Insulin treatment attenuates diabetes-increased atherosclerotic intimal lesions and matrix metalloproteinase 9 expression in apolipoprotein E-deficient mice

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Abstract

Patients with diabetes mellitus have increased mortality and morbidity of cardiovascular diseases compared with non-diabetic patients. Although clinical studies have shown that effective glycemic control with insulin treatment in patients with type 1 diabetes is associated with reduced cardiovascular events, the underlying mechanisms have not been well understood. In this study, we treated diabetic apolipoprotein E-deficient (apoE^{−/−}) mice with insulin for 20 weeks and studied the effect of insulin treatment on intimal lesion size and matrix metalloproteinase (MMP) 9 expression known to be involved in plaque destabilization. Results showed that insulin treatment, which effectively reduced plasma glucose

level in diabetic mice, attenuated diabetes-increased intimal lesion size and significantly inhibited diabetes-increased MMP9 expression, but had no effect on tissue inhibitor of metalloproteinase 1 in atherosclerotic plaques. Furthermore, we observed that insulin treatment did not reduce diabetes-increased macrophage content but inhibited interleukin 6 expression, a stimulator for MMP expression. Taken together, this study has shown for the first time that insulin treatment in diabetic apoE^{−/−} mice changes atherosclerotic lesions and gene expression to a state that favors plaque stability.

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Introduction

Epidemiological studies have consistently shown that patients with diabetes mellitus have increased mortality and morbidity of cardiovascular disease compared with the general population (Soedamah-Muthu & Stehouwer 2005). To determine whether the control of hyperglycemia, the hallmark of diabetes, reduces the risk of cardiovascular events, a clinical study entitled 'Diabetes Controls and Complication Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC)' was conducted on 1441 patients with type 1 diabetes (Nathan *et al.* 2005). After giving patients either conventional or intensive insulin treatment, which lowered HbA1c level to 9·1 or 7·4% respectively, for 6·5 years and following up patients for 17 years, the study group reported that the intensive treatment significantly reduced the risk of any cardiovascular disease event by 42% ($P=0\cdot02$) and the risk of nonfatal myocardial infarction, stroke, or death from cardiovascular disease by 57% ($P=0\cdot02$). Furthermore, the study showed that the decrease in HbA1c values was significantly associated with most of the positive effects of intensive treatment on the risk of cardiovascular disease.

Clearly, this study provided evidence to support an essential role of glycemic control in reduction in mortality and morbidity of cardiovascular disease in patients with type 1 diabetes. However, it remains unclear how glycemic control decreases cardiovascular events and, in particular, the effect of glycemic control on atherosclerotic plaque stability remains unclear.

It has been established that atherosclerotic plaque rupture is a critical event triggering thrombus formation and subsequent acute coronary syndrome (Libby 1995, Libby & Theroux 2005). Pathologically, plaques that are prone to rupture consist of a larger intimal lesion and an increased number of macrophages. Analysis of gene expression in atherosclerotic plaques showed that when compared with stable plaques, vulnerable plaques have higher expression of matrix metalloproteinases (MMPs) that weaken atherosclerotic plaques (Richardson *et al.* 1989, Falk *et al.* 1995). Autopsy studies have demonstrated that atherosclerosis in diabetic patients is more extensive and accelerated than that in nondiabetic patients (Jarrett 1981). Furthermore, studies have also shown that atherosclerotic lesions in diabetic patients were more vulnerable as they had larger intimal lesions, more

macrophage infiltration and thrombosis compared with those in nondiabetic patients (Moreno *et al.* 2000). Given that plaque rupture is a key event in triggering cardiovascular events, these studies suggest that glycemic control may decrease the mortality and morbidity of cardiovascular disease in diabetic patients by stabilizing atherosclerotic plaques.

MMPs are a family of zinc-dependent endopeptidases capable of degrading extracellular matrix components and play an essential role in vascular tissue remodeling (Galis & Khatri 2002). It is known that MMPs are involved in plaque destabilization and ultimate plaque rupture (Galis & Khatri 2002). Among various MMPs, plasma MMP9 has been associated with the presence of coronary atherosclerosis and is an independent risk factor for atherothrombotic events (Loftus *et al.* 2001, Blankenberg *et al.* 2003, Montaner *et al.* 2003). Furthermore, it has been reported that the plasma MMP9 level in patients with both diabetes and coronary artery disease was significantly higher than that in age-, sex-, and body mass index-matched patients with coronary artery disease alone (Marx *et al.* 2003), indicating an important role of MMP9 in diabetes-associated atherosclerosis. Given the importance of MMP9 in diabetes-associated atherosclerosis, we proposed that glycemic control might reduce cardiovascular events by inhibiting MMP9 expression in atherosclerotic plaques and thus stabilizing atherosclerotic plaques.

