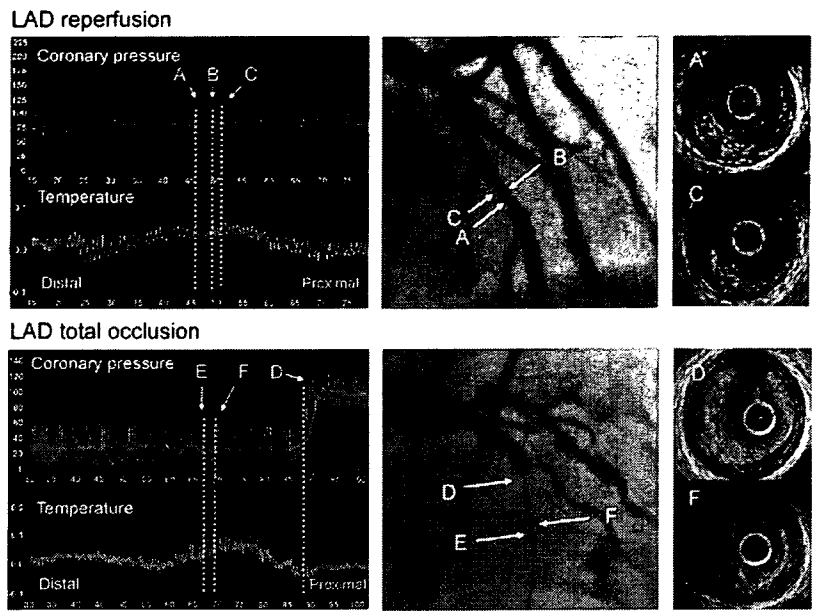

Figure 4 **Representatives of Coronary Pressure and Temperature With IVUS Images**

Coronary plaque temperature and coronary pressure by the pressure/temperature guidewire and intravascular ultrasound (IVUS) images in patients with left anterior descending coronary artery (LAD) reperfusion **(top panel)** and total occlusion **(bottom panel)**. Angiographically most stenotic site **(A)**, the maximal temperature (Tmax) site **(B)**, and the site of culprit plaque by IVUS **(C)** were closely located to each other **(top panel)**. The angiographic occlusive site **(D)** was considerably more proximal by 9 mm, compared with both the Tmax site **(E)** and the site of culprit plaque by IVUS **(F)**, and the Tmax site and the site of culprit plaque by IVUS were located close to each other **(bottom panel)**.

but coincided with the Tmax site; thus, the Tmax site was regarded as the culprit plaque site. A previous study reported that vulnerable plaques are characterized by large lipid cores, larger plaque burden, superficial calcified nodules, and a larger remodeling index (26). In the present study, findings that reflect vulnerable plaque were clearly observed at the Tmax site but not at the angiographic occlusive site, which suggests that the Tmax site was the culprit plaque site in patients without distinct ruptured plaque demonstrated by IVUS.

Previous pathological studies have reported that coronary arterial thrombi responsible for ST-segment elevated myocardial infarction are approximately 10 mm (27), and Brousius et al. (5) have reported that the length of occlusive thrombi in the LAD, left circumflex, and right coronary artery was 14, 11, and 24 mm, respectively. In this study, the proximal development of the arterial thrombosis was approximately 9 mm, which was comparable to these studies.

In patients with reperfused AMI, the culprit plaque by IVUS was observed close to the angiographically most stenotic site, and the Tmax site coincided with the culprit plaque in this study. Similarly, Maehara et al. (25) have reported that a ruptured plaque is present close to the site of minimum lumen area in IVUS study. These findings indicated that the site nearest to the most stenotic site is the culprit plaque in patients with reperfused AMI. Temperature elevations in coronary plaques were also observed in patients with reperfusion, although the magnitude of the temperature elevation was less than that seen in patients with AMI and total occlusion. This likely results from the heat-lowering convection effect of residual coronary blood flow (28,29).

Recently, IVUS has been widely used for detection of the culprit plaque, but there are some cases where it does not apply, owing to coronary tortuosity and calcification. Temperature measurement of coronary plaque by the P/T guidewire is able to be used even in these cases, and it is considered to be an effective option to identify the culprit plaque in patients with AMI and total occlusion. When performing PCI, use of the P/T guidewire might help determine the optimal site for interventional treatment.

Study limitations. Several limitations of this study must be considered. First, the use of a thermography catheter to measure temperature of coronary plaques has previously been described (10–13,28,29). However, the present study used a P/T guidewire, which is a device designed to measure blood temperature rather than plaque surface temperature. Although we clearly showed the reproducibility of the temperature measurements with this P/T guidewire, internal validity does not guarantee external validity. This study contained a relatively small number of patients. Future investigations using the present methodology would benefit from inclusion of a larger patient population. In addition, the guidewire was not always in close contact to the coronary artery wall, and the degree of coronary stenosis and tortuosity might have affected temperature measurement.

