrine manners in M1/M2 macrophages, endothelial cells, and fibroblasts, thereby contributing to atherosclerosis development and plaque instability (Kushiyama et al., 2013). Resistin is a well-known adipokine that was originally identified in the adipose tissue with physiological roles in promoting insulin resistance and linked to obesity with insulin resistance (Kushiyama et al., 2013). However, the function of RELM-α in diabetic atherosclerosis has been less studied.

1.4. Previous studies

Recent evidence has revealed that RELM-α may have some role in inflammatory processes in tissue associated with atherosclerosis (Lee et al., 2014) and RELM-β/resistin are implicated as mediators of inflammation and abundantly expressed in macrophages of atheroma related to atherosclerosis (Kushiyama et al., 2013; Lee et al., 2014). In particular, recent studies have shown RELM-α deficiency promotes macrophage accumulation in atheroma and increases cell death in the atherosclerotic lesions (Lee et al., 2014). RELM-β expression in the colon was reportedly induced by a high-fat diet (Kushiyama et al., 2005), and RELM-β expression in

the human colonic cell line LS174T was upregulated by saturated free fatty acid (SFA) or tumor necrosis factor-α (TNFα) stimulation (Fujio et al., 2008). Thus, RELM-β seems to contribute to local immune system function in the gut by acting against bacteria and nematodes (De'Broski et al., 2009; He et al., 2003). It was also documented that RELM-β augments interferon ɣ-induced TNF α secretion in thioglycolate-isolated macrophages and infection-induced intestinal inflammation (Nair et al., 2008). In addition, RELM family has been reported to be implicated in insulin resistance. Resistin links obesity to insulin resistance and diabetes (Rajala, Obici, Scherer, & Rossetti, 2003) and the function of RELM-β might be similar to that of resistin by impairing insulin action in the liver. Indeed, RELM-β- overexpressing mice reportedly exhibited insulin resistance with hyperinsulinemia, hyperglycemia, hyperlipidemia, and fatty liver when consuming a high-fat diet (Kushiyama et al., 2013). In terms of a close relationship between inflammation and insulin resistance in diabetes (Elsner et al., 2000), RELM family may have interactive roles linking inflammation and insulin resistance, both of which major involvement in the progression of atherosclerosis.

1.5. Purpose of this study

Considering the attractive effects of RELM-α in hypercholesterolaemia-induced atherosclerosis (Lee et al., 2014), we hypothesized that RELM-α may reduce diabetic atherosclerosis caused by a metabolic defect, especially diabetes mellitus. To investigate the effect of RELM-α in diabetic atherosclerosis, we used LDLR $^{-/-}$ mice that overexpress RELM-α. The present study is expected to illustrate potential role of RELM-α, showing prominent association with diabetic atherosclerosis.

2. Materials and Methods

2.1. Animal Studies and Diet

All animal experiments were approved by the Institutional Animal Care and Use Committees (IACUC) of Ewha Womans University and followed National Research Council Guidelines. All mice were maintained under specific pathogen-free (SPF) conditions with isolated ventilation cages in an air-conditioned room with a 12 hours light/dark cycle and water ad libitum. LDLR null (C57BL/6J background) male mice were purchased from Jackson Laboratories (Bar Harbor, ME) and cross-bred with RELM-α transgenic mice (0011 line, C57BL/6J background) to generate LDLR $^{-/-}$/ RELM-α transgenic (Tg) double-mutant mice. For the diabetic atherosclerosis studies, mice were switched from a normal chow (11.4% fat, 62.8% carbohydrate and 25.8% protein) to a high fat diet (HFD) at 27 and 40 weeks of age and maintained on that diet (Research Diets D13052906, 60 kcal% fat, 0% cholesterol) for 8 weeks in young adult and adult group respectively.

2.2. Genotyping

Genotyping was performed to confirm LDLR deficiency and RELM-α transgene. Mice were sacrificed with isoflurane and 1cm section

of mice tails were incubated with lysis buffer (50mM Tris-HCl pH8.0, 100mM EDTA, 100mM NaCl, 1% SDS) and proteinase K digestion overnight at 55°C on water bath. Tail DNA was vigorously mixed with saturated NaCl and centrifuged at 12,000 rpm at RT for 20min. Supernatant decanted into new tube was mixed with Isopropanol and inverted to precipitate genomic DNA. After centrifugation at 12,000 rpm at RT for 10min, supernatant was removed and DNA pellet was washed with 70% ethanol. Pellet was dried at RT for 10min and dissolved in distilled water. PCR was performed from 1ug DNA using PCR kit (Bioneer, Accupower TM PCR premix, Korea). Primers for LDLR deficiency used to amplify DNA fragments were 5'-ACCCCAAGACGTGCTCCCAGGATGA-3' (Ldlr forward), 5'-CGCAGTGCTCCTCATCTGACTTGT-3' (Ldlr reverse) and 5'-AGGTGAGATGACAGGAGATC-3' (Neo reverse). Primers for RELM-α transgene used to amplify DNA fragments were 5'-GAGCCTGCCAGAGTCTGATACTCAC-3' (RELM-α forward), 5'-GTGTTATCTCCTTTCCTGCCCCC-3' (RELM-α reverse) and 5'-TGTGGCGGACCGCTATCAGGA-3' (Neo reverse).

2.3. Antibodies

For preparation of anti-RELM-α antibody, a GST-RELM-α fusion protein construct containing an 89-amino acid peptide

corresponding to amino acid residues 24-112 of RELM-α was prepared by cloning the EcoRI-XhoI fragment of the RELM-α cDNA in the pGEX-4T1 GST fusion vector and was expressed in the bacterial strain BL21 (DE3). Anti-RELM-α polyclonal antibody was raised in rabbit against the GST-RELM-α fusion protein and further purified by Affinity chromatography (Young In Frontier, Seoul, Korea).