In this study, we employed apolipoprotein E-deficient (apoE^{−/−}) mice with streptozotocin (STZ)-induced diabetes and studied the effect of insulin treatment on diabetes-increased intimal lesion and expression of MMP9 and interleukin (IL) 6, a stimulator for MMP9, in atherosclerotic lesions.

Materials and Methods

Animals and diets

Male C57BL/6 mice and apoE^{−/−} mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and housed at the animal facility in the Veterans Affairs Medical Center in Charleston, SC, USA. The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC). The C57BL/6 mice served as nonatherosclerosis control (group 1) and apoE^{−/−} mice were randomly divided into three groups (groups 2–4). All mice were maintained on a 12 h light:12 h darkness cycle in a pathogen-free environment and had *ad libitum* access to regular chow (Harlan Teklad 8604, Indianapolis, IN, USA) and water. At 12 weeks of age, the mice in groups 3 and 4 were rendered diabetic by i.p. administration of single dose of STZ (Sigma–Aldrich), 200 mg/kg in citrate buffer, pH 4.5. At 4 weeks after the STZ treatment, the mice in group 4 were treated with long-acting Lantus insulin (Aventis Pharmaceuticals, Bridgewater, NJ, USA) by daily s.c. injection of 1 unit/mouse for 20 weeks.

Metabolic measurements

Blood samples were obtained under the fasting condition, and glucose level was determined using a Precision QID glucometer (MediSense, Inc., Bedford, MA, USA). Plasma cholesterol and triglycerides were measured by enzymatic methods (Wako Diagnostics, Richmond, VA, USA).

Histological analysis of atherosclerotic lesions

The tissues of aortic root were dissected and frozen in Tris-buffered saline (TBS) freezing media and sectioned with a cryostat. Sections with 5 µm thickness were mounted on slides before being placed in 95% ethanol for 10 min and washed in PBS. Fixed hydrated slides were stained with Harris modified hematoxylin (Fisher Scientific, Pittsburgh, PA, USA) for 3 min and then rinsed in deionized water. Staining of hematoxylin was developed in tap water for 5 min, and the slides were dipped in acid ethanol to stop the development. Slides were stained with eosin in Harleco 1% alcohol solution (EMD Chemicals, Gibbstown, NJ, USA) for 30 s. Slides were placed in Coplin jars with 95% ethanol three times for 5 min each, and then this was repeated with 100% ethanol. Slides were further dehydrated in 99.5% xylene (Sigma–Aldrich) and mounted in xylene-based Cytoseal-XYL mounting media (Fisher Scientific). Photomicrographs of tissue sections were taken using a Nikon Eclipse 90i digital microscope with the NIS Elements 3.10. image analysis system (Nikon Instruments, Inc., Lewisville, TX, USA). The area of intima was presented as the percentage of the total aortic area including intima, media, and lumen (Collins *et al.* 2001).

Immunohistochemistry for MMP9 and IL6

Frozen tissue sections were exposed to 2% normal goat serum for 20 min. Primary monoclonal antibodies to MMP9 or IL6 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were applied to the sections at 1:40–1:100 dilutions for 45 min. Sections were then incubated with secondary biotinylated antibody from the ABC Elite kit (Vector Laboratories, Burlingame, CA, USA) for 30 min at 1:250 dilutions. After being exposed to avidin–biotin solution from the ABC Elite kit for 30 min, slides with the tissue sections were placed in 30% methanol peroxide. The tissue sections were covered with diaminobenzidine peroxidase substrate solution from the Impact DAB kit (Vector Laboratories) for 3 min. Slides were rinsed in water and counterstaining was performed with hematoxylin. Slides were then dehydrated using increasing concentrations of ethanol and xylene and mounted. Staining with normal rabbit IgG was used as a negative control. Photomicrographs of atherosclerotic lesions with positively immunostained cells were taken using the computer-based image analysis system as described above in the histological study.

Image analysis

Images were analyzed using the Photoshop software (version 10; Adobe Systems). The method of using the 'Similar' feature to select a particular color staining on a digitized immunohistochemical image has previously been described in detail (Lehr *et al.* 1997). Briefly, a standard was created by selecting an area of 0.5 × 0.5 cm from a tissue section that had desired brown color from immunostaining. The cursor of the Magic Wand tool was clicked on the standard to make a selection and the area of the standard was highlighted. To specify how broad a range of color the Magic Wand tool should include in the selection, the tolerance value in the Magic Wand Options palette was set to 100. Using the 'Similar' command, all the areas with the brown color that is similar to the standard on an image being determined were highlighted. The quantification was done using the histogram command in the Image menu, which showed the pixels of the highlighted area. The extent of the positive immunostaining was presented as average pixels per cross-section of atherosclerotic lesions.

Isolation of RNA from aortic tissue

The vascular tissues of aortic root were homogenized in TRIzol solution (Invitrogen) on ice using an ultrasonic processor (GE130, Sonics & Materials, Inc., Newtown, CT, USA). After homogenization, 0.75 ml homogenate was mixed with 0.2 ml chloroform and centrifuged at 12 000 *g* for 15 min. The aqueous phase was removed, mixed with equal volume of 70% ethanol, and loaded into the RNeasy column (Qiagen) for RNA isolation using the RNeasy mini kit by following the instructions from the manufacturer.

Quantitative real-time PCR

First-strand complementary DNA (cDNA) was synthesized using the iScript cDNA synthesis kit (Bio-Rad Laboratories) using 20 µl reaction mixture containing 0.25 µg total RNA, 4 µl 5× iScript reaction mixture, and 1 µl iScript reverse transcriptase. The complete reaction was cycled for 5 min at 25 °C, 30 min at 42 °C, and 5 min at 85 °C using a PTC-200 DNA Engine (MJ Research, Waltham, MA, USA). The reverse transcription (RT) reaction mixture was then diluted

1:10 with nuclease-free water and used for PCR amplification of cDNA in the presence of the primers. The Beacon designer software (PREMIER Biosoft International, Palo Alto, CA, USA) was used for primer designing (Table 1). Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA), and real-time PCR was performed in duplicate using 25 µl reaction mixture containing 1.0 µl RT mixture, 0.2 µM both primers, and 12.5 µl iQ SYBR Green Supermix (Bio-Rad Laboratories). Real-time PCR was run in the iCycler real-time detection system (Bio-Rad Laboratories) with a two-step method. The hot-start enzyme was activated (95 °C for 3 min), and cDNA was then amplified for 40 cycles consisting of denaturation at 95 °C for 10 s and annealing/extension at 53 °C for 45 s. A melt curve assay was then performed (55 °C for 1 min and then temperature was increased by 0.5 °C every 10 s) to detect the formation of primer-derived trimers and dimers. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. Data were analyzed using the iCycler iQ software. The average starting quantity (SQ) of fluorescence units was used for analysis. Quantification was calculated using the SQ of targeted cDNA relative to that of GAPDH cDNA in the same sample.

Cell culture

U937 mononuclear cells (Sundstrom & Nilsson 1976) were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in a 5% CO₂ atmosphere in RPMI 1640 medium (GIBCO, Invitrogen Corp.) containing different glucose concentrations (5–25 mM), 10% FCS, 1% MEM nonessential amino acid solution, and 0.6 g/100 ml HEPES.

ELISA

MMP9 and tissue inhibitor of metalloproteinase (TIMP)1 in conditioned medium were quantified using sandwich ELISA kits according to the protocol provided by the manufacturer (R&D System, Minneapolis, MN, USA).

Statistical analysis

Data are presented as mean ± S.D. Comparison between treatments was performed by the one-way ANOVA. A value of *P* < 0.05 was considered significant.