2.4. Immunoblotting

For the detection of RELM-α overexpression in multiple tissues of transgenic mice, mice were euthanized by CO2 inhalation and their plasma, brain, heart, fat, lung, liver, skeletal muscle, intestine, tongue, stomach, kidney and aorta tissues was stored at -70℃ before experiment. Tissues were homogenized with RIPA buffer (25mM Tris-HCl pH 7.6, 150mM NaCl, 1mM EGTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Roche) and beta-mercaptoethanol. Tissue homogenate was sonicated on the ice (1sec ON/ 1sec OFF, 10 cycles) to break the tissues further and washed with phosphate-buffered saline (PBS). After standing the samples on the ice for 10min, the supernatant was transferred to a fresh tube without disturbing pellet and centrifuged at 13,000rpm at 4℃ for 20min to remove any

remaining insoluble material. Protein concentration of the lysate was determined with BSA by Bradford protein assay. Aliquots of samples was frozen at −70℃ for the further analysis. 50ug of protein from each tissues was loaded into the wells of the 15% SDS−PAGE gel, along with molecular weight marker (ELPIS, EBM−1032). Blots were incubated overnight at 4 ℃ with the primary antibodies: RELM−α (1:2,000 dilution), glyceraldehyde−3−phosphate dehydrogenase (1:1,000 dilution). HRP−coupled secondary antibody (Milipore Corporation) was used at a 1:5,000 dilution at room temperature for 1h, followed by detection using the ECL system.

2.5. Streptozotocin Induced Diabetic Model and Mice Monitoring

Young adult and adult male mice were randomly divided into two groups. One group received intraperitoneal injection of STZ (Sigma−Aldrich, S0130−1G) per day for five days (50mg/kg per day in 200ul sodium citrate buffer, pH 4.5, 20mM, Sigma, S−4641) to induce diabetes. As a control, vehicle group received similar injections of sodium citrate buffer alone. Body weight and blood glucose concentration was monitored after STZ injection until mice were sacrificed. Blood was obtained with retro−orbital phlebotomy

and blood glucose concentration was determined by glucometer (One Touch Ultra®, Johnson and Johnson). Mice with a glucose concentration exceeding 300mg/dl were considered diabetic. Mice were inspected at least couple days a week for signs of pain or distress such as urine output, excessive thirst and weight loss.

2.6. Blood Analysis

Mice were fasted for 4hours after the indicated feeding regimen. Blood was collected from the retro-orbital sinus into non-heparinized capillary tubes (Scientific Glass, Inc) before sacrifice. Thereafter serum was obtained by centrifugation at 13000 rpm for 20 minutes at 4℃ and stored at −70℃ before analysis. GOT (glutamic oxaloacetic transaminase), GPT (glutamic pyruvic transaminase), total cholesterol, triglyceride, HDL, LDL cholesterol and glucose levels were measured.

2.7. Assessment of Atherosclerosis

After mice were euthanized, hearts and aortas were perfused with (PBS) through the left ventricle. The aortas were dissected from the proximal ascending aorta to the bifurcation of the iliac artery, and adventitial fat was removed. After aortas were opened longitudinally, these were pinned onto a flat black silicone plate with

2 cm needles.

For lesion quantification in the aortic root, the hearts were removed at the proximal aorta and the upper portion was embedded without bubbles in OCT compound (Tissue-Tek) and frozen at -70℃. Ventricular tissue was sectioned into 10 μm sections by a cryostat microtome (Leica CM18050 XL). Section slides were dried for 30-60 minutes at room temperature and then fixed in ice cold 10% formalin for 5-10 minutes, which was dried again for another 30-60 minutes or rinsed immediately in 3 changes of distilled water. Sections and fixed aortas were immersed in absolute propylene glycol (Duchefa Biochemie) for 1 minutes and stained with oil red O (Sigma Aldrich) for 16hours. The samples were immersed in 85% propylene glycol for 2 minutes, washed with PBS, and then digitally photographed at a fixed magnification. The area occupied by the lesion in the aortic root was measured using Axiovision AC (Carl Zeiss, Germany). To quantify en face lesions, the lesion area was evaluated as a percentage of total aortic area.

2.8. Statistical Analysis

Data expressed as the mean ±SD. Statistical significance was determined by Student's t test and Mann-Whitney U-test. Throughout all statistical analyses, P values of <0.05 were

considered to be significant.

3. Results

3.1. The mice model of RELM-α overexpression

Vascular inflammation by facilitating stress and damage to endothelial cells and smooth muscle cells contributes to the pathogenesis of diabetic atherosclerosis (Figure 1). To define the role of RELM-α in vivo, we used RELM-α -overexpression mice generated by using a pCAGGS expression vector containing a cytomegalovirus enhancer fused to the ubiquitously expressed chicken beta-actin promoter. RELM-α Tg mice were healthy and fertile and did not exhibit detectable impairment. For the diabetic atherosclerosis studies, RELM-α Tg mice were cross-bred with LDLR $^{-/-}$mice to produce LDLR $^{-/-}$ /RELM-α Tg double-mutant mice since RELM-α Tg mice do not develop measureable atherosclerotic plaque lesion on a HFD diet. The offspring that did not inherit the RELM-α transgene were used as control. Confirmation of RELM-α transgene and LDLR gene deficiency that were used in the present study were determined by genotyping PCR analysis (Figure 2A). Expression of the mutant allele was confirmed by immunoblotting using protein extracted from brain, heart, abdominal fat, lung, liver, skeletal muscle, intestine, tongue, stomach, kidney and aorta with plasma as control. The highest

expression was observed in skeletal muscle, intestine, tongue, and stomach, whereas the lowest expression was detected in lung and liver. Expression of RELM-α transgene was rarely detected in fat and kidney compared to control (Figure 2B). Accordingly, compared with nontransgenic mice, RELM-α Tg mice displayed a greater expression in most analyzed tissues though there was difference depending on the tissues.