Table 1 Primer sequences for real-time PCR

Genes	5' Primer sequence	3' Primer sequence
MMP9	GCTGACTACGATAAGGACGG	CTCAAAGATGAACGGGAACAC
IL6	ATGAAGTTCCTCTCTGCAAGAGACT	CACTAGGTTTGCCGAGTAGATCTC
TNFα	TTCTGTCTACTGAAC TTCGGGGTGATCGGTCC	GTATGAGATAGCAAATCGGCTGACGGTGTGGG
MCPI	CTTCTGGGCCTGCTGTTCA	CCAGCCTACTCATTTGGGATCA
M-CSF	CATCCACGCTCGGTGAAG	GGGATTCGGGTGTCGCAATAT
GAPDH	GDDTTCGCTGTTCTACC	GCCTGCTTCACCACCTTC

Figure 1 Plasma glucose (A), cholesterol (B), and triglyceride levels (C) in nondiabetic and diabetic mice. The plasma glucose, cholesterol, and triglycerides in C57BL/6 ($n=8$), nondiabetic apoE^{-/-} ($n=8$), diabetic apoE^{-/-} ($n=10$), and insulin-treated diabetic apoE^{-/-} mice ($n=10$) were quantified at the end of the study.

Results

The effect of insulin treatment on the metabolic parameters

The plasma levels of glucose, cholesterol and triglycerides were determined in nondiabetic and diabetic mice treated with or without insulin. Results showed that glucose level in nondiabetic apoE^{-/-} mice was similar to that in C57BL/6 mice. As expected, glucose level was markedly increased in STZ-treated mice, but the increase was reduced by insulin treatment by 76% (Fig. 1A). Lipid analysis showed that the total cholesterol level was increased in nondiabetic apoE^{-/-} mice by threefold compared with that in C57BL/6 mice. Induction of diabetes in apoE^{-/-} mice further increased total cholesterol level by 43%, but insulin treatment abolished the effect of diabetes on cholesterol (Fig. 1B). For triglycerides, no difference was found among four groups (Fig. 1C). Taken together, these data indicate that insulin treatment effectively reduced diabetes-increased hyperglycemia and cholesterol.

Reduction in intimal lesions by insulin treatment

The image analysis of aortic atherosclerotic lesions showed that C57BL/6 mice had little neointima formation in the aortic origin (Fig. 2A, panel a and B), whereas nondiabetic apoE^{-/-} mice had well-developed atherosclerotic plaques (Fig. 2A, panel b and B). In diabetic apoE^{-/-} mice, intimal lesions were more extensive than those in nondiabetic

apoE^{-/-} mice (33 ± 7 vs $10 \pm 6\%$, $P < 0.01$; Fig. 2A, panel c and B). Furthermore, insulin treatment in diabetic mice significantly attenuated diabetes-increased intimal lesions by 60% (Fig. 2A, panel d and B).

Inhibition of MMP9, but not TIMP1, by insulin treatment

The expression of MMP9 in the intimal lesions of atherosclerotic plaques was detected by immunohistochemistry. Results showed that MMP9 was markedly increased in the intimal lesions in diabetic apoE^{-/-} mice compared with that

Figure 2 Inhibition of diabetes-increased atherosclerotic intimal lesion by insulin treatment. The vascular tissues of the aortic roots were dissected from C57BL/6 mice, apoE^{-/-} mice with or without STZ-induced diabetes, and diabetic apoE^{-/-} mice treated with insulin. Frozen sections were made from the tissues and stained with hematoxylin and eosin. (A) Representative photomicrographs of atherosclerotic plaques of C57BL/6 (a), nondiabetic apoE^{-/-} mice (b), diabetic apoE^{-/-} mice (c), and diabetic apoE^{-/-} mice treated with insulin (d). (B) Quantification of intimal lesion area of atherosclerotic plaques. The intimal lesions were quantified and presented as the percentage of the total aortic area including intima, media, and lumen ($n=8-10$).

Figure 3 Inhibition of diabetes-increased MMP9 quantification in atherosclerotic plaques by insulin treatment. MMP9 expression in atherosclerotic plaques was detected by immunostaining and presented as pixels per plaque. (A) Representative photomicrographs of MMP9 immunostaining of atherosclerotic plaques from nondiabetic apoE^{-/-} mice (a), diabetic apoE^{-/-} mice (b), and diabetic apoE^{-/-} mice treated with insulin (c). The detailed images in the areas as indicated are enlarged in the insets. (B) Quantification of MMP9 in atherosclerotic plaques ($n=8-10$).

in nondiabetic apoE^{-/-} mice (Fig. 3A, panels a and b and B). However, MMP9 expression was significantly reduced in diabetic apoE^{-/-} mice treated with insulin (Fig. 3A, panel c and B). MMP9 expression was localized not only in the lipid core (Fig. 3A, insets of panel b and c), but also in the area near surface of atherosclerotic plaque (Fig. 3A, inset of panel a). Although the expression of MMP13 and MMP2 in atherosclerotic plaques was also increased in diabetic mice, insulin treatment did not inhibit the expression (data not shown), suggesting that insulin treatment had a specific inhibition on MMP9 expression. Furthermore, although TIMP1 expression appeared to be increased in diabetic mice with insulin treatment when compared with that in nondiabetic and diabetic mice without insulin treatment, the increase did not reach significance statistically (Fig. 4).

In support of the findings from the above immunohistochemical studies, experiments using quantitative real-time PCR showed that *MMP9* mRNA expression in vascular tissues of the aortic root was significantly increased in diabetic apoE^{-/-} mice compared with that in nondiabetic mice, and that the increase was completely abolished by insulin treatment (Fig. 5).

Insulin treatment inhibits IL6 expression in atherosclerotic plaques

It is known that IL6 is expressed in atherosclerotic plaques and involved in MMP expression (Li *et al.* 2010). To determine the effect of diabetes and insulin treatment on the expression of

IL6, we assessed IL6 expression in atherosclerotic lesions by immunohistochemistry. Results showed that diabetes significantly increased IL6 expression (Fig. 6A, panels a and b and B), but insulin treatment markedly attenuated diabetes-increased IL6 expression (Fig. 6A, panel c and B). Similar findings were observed from studies using quantitative real-time PCR technique, which showed a significant increase in IL6 mRNA in the vascular tissues of aortic root of diabetic mice compared with that of nondiabetic mice and the attenuation of IL6 upregulation by insulin treatment (Fig. 7A).

In addition to IL6, we also determined mRNA expression of tumor necrosis factor (TNF) α , monocyte chemotactic protein (MCP) 1, macrophage colony-stimulating factor (M-CSF), and CD11B, which are all involved in macrophage recruitment and plaque destabilization (Gray & Shankar 1995, Yakubenko *et al.* 2008, McKellar *et al.* 2009, Yadav *et al.* 2010, Brocheriou *et al.* 2011). Results (Fig. 7B) showed that diabetes increased *TNF α* and *MCP1* mRNA expression, but insulin treatment only inhibited *MCP1* mRNA expression. Diabetes decreased *M-CSF* mRNA expression and insulin treatment did not change the effect of diabetes. In addition, we found that diabetes had no effect on *CD11B* mRNA expression (data not shown).

Reduction in glucose attenuates IL6-stimulated MMP9 by mononuclear cells in vitro

Figure 3 showed that insulin treatment reduced MMP9 expression in atherosclerotic plaques in diabetic mice. Given

Figure 4 Effect of diabetes and insulin treatment on TIMP1 quantification in atherosclerotic plaques. TIMP1 protein expression in atherosclerotic plaques was detected by immunostaining and presented as pixels per plaque. (A) Representative photomicrographs of TIMP1 immunostaining of atherosclerotic plaques from nondiabetic apoE^{-/-} mice (a), diabetic apoE^{-/-} mice (b), and diabetic apoE^{-/-} mice treated with insulin (c). (B) Quantification of TIMP1 protein in atherosclerotic plaques ($n=8-10$).

Figure 5 Inhibition of diabetes-increased *MMP9* mRNA quantification in atherosclerotic plaques by insulin treatment. Total RNA was isolated from vascular tissue of aortic root of nondiabetic apoE^{-/-} mice, diabetic apoE^{-/-} mice, and diabetic apoE^{-/-} mice treated with insulin and subjected to real-time PCR to quantify *MMP9* and *GAPDH* mRNA. Data presented are mean ± s.d. of the ratio of *MMP9* mRNA versus *GAPDH* mRNA ($n=7$).

that insulin treatment lowered serum glucose in diabetic mice, we hypothesized that glucose reduction downregulates *MMP9* expression in atherosclerotic plaques. To test this hypothesis, we pre-exposed U937 cells with different concentrations of glucose and then treated with IL6, a stimulator for *MMP9* expression. After the treatment, *MMP9* in the culture medium was quantified by ELISA. Results showed that IL6-increased *MMP9* secretion by cells pre-exposed to 25, 15, and 5 mM of glucose was 1.8, 1.2, and 0.8 ng/ml respectively (Fig. 8A), suggesting that reduction in glucose from 25 or 15 to 5 mM significantly attenuated IL6-increased *MMP9* secretion. These *in vitro* findings suggest that glucose reduction by insulin treatment may play an important role in insulin treatment-attenuated *MMP9* expression in atherosclerotic lesions.