3.2. RELM-α overexpression reduce cholesterol in of diabetic atherosclerosis mice

To study the effects of RELM-α on diabetic atherosclerosis, mice in young adult and adult group were fed a HFD for 8weeks after STZ injection (Figure 3). RELM-α transgenic mice from both groups were similar in regard to glucose level compare to that of control animals with no significant difference by RELM-α overexpression. STZ-induced diabetic mice maintained higher glucose level (Figure 4A and 4B). Weight gain was also similar in RELM-α Tg and non-Tg mice of both groups on HFD diet after STZ injection. Given that the daily food intake was similar between RELM-α Tg and non-Tg mice of both groups, RELM-α was unlikely to contribute to weight gain in diabetic and non-diabetic state (Figure 5A and 5B). However, the time to reach the diabetic

state and the average glucose level after STZ injection differed slightly between young adult and adult group. Mice from young adult group developed diabetes within 1week after treatment, whereas achieving this state took on average 3weeks in adult group. Blood analysis result showed RELM-α does not affect plasma glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) including blood glucose level (Table 1). However,total plasma cholesterol levels were decreased by 17% and 24%,respectively, in RELM-α Tg mice in both non-diabetic and diabetic group. (Table 2). These favorable results observed in LDLR $^{-/-}$ /RELM-α Tg mice further support a protective role of RELM-α in diabetic atherosclerosis.

3.3. RELM-α overexpression reduces aortic arch plaque size

We analyzed atherosclerotic lesions in aortic arch to whether RELM-α overexpression influences inflammatory plaque phenotype. After 8 weeks on a HFD, the total aortic arch lesion Oil Red O positive area was decreased by 7 and 9% in non-diabetic and diabetic LDLR $^{-/-}$/ RELM-α mice, respectively (Figure 6). Plaque area reduction in aortic arch was higher with diabetic RELM-α Tg mice than non-diabetic group. Plaque area in non-diabetic group

also showed decreasing trend in LDLR $^{-/-}$/ RELM-α mice compared with control mice, though it did not show significant difference according to diabetic state. This results indicate that RELM-α has a further support in STZ-induced diabetic atherosclerosis.

3.4. RELM-α overexpression decreases aortic root plaque size

Aortic root lesions were also decreased by 35 and 37% in non-diabetic and diabetic LDLR $^{-/-}$/ RELM-α Tg mice, respectively (Figure. 7). Plaque area reduction in aortic root was also higher with diabetic RELM-α Tg mice than non-diabetic group.

Collectively, these results indicate that RELM-α ameliorates HFD-induced diabetic atherosclerosis.

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Table 1. Plasma GOT, GPT, GLU and ALB levels

		GOT	GPT	GLU	ALB
Non diabetic	*Ldlr* $^{-/-}$	253.8±213.7	144.0±64.2	252.6±68.1	2.0±0.2
	Ldlr $^{-/-}$ *RELM-α Tg*	276.6±93.4	177.6±77.2	249.6±96.7	2.0±0.2
	p-value	0.83	0.48	0.96	1.0
		GOT	GPT	GLU	ALB
Diabetic	*Ldlr* $^{-/-}$	252.0±237.4	157.8±131.7	468.6±68.0	2.7±0.7
	Ldlr $^{-/-}$ *RELM-α Tg*	228.0±69.6	142.8±43.2	476.4±143.4	2.6±1.4
	p-value	0.83	0.81	0.92	0.90

After mice were fasting for 4h, blood was collected from the retro-orbital sinus for verification of hepatic toxicity.

All the values are expressed as mean [standard deviation].

Abbreviations: GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; GLU, glucose; ALB, albumin.

Table 2. Lipid profile in control and STZ-induced diabetic mice.

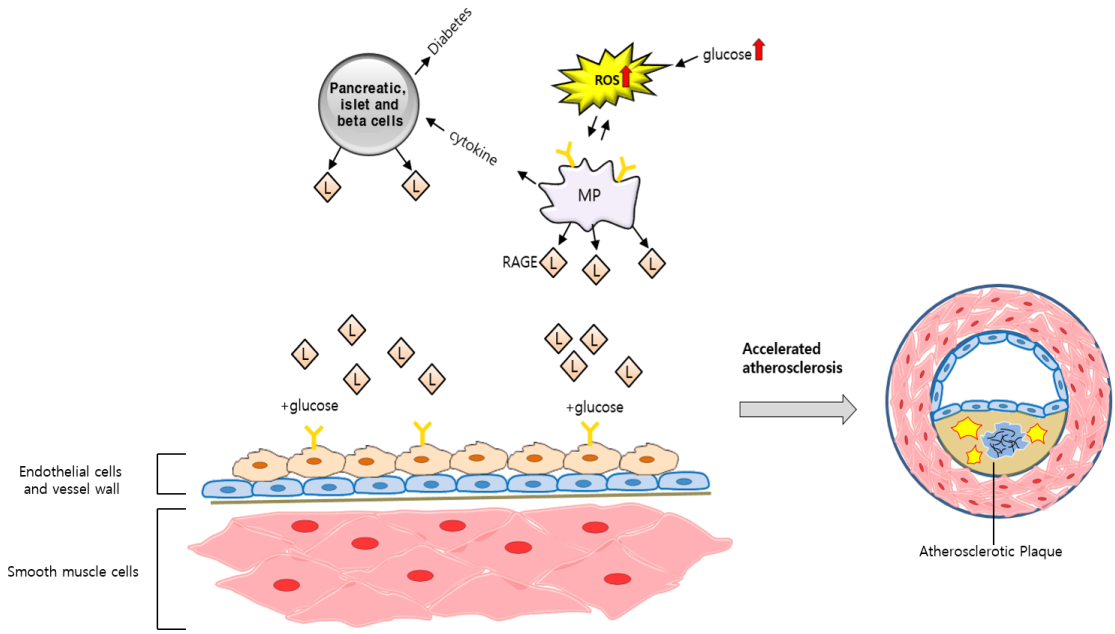
		CHO	TG	HDL	LDL
Non diabetic	*Ldlr* $^{-/-}$	1750.6±94.1	147.0±72.3	123.6±14.0	442.8±153.6
	Ldlr $^{-/-}$*RELM-α Tg*	1461.6±354.2	158.4±90.0	108.6±28.7	514.8±116.1
	p-value	0.10	0.84	0.32	0.43
		CHO	TG	HDL	LDL
Diabetic	*Ldlr* $^{-/-}$	1983.7±431.0	734.4±371.5	95.4±21.0	896.4±281.6
	Ldlr $^{-/-}$*RELM-α Tg*	1499.9±231.9	837.0±969.5	104.4±10.7	719.4±223.1
	p-value	0.04	0.83	0.42	0.3

After mice were fasting for 4h, blood was collected from the retro-orbital sinus for lipid profile analysis.

All the values are expressed as mean [standard deviation].

Abbreviations: CHO, cholesterol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Figure 1.

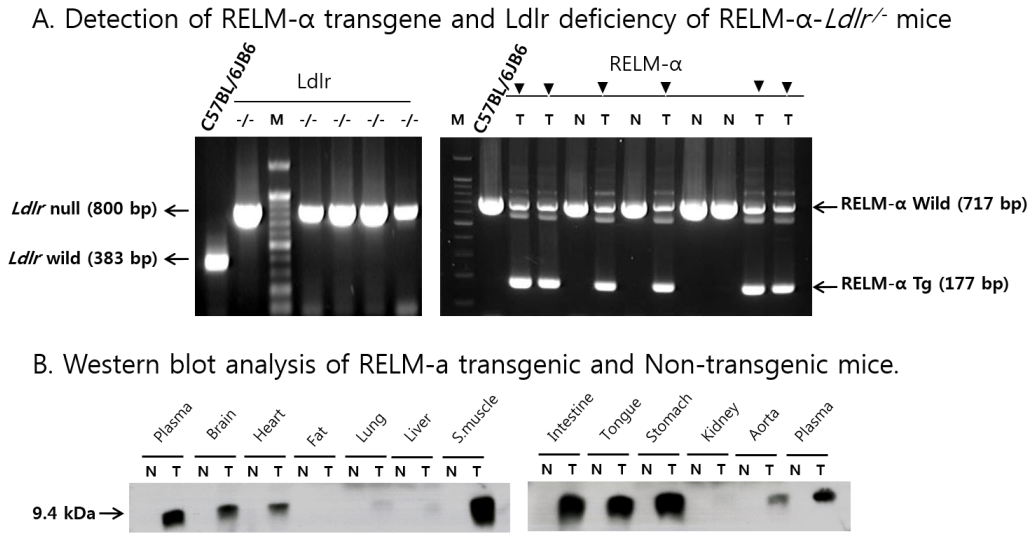


Mechanism of Diabetic atherosclerosis

Oxidative stress has a central role in the pathogenesis of diabetic complications. ROS induced by high glucose concentrations leads to enhanced production of AGEs. RAGE is expressed on key cells implicated in the inflammatory response in parallel with increased expression of its ligands. RAGE ligand which inflammatory cells release might reach the periphery. A first target is the vascular endothelium. Engagement of RAGE by its ligands generates ROS in vascular cells affected by diabetes and contribute to vascular inflammation by facilitating stress and damage to endothelial cells and smooth muscle cells. Such injury contributes to the pathogenesis of diabetic accelerated atherosclerosis.

Abbreviations: ROS, reactive oxygen species; L, ligands of the receptor for advanced glycosylation end-products; MP, mononuclear phagocyte; RAGE, receptor for advanced glycosylation end-products.

Figure 2.

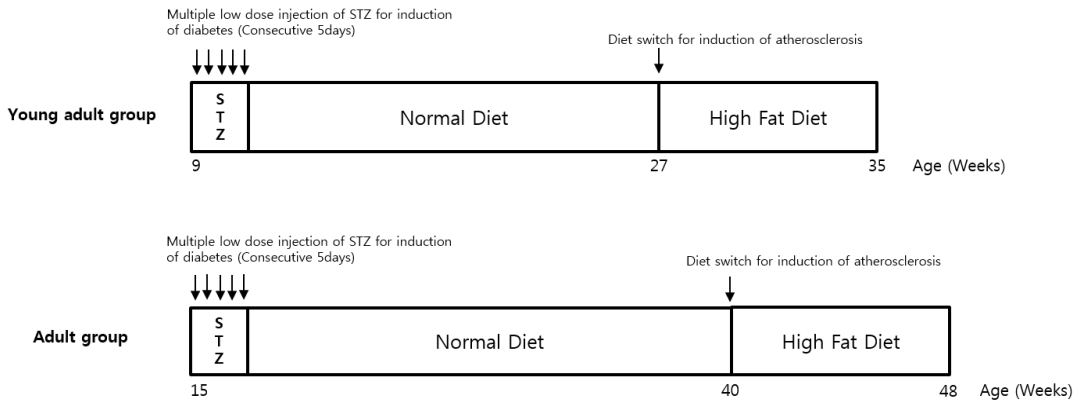


RELM-α overexpression in transgenic mice

(A)PCR analysis result of Ldlr deficiency and RELM-α transgene. (B) Relative protein expression levels for RELM-α in tissues of RELM-α transgenic and non-transgenic mice. Expression of the mutant allele was confirmed by immunoblotting using protein extracted from brain, heart, abdominal fat, lung, liver, skeletal muscle, intestine, tongue, stomach, kidney and aorta with plasma as control.

Abbreviations: M, size marker; N: RELM-α Non-Transgenic mice; T, RELM-α Transgenic mice.