The effect of high glucose (25 mM) on TIMP1 was also studied. Results showed that high glucose alone had no effect on TIMP1 secretion compared with normal glucose (5 mM; Fig. 8B). Furthermore, the combination of high glucose and IL6 also had no significant stimulation on TIMP1 secretion compared with the combination of normal glucose and IL6 (Fig. 8B), indicating that reduction in glucose from 25 to 5 mM does not significantly decrease TIMP1 secretion by mononuclear cells in response to IL6.

Effect of insulin treatment on macrophage content in atherosclerotic lesions

Macrophages in atherosclerotic plaques were detected by immunostaining with antibody against mouse CD68, a

surface marker for mouse macrophages. Results showed that diabetes increased macrophage content markedly (Fig. 9A, panels a and b and B). Interestingly, insulin treatment did not reduce macrophage content (Fig. 9A, panel c and B).

Discussion

Clinical trials have shown that a good glycemic control in patients with type 1 diabetes significantly reduces cardiovascular disease mortality and morbidity (Soedamah-Muthu & Stehouwer 2005). To understand how glycemic control in diabetic subjects reduces cardiovascular events, we focused on the effect of glycemic control on atherosclerotic lesions and gene expression that affects plaque stability in this study. With long-acting Lantus insulin, we successfully reduced hyperglycemia in diabetic apoE^{-/-} mice. The metabolic studies showed that STZ injection markedly increased plasma glucose level by threefold (591 vs 145 mg/dl), but insulin treatment led to a 76% reduction in diabetes-increased glucose. Therefore, this study established an effective treatment on hyperglycemia using long-acting insulin in a mouse model for atherosclerosis and diabetes.

Previous studies have shown that induction of diabetes in apoE^{-/-} mice increases not only plasma glucose but also cholesterol (Park *et al.* 1998, Tse *et al.* 1999, Candido *et al.* 2004, Hayek *et al.* 2005). However, the levels of cholesterol increase varied remarkably between these studies. For

Figure 6 Inhibition of diabetes-increased IL6 expression in atherosclerotic plaques by insulin treatment. IL6 protein expression in atherosclerotic plaques was detected by immunostaining and presented as pixels per plaque. (A) Representative photomicrographs of IL6 immunostaining of atherosclerotic plaques from nondiabetic apoE^{-/-} mice (a), diabetic apoE^{-/-} mice (b), and diabetic apoE^{-/-} mice treated with insulin (c). (B) Quantification of IL6 protein expression in atherosclerotic plaques ($n=8-10$).

Figure 7 Effect of insulin treatment on *IL6* (A), *TNFα*, *MCP1*, and *M-CSF* mRNA (B) expression in atherosclerotic plaques. Total RNA was isolated from vascular tissue of aortic root of nondiabetic apoE^{-/-} mice, diabetic apoE^{-/-} mice, and diabetic apoE^{-/-} mice treated with insulin and subjected to real-time PCR to quantify *IL6*, *TNFα*, *MCP1*, *M-CSF*, and *GAPDH* mRNA. Data presented are mean $\pm$ s.d. of the ratio of *MMP9* mRNA versus *GAPDH* mRNA ($n=7$).

example, the study by Park *et al.* (1998) reported that the average cholesterol level in apoE^{-/-} mice with STZ-induced diabetes was 1359 mg/dl, which was 2.7-fold of that in nondiabetic apoE^{-/-} mice, while the study by Hayek *et al.* (2005) reported that the average cholesterol level in diabetic apoE^{-/-} mice was 545 mg/dl, only 13% increase (not significant) over that in nondiabetic apoE^{-/-} mice. Interestingly, the glucose levels in these two studies were similar, 350–500 mg/dl in the study of Park *et al.* and 415 $\pm$ 223 mg/dl in the study of Hayek *et al.*, suggesting that the large difference in cholesterol increase is not due to glucose increases. An apparent difference between these two studies was the method in STZ treatment. Park *et al.* treated mice with six daily i.p. injections of STZ at the dose of 55–65 mg/kg, whereas Hayek *et al.* treated with only one injection at the dose of 200 mg/kg. It remains unclear whether repeated

injection with lower doses of STZ increased plasma cholesterol to a greater extent compared with a single injection with higher dose. In this study, we employed the method of Hayek *et al.* in STZ treatment, and found that STZ treatment led to a threefold increase in glucose with a moderate 43% increase in cholesterol. We also found that insulin treatment led to an effective glycemic control and significant reduction in cholesterol.