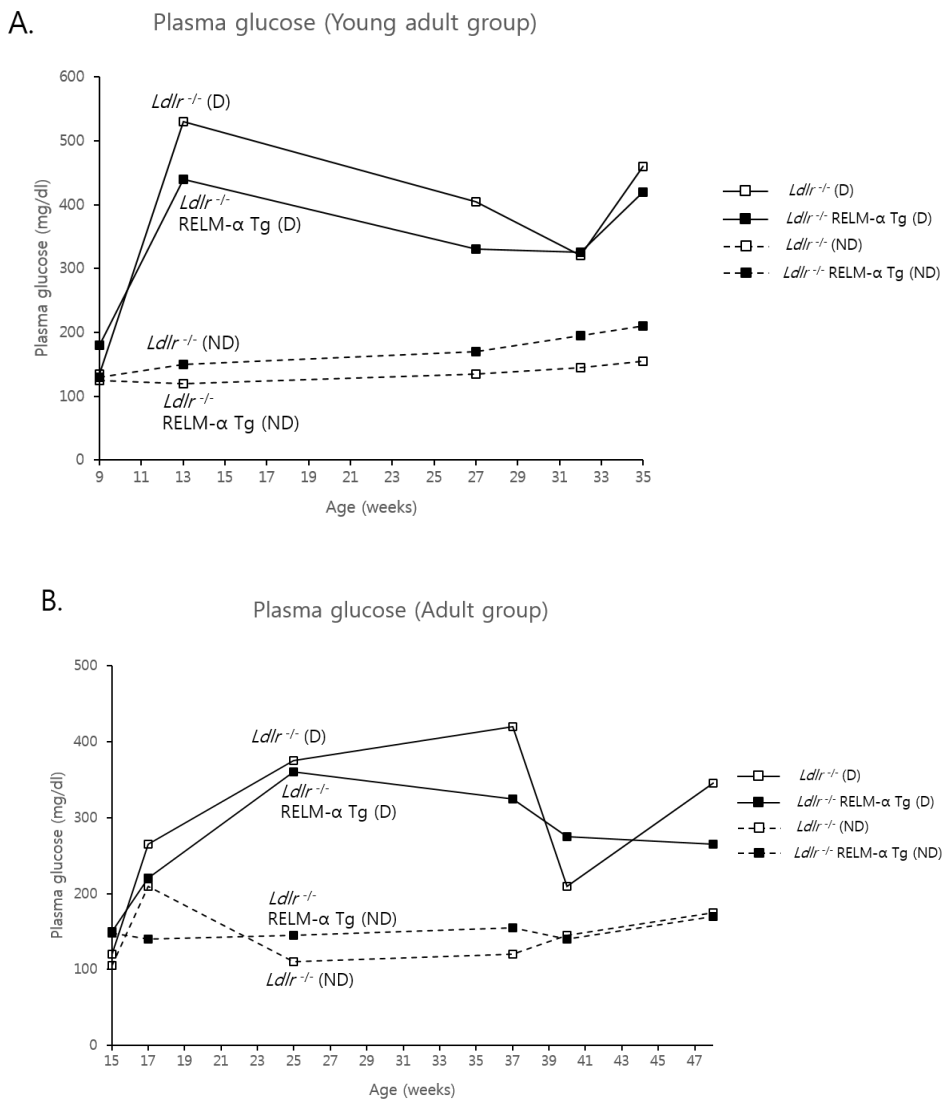
Figure 3.



Schematic diagram showing the experimental course

Young adult and adult male mice were randomly divided into two groups. Mice were injected with STZ (50mg/kg) for consecutive 5 days to produce diabetic model. For the diabetic atherosclerosis studies, mice were switched from a normal chow to a high fat diet (HFD) at 27 and 40 weeks of age and maintained on that diet for 8 weeks in young adult and adult group respectively.

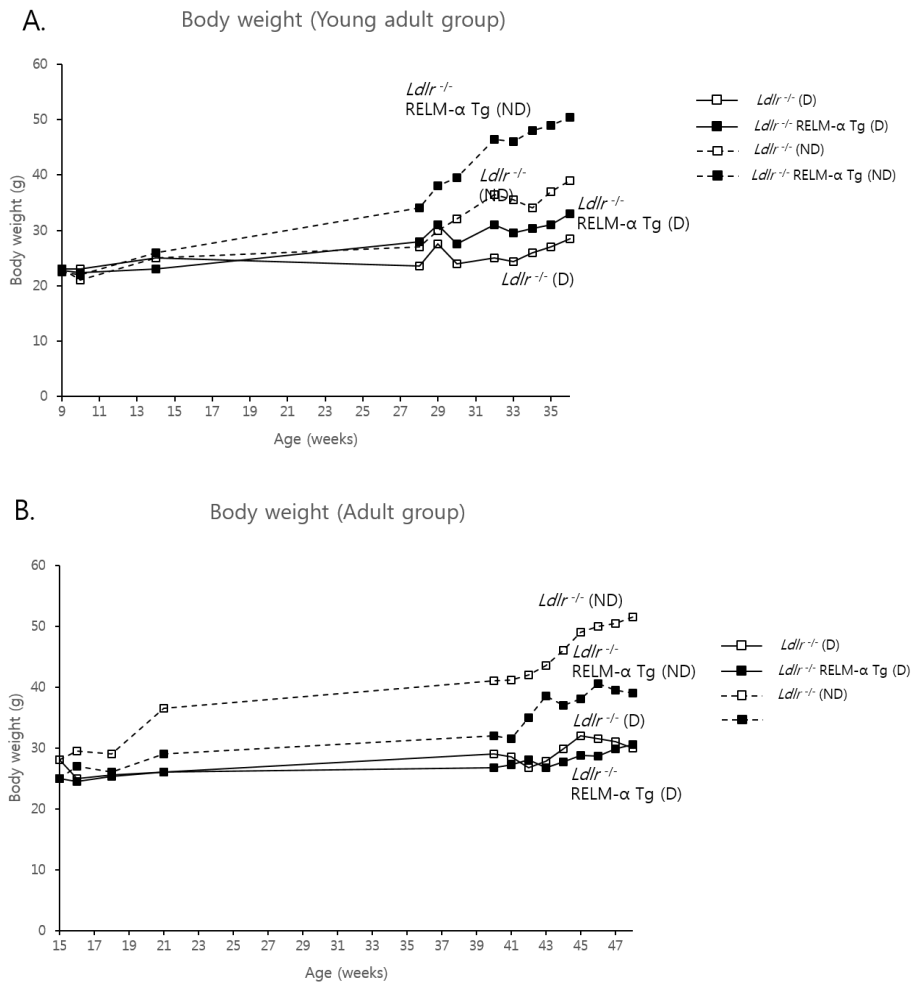
Figure 4.



Plasma glucose level in control and STZ-induced diabetic mice

Plasma glucose level was measured after STZ injection until mice of both young adult (A) and adult group (B) were sacrificed. Abbreviations: ND, non-diabetic; D, diabetic.

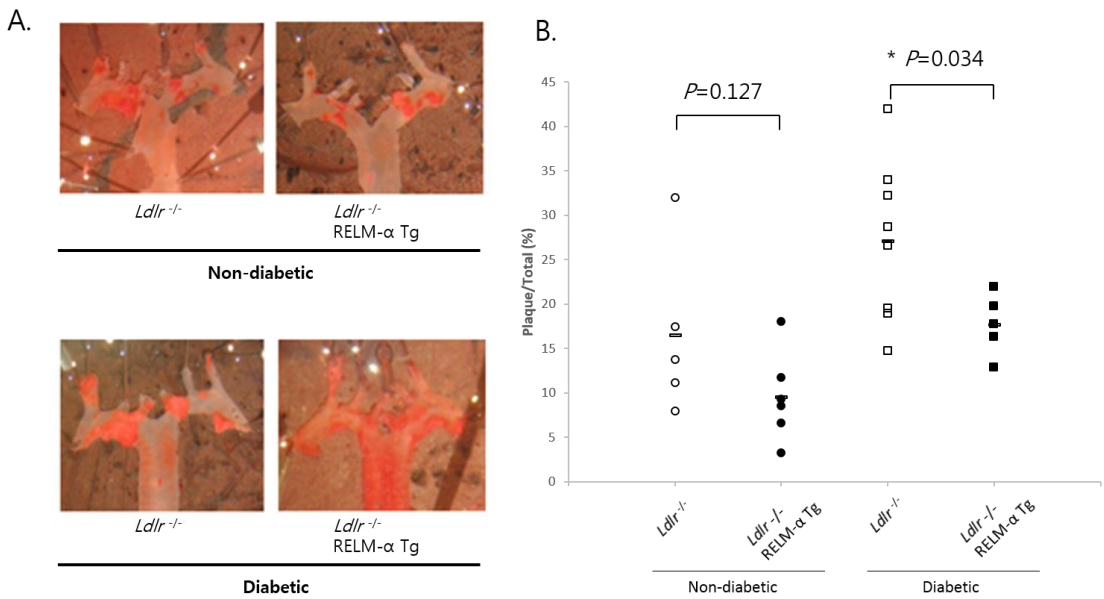
Figure 5.



Body weight in control and STZ-induced diabetic mice.

Body weight was measured after STZ injection until mice of both young adult (A) and adult group (B) were sacrificed. Abbreviations: ND, non-diabetic; D, diabetic.

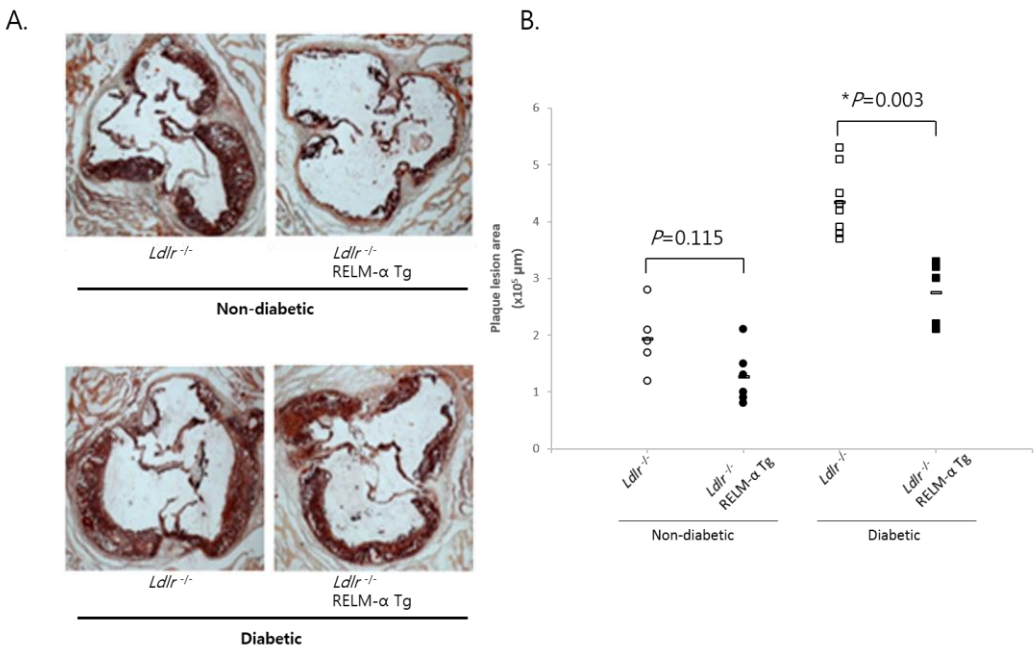
Figure 6.



RELM-α overexpression reduces aortic arch plaque size

Oil red O-stained aortic en face view of *Ldlr* $^{-/-}$ and *Ldlr* $^{-/-}$ RELM-α Tg mice fed a HFD for 8 weeks of non-diabetic (vehicle) and diabetic (STZ-induced) group. Representative Oil red O staining of atherosclerotic lesions in each group is shown. Quantitative data in the right graph represent plaque area. $*P<0.05$ compared with control.

Figure 7.