In humans, it is known that hyperglycemia is not associated with hypercholesterolemia, and glycemic control is not associated with a decrease in cholesterol level. However, an inverse relationship between HbA1C and high-density lipoprotein (HDL) cholesterol levels in type 2 diabetic patients has been reported (Khan *et al.* 2007). Gatti *et al.* (2009) have recently reported that after adjustments for obesity and hypertriglyceridemia in multivariate analysis, poor glycemic control was found to be an independent risk factor for low HDL cholesterol in patients with type 2 diabetes. These studies, therefore, indicate that hyperglycemia in humans also affects cholesterol metabolism. The difference is that hyperglycemia in apoE^{-/-} mouse increases total cholesterol level, whereas hyperglycemia in humans decreases HDL cholesterol. Given the critical role of cholesterol in atherosclerosis, it is likely that both hyperglycemia and hypercholesterolemia in apoE^{-/-} mice in this study contributed to diabetes-promoted progression of atherosclerosis. On the other hand, the reduction in both glucose and cholesterol by insulin treatment is also likely responsible for the attenuation of diabetes-promoted atherosclerosis.

It has been shown that apoE^{-/-} mice on a normal chow diet first manifest foam-cell lesions in aorta at 8 weeks of age, and develop advanced and complex lesions after 15 weeks (Nakashima *et al.* 1994). In this study, apoE^{-/-} mice were grown for 32 weeks, and therefore we considered that these mice had developed advanced atherosclerotic plaques. It is known that large intimal lesion is one of the features for unstable plaques in the advanced stage

Figure 8 Effect of glucose concentration on MMP9 and TIMP1 secretion by U937 mononuclear cells in response to IL6. (A) U937 mononuclear cells were pre-exposed to different concentrations of glucose (5–25 mM) for 2 days and then treated with 100 ng/ml IL6 for 24 h. MMP9 released by cells into medium was quantified by ELISA. (B) U937 cells were pre-exposed to normal (5 mM) or high glucose (25 mM) for 2 days and then treated with or without 100 ng/ml IL6 for 24 h. After the treatment, TIMP1 in culture medium was quantified by ELISA.

Figure 9 Effect of diabetes and insulin treatment on CD68 expression in atherosclerotic plaques. CD68 protein expression in atherosclerotic plaques was detected by immunostaining and presented as pixels per plaque. (A) Representative photomicrographs of CD68 immunostaining of atherosclerotic plaques from nondiabetic apoE^{-/-} mice (a), diabetic apoE^{-/-} mice (b), and diabetic apoE^{-/-} mice treated with insulin (c). (B) Quantification of CD68 protein expression in atherosclerotic plaques (n=8-10).

(Richardson *et al.* 1989, Falk *et al.* 1995). Autopsy studies have shown that diabetic patients have more extensive atherosclerotic plaques in coronary arteries than nondiabetic patients (Jarrett 1981, Moreno *et al.* 2000). Consistent with these clinical findings, we observed that diabetes increased intimal lesion area in apoE^{-/-} mice by 3.3-fold when compared with nondiabetic mice. This is a striking finding and has been reported consistently by other laboratories. For example, Candido *et al.* (2004) showed that the atherosclerotic plaque area in apoE^{-/-} mice that had diabetes for 20 weeks was fivefold of that in nondiabetic mice. Park *et al.* (1998) also reported similar. In this study, in addition to the effect of hyperglycemia on intimal lesions, we demonstrated that insulin treatment significantly reduced diabetes-increased lesion size. Thus, this study further underscores an important role of hyperglycemia in atherosclerosis.

It has been well established that MMPs are upregulated in unstable plaques and play a critical role in plaque destabilization (Galis & Khatri 2002). It has been also reported that although TIMPs that inhibit MMP activities are upregulated in unstable plaques as well, MMPs are stimulated to a much higher extent than TIMPs, leading to an imbalance between MMP and TIMP expression (Galis *et al.* 1994, Galis & Khatri 2002). In this study, we demonstrated that diabetes significantly increased the expression of MMP9, but not of TIMP1, in atherosclerotic plaques. Furthermore, we demonstrated that insulin treatment significantly reduced diabetes-increased MMP9 expression but had no effect on TIMP1 expression. In support of these *in vivo* observations, our

in vitro studies also showed that high glucose augmented IL6-stimulated MMP9 secretion in a concentration-dependent manner. Reduction in glucose concentration from 25 to 5 mM decreased IL6-stimulated MMP9 secretion by 56%. Taken together, these studies showed that induction of diabetes in apoE^{-/-} mice increased the ratio of MMP9 versus TIMP1 expression, but insulin treatment led to a rebalance between MMP9 and TIMP1 expression.

The mechanisms involved in the stimulation of MMP9 by high glucose have been explored in recent years (Bai *et al.* 2005, Lee *et al.* 2007, Nareika *et al.* 2009). Our laboratory showed that high glucose increased the expression of c-Jun and c-Fos, which are the major subunits of AP-1 transcription factor (Li *et al.* 2010). Given that AP1 is a key transcription factor for MMP9 expression (Aljada *et al.* 2001), it is most likely that high glucose stimulates MMP9 expression by increasing AP1 level. Furthermore, under the pathological conditions such as diabetes, multiple stimulators such as high glucose and proinflammatory cytokines are present in atherosclerotic plaques. All these stimulators are likely to act in concert to stimulate MMP9 expression. Our findings on the coordinated stimulation on MMP9 by high glucose and IL6 (Fig. 8A) have provided evidence indicating the effect of multiple stimulators on MMP9 upregulation. Since IL6 also increased c-Jun and c-Fos expression (Li *et al.* 2010), it is likely that high glucose and IL6 synergistically increase MMP9 expression by their coordinate action on AP1 activity.

In addition to the stimulation of MMP9 expression by IL6 and other proinflammatory cytokines, previous studies have shown that nitric oxide (NO) inhibited MMP2 and MMP9 expression by smooth muscle cells (Gurjar *et al.* 1999) and enhancement of eNOS expression in rat heart reduced MMP9 expression (Liu *et al.* 2011). Thus, MMP9 expression is associated with endothelial dysfunction. Given that chronic exposure to high glucose reduces NO generation in endothelial cells, probably by impairing phospholipase-C-mediated Ca²⁺ signaling due to excess protein kinase C activation (Tang & Li 2004), it is obvious that diabetes downregulates MMP9 expression via endothelial dysfunction and subsequent reduction in NO production from endothelial cells.

Increased macrophage content is another important feature for unstable plaques (Moreno *et al.* 2000). Pathological studies have shown that diabetes is associated with an increase in macrophage number in atherosclerotic plaques (Jarrett 1981), suggesting that the increased macrophage content in plaque destabilization in diabetes may contribute to plaque instability. Consistent with these reports, this study showed that macrophage content in atherosclerotic plaques as detected by CD68 immunostaining was significantly increased in diabetic apoE^{-/-} mice. Surprisingly, our data showed that insulin treatment did not reduce diabetes-increased macrophage content. It is possible that in addition to hyperglycemia, other diabetes-associated factors may also influence macrophage content in atherosclerotic plaques, and glycemic control alone is insufficient to reduce macrophage content.

Verhoeven *et al.* (2006) reported their interesting findings on macrophage content in carotid atherosclerotic plaques from a clinical study in 378 patients with or without statin treatment. They found that although serum cholesterol, low-density lipoprotein, C-reactive protein, and carotid atheromatous plaques were reduced in patients treated with statins, CD68-positive cells were increased in atherosclerotic lesions. Furthermore, they found that the expression of IL6, a major proinflammatory cytokine expressed by macrophages (Li *et al.* 2010), was lower in atherosclerotic plaques in patients treated with statins. This study suggests that statin treatment attenuates macrophage activation, but not the recruitment of macrophages. Indeed, we also found that insulin treatment inhibited diabetes-increased IL6 expression.

In conclusion, this study showed that insulin treatment in diabetic apoE^{−/−} mice led to a reduction in intimal lesions and rebalance between MMP9 and TIMP1 expression. This study also showed that insulin treatment inhibited proinflammatory cytokine IL6 expression in atherosclerotic lesions but did not decrease diabetes-increased macrophage content. These findings indicate that insulin treatment may reduce cardiovascular events by stabilizing advanced atherosclerosis.

Declaration of interest

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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