RELM-α overexpression decreases aortic root plaque size

Oil red O-stained aortic root of *Ldlr* $^{-/-}$ and *Ldlr* $^{-/-}$ RELM-*α* Tg mice fed a HFD for 8 weeks of non-diabetic (vehicle) and diabetic (STZ-induced) group. Representative Oil red O staining of atherosclerotic lesions in each group is shown. Quantitative data in the right graph represent plaque area. $*P<0.05$ compared with control.

4. Discussion

RELM-α was recently suggested to be a link between hypercholesterolaemia and atherosclerosis in rodents (Lee et al., 2014; Miyake et al., 2002), but evidence to confirm this in humans is not sufficient because RELM-α has been identified only as mouse gene. Human resistin shows more similar expression pattern to mouse RELM-α than to mouse resistin, suggesting that the functional role of RELM-α may be associated with that of human resistin. Instead, human resistin expression was found to be abundant in monocytes/macrophages, which play an important role in atherosclerosis. These cells infiltrate arteries and initiate or promote atherogenesis by secreting various pro-inflammatory cytokines (Jung et al., 2006).

In this study, we found that RELM-α has anti-atherogenic effects and protects against diabetic atherosclerosis. RELM-α overexpression significantly reduces plaque size of both aortic arch and aortic root in diabetic LDLR $^{-/-}$ mice (Figure. 6-7) with total plasma cholesterol lowering effect (Table 1-2). As shown in Figure. 4 and 5, RELM-α overexpression did not affect plasma glucose level and weight gain in both non-diabetic and diabetic mouse model. These findings are similar with previous study (Lee et al.,

2014) showed significant differences in serum cholesterol and atherosclerotic lesion size by RELM-α overexpression in non-diabetic mouse model. However, these were caused by much longer HFD feeding period (28weeks) compared with this study (8weeks). In this study, we subjected age-matched RELM-α overexpression mice and control mice to a HFD for 8weeks to examine the effect of RELM-α overexpression in diabetic atherosclerosis. Our findings suggest that RELM-α overexpression contributes to attenuation of atherogenesis with no significant changes in direct regulation of glucose concentration, while previous studies showed RELM-α deficiency protected against impaired glucose tolerance and colitis in a model of dextran sodium sulphate-induced colitis (Munitz et al., 2009; Munitz et al., 2008). It is also consistent with another report, the beneficial and detrimental roles of RELM-α in inflammatory diseases are likely influenced by the type of immune stimulus, the duration of the stimulus exposure and the tissue type (Nair et al., 2009; Osborne et al., 2013; Pesce et al., 2009). To date, no report has been issued on RELM-α binding proteins or RELM-α receptors. Instead, previous studies suggest the presence of a receptor of RELM-α in hepatocytes (Lee et al., 2014). Given that affinity of hepatocyte metabolism for glucose and resistin regulates blood glucose levels (Brocklehurst et al., 2004; Munitz et al., 2009),

RELM-α may have a role in glucose metabolism for preventing atherosclerosis.

Although RELM-α is strongly associated with atherosclerosis, clear causal relationship has not been established. Recent study identified RELM-α as a new atheroprotective adipokine having a cholesterol-lowering effect caused by enhanced cholesterol excretion in the form of bile acids under hyperlipidemic states (Graham et al., 2011). Thus our findings in the role of RELM-α for atherosclerosis are consistent with those of previous studies, because we observed RELM-α overexpression reduces plaque size of both aortic arch and aortic root in mice models. Further studies on RELM-α in the inflammatory process of atherosclerosis are needed to understand the exact role of RELM-α with the specific molecular and cellular mechanisms leading to the effects by RELM-α we observed.

Atherosclerosis is a chronic inflammatory disease characterized by endothelial dysfunction and slow thickening of arterial walls due to the lesion formation in the arteries of lesions containing lipid accumulation, cell death and fibrosis (Hansson & Libby, 2006; Stoll & Bendszus, 2006). Endothelial dysfunction includes altered anticoagulant and anti-inflammatory properties of the endothelium, the aberrant modulation of vascular growth, and the dysregulation

of vascular remodeling (Jung et al., 2006). Daley et al. demonstrated that pulmonary arterial remodeling is associated with the recruitment of RELM-α + macrophages in a model of antigen-specific airway inflammation (Daley et al., 2008). Angelini et al. also showed that RELM-α induces bone marrow derived (BMD) cell recruitment to the remodeling pulmonary vasculature (Angelini et al., 2010). BMD cells have been shown to be localized in atherosclerotic lesions of the vasculature. Additionally, previous study showed that recombinant RELM-α induced the expression of angiogenic factors such as vascular endothelial growth factor and vascular endothelial cell adhesion molecule-136, leading to the hypothesis that RELM-α may mediate vascularization associated with inflammation and involve atherosclerosis.

Hyperglycemia and insulin resistance are major consequences of diabetes mellitus responsible for cardiovascular disorders in patients with diabetes mellitus, which are associated with the multiple mechanisms (Lenzen et al., 1987; Wang & Gleichmann, 1998). Aldose reductase catalyzes the reduction of a variety of carbonyl-containing compounds—including glucose and several glycolytic intermediates—to their corresponding alcohols. In some cell types, glucose is converted to the sugar alcohol sorbitol which is then converted to fructose by sorbitol dehydrogenase (Wang &

Gleichmann, 1998). This series of reactions, termed the polyol pathway, has been implicated in the pathogenesis of diabetic cardiovascular disease. Previous studies showed that overexpression of human aldose reductase accelerated atherosclerosis with activated VCAM-1 and increased lesion size in STZ induced diabetic mice (Lenzen et al., 1987; Vedantham et al., 2011). The post-translational modifications of proteins called advanced glycation end-products (AGEs) are formed by glucose-derived compounds and increased in diabetic tissues and expressions of both the pattern recognition receptor for AGEs (RAGEs) and its ligands are all increased by high levels of glucose and ROS (Lenzen et al., 1987; Wang & Gleichmann, 1998). Recent study has identified increased AGE precursor methylglyoxal as an important element in the pathogenesis of both diabetic atherosclerosis and diabetic cardiomyopathy (Brownlee, 2001; Wang & Gleichmann, 1998). Our results supported a role of RELM-α in diabetic atherosclerosis, as reduction of atherosclerotic lesion size by RELM-α overexpression was higher in diabetic mice compared to non-diabetic mice. Thus, it is possible that RELM-α exerts anti-atherosclerotic effects by involving in pathophysiology of hyperglycemia-induced atherosclerosis. Indeed, it is assumed that resistin and the RELM protein family may have a role in the

metabolism and energy balance including regulation of glucose (Munitz et al., 2009).

The role of lipid modification as a means to decrease cardiovascular risk in diabetes has been clarified by a number of clinical trials (Ali, Jamil, Anwar, & Wajid, 2015; Farmer, 2008; Nicholls et al., 2011). It is generally accepted that the diabetic vascular complications are diseases caused by multiple risk factors including chronic hyperglycemia, hyperinsulinemia, lipid metabolism disorders, etc. In addition, there may be complex interactions between these factors, contributing to the occurrence and development of vascular lesions under diabetic condition (Zheng et al., 2016). Fujii et al have demonstrated that dietary 1,3-diacylglycerol-rich oil reduced atherosclerosis in diabetic apoE-deficient mice, and was associated with reduction in plasma cholesterol especially (Fujii, Allen, & Nestel, 2007). Therefore, our findings suggest RELM-α is associated with cholesterol reduction which ameliorates diabetic atherosclerosis.

The association between resistin and atherosclerosis remains controversial. Diez et al. were unable to show any difference in the serum concentrations of resistin between end-stage renal disease patient with or without vascular disease (Díez et al., 2005; Jung et al., 2006). On the other hand, in a prediabetic/diabetic patient

population Shetty et al. found a significant positive correlation between resistin and CRP (C-Reactive Protein) serum concentrations, and a significant negative correlation between resistin and HDL serum concentrations (Shetty, Economides, Horton, Mantzoros, & Veves, 2004). CRP is an acutephase reactant that is elevated in inflammatory states and present in the atherosclerotic lesion, more specifically in the vascular intima, where it co-localizes with monocytes, monocyte-derived macrophages and lipoproteins (Shrivastava, Singh, Raizada, & Singh, 2015). Higher levels of CRP predict cardiovascular disease in the general population (Shrivastava et al., 2015). Previous studies showed that coronary artery calcium score increased with increasing serum resistin concentration in men (Lenzen et al., 1987). In this study of inherited risk of coronary atherosclerosis (SIRCA) substudy, plasma levels of resistin, in contrast to CRP, were of incremental value in the association between coronary artery disease and metabolic syndrome (Jung et al., 2006; Reilly et al., 2005). However, resistin is more likely to affect atherosclerosis as a local inflammatory factor, although its systemic effects cannot be excluded (Reilly et al., 2005). It remains to be elucidated whether high levels of resistin is a marker of inflammation or whether it has systemic effects on atherosclerosis.

In summary, consistent with a previous report (Lee et al., 2014), we showed that reduction of plasma cholesterol levels in RELM-α Tg mice. Furthermore, we also observed that RELM-α overexpression has a higher anti-atherogenic effect in diabetic atherosclerosis compared with non-diabetic group. Given that affinity of glucose metabolism and atherosclerosis for RELM family, we conclude that RELM-α plays important role to ameliorate atherosclerosis accelerated by diabetes mellitus as a novel therapeutic target.

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국문초록

Ldlr 유전자가 제거된 마우스 모델에서 RELM-α 의한 당뇨성 동맥경화 감소효과

Resistin과 유사한 단백질 RELM-α는 포유류에서 분비되는 단백질 계열에 속하고 면역성 조절 기능이 있다고 추정된다. 최근 연구는 고지혈증에 의해 유발된 동맥경화의 발생 기전에서 RELM-α의 역할을 발표한 바 있다. 그러나, RELM-α가 당뇨성 동맥경화를 조절하는지에 대한 기전은 알려져 있지 않다. 본 연구를 통해 RELM-α가 Ldlr 유전자가 결손된 마우스 (*Ldlr*$^{-/-}$)에서 항 동맥 경화 효과를 가지고 있을 뿐만 아니라 당뇨성 동맥경화를 억제시키는 효과가 있음을 확인하였다. 마우스에 유도된 당뇨의 심각도는 혈당 수준과 몸무게를 측정함으로써 확인되었다. Lldr 유전자가 결손된 동시에 RELM-α를 과발현시킨 마우스 (*Ldlr* $^{-/-}$ RELM-α Tg)는 당뇨를 유발하지 않은 그룹과 당뇨를 유발한 그룹에서 모두 대조군 마우스 (*Ldlr* $^{-/-}$)보다 혈중 콜레스테롤 수준이 감소하였고, 당뇨 그룹에서 비 당뇨 그룹보다 더 큰 감소를 보였다. 8주간의 고지질 사료를 섭취한 마우스 중 당뇨를 유발하지 않은 그룹의 대동맥누와 대동맥궁에서 병변의 크기가 *Ldlr* $^{-/-}$ 마우스에서보다 *Ldlr* $^{-/-}$ RELM-α Tg에서 감소한 것을 확인하였고, 당뇨를 유발한 그룹에서도 RELM-α Tg를 과발현 시킨 마우스가 그렇지 않은 마우스보다 병변의 크기가 감소한 것을 확인하였다. 본 연구에서는 비 당뇨성 그룹과 비교

하였을 때 RELM-α 의 과발현은 당뇨성 동맥경화에서 콜레스테롤 감소와 함께 더 큰 항 동맥 경화 효과를 가짐을 확인할 수 있었다. 이러한 결과는 RELM-α가 당뇨성 동맥경화에 대한 새로운 가망성 있는 치료 대상으로서 연구할 필요성이 있다고 사료된다.

주요어: RELM-α, 당뇨성 동맥경화, 저밀도 리포 단백질 유전자 결손 마우스, 대동맥 병변

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