Furthermore, the presence of thrombus, itself, might have affected temperature measurements. Indeed, the degree of ΔT in this study was smaller than that in previous studies by thermography catheter (10–13), which might be attributed to the use of the P/T guidewire. However, attenuated ΔT is not expected to influence determination of the site of Tmax.

This study was limited to patients with an anterior AMI of <12 h from onset who potentially required emergent PCI (30). Thus, it is not clear whether these results can be generalized to those who present with AMI of >12 h from symptom onset.

Conclusions

The identification of the culprit plaque by coronary angiography is of limited utility, owing to the thrombosis with development to the proximal region, whereas temperature measurement of coronary atherosclerotic plaque enables accurate localization of the culprit plaque in the acute phase in AMI with coronary total occlusion.

Acknowledgments

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Original article

Sirt1 modulates premature senescence-like phenotype in human endothelial cells

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Abstract

Yeast Sir2 plays critical roles in gene silencing, stress resistance and longevity. Mammalian Sirt1 NAD^+-dependent protein deacetylase, the closest homolog of Sir2, regulates cell cycle, cellular senescence, apoptosis and metabolism, by functional interactions with a number of biological molecules such as p53. To investigate a role of Sirt1 in endothelial dysfunction and premature senescence, we examined the effects of Sirt1 inhibition in human umbilical vein endothelial cells (HUVEC). Sirt1 inhibition by sirtinol, which is a 2-hydroxy-1-napthaldehyde derivative, or siRNA for Sirt1-induced premature senescence-like phenotype, as judged by increased senescence-associated β-galactosidase (SA-β-gal) activity, sustained growth arrest and enlarged and flattened cell morphology at 10 days after the treatment. Sixty-four percent of sirtinol (60 μmol/L)-treated HUVEC was SA-β-gal-positive, whereas only 17% of vehicle-treated cells were positive. Sirt1 inhibition by sirtinol or Sirt1 siRNA increased PAI-1 expression and decreased both protein expression and activity of eNOS. Treatment with sirtinol or Sirt1 siRNA increased acetylation of p53, while p53 expression was unaltered. Impaired epidermal growth factor-induced activation of mitogen-activated protein kinases was associated with Sirt1 inhibition-induced senescence-like growth arrest. Conversely, overexpression of Sirt1 prevented hydrogen peroxide-induced SA-β-gal activity, morphological changes and deranged expression of PAI-1 and eNOS. These results showed that Sirt1 inhibition increased p53 acetylation and induced premature senescence-like phenotype in parallel with increased PAI-1 and decreased eNOS expression. Our data suggest that Sirt1 may exert protective effects against endothelial dysfunction by preventing stress-induced premature senescence and deranged expression of PAI-1 and eNOS.

Keywords: Sirt1; Sirtinol; Premature senescence; Endothelial dysfunction; Oxidative stress

1. Introduction

Replicative senescence is the limited ability of mammalian cells to divide when cultured in vitro [1]. Cessation of cell division after extended propagation in weeks - or months - culture results from the attrition of telomeres and is termed replicative senescence. In contrast to replicative senescence, stressors such as oxidative stress elicit irreversible growth arrest within just a few days, which is referred to as stress-induced premature senescence (SIPS) [2,3]. Although cellular phenotypes of replicative senescence and SIPS are quite similar or indistinguishable, unlike replicative senescence, SIPS can be induced independent of telomere attrition. In either type of cellular senescence, it is accompanied by a specific set of changes in cell function, morphology and gene expression [4,5]. Well-established biomarkers of cellular senescence include staining for senescence-associated β-galactosidase (SA-β-gal) at pH 6.0 as opposed to endogenous lysosomal enzyme detected at pH 4.0 in normal cells.

Recently, premature senescence of endothelial cells has been proposed to be involved in endothelial dysfunction and atherogenesis [6]. Increased plasminogen activator inhibitor-1 (PAI-1) expression and reduced endothelial nitric oxide synthase (eNOS) activity, which play key roles in endothelial dysfunction and atherogenesis [7], are associated with premature senescence of vascular endothelial cells. Indeed, previous studies have shown that senescence of endothelial cells leads to endothelial

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dysfunction and may contribute to the progression of atherosclerosis [8,9]. Human aortae with atherosclerotic lesion exhibited premature senescence-like phenotype, including SA-β-gal activity and increased expression of PAI-1 [10], in association with decreased production of nitric oxide (NO) in endothelial cells [6]. In addition to the role as a prothrombotic factor, the induction of PAI-1 is part of cellular senescence program and has been used as an indicator of cellular senescence in various cell types, including endothelial cells [11,12]. Moreover, NO production [13] and eNOS expression [14,15] are markedly attenuated in senescent endothelial cells. However, the molecular mechanisms underlying premature senescence and endothelial dysfunction remain to be clarified.

Sir2 (silent information regulator-2), an NAD^+-dependent histone deacetylase, is highly conserved in organisms ranging from archaea to humans [16,17]. In yeast, Sir2 is responsible for silencing at repeated DNA sequences in mating-type loci, telomeres and rDNA and plays critical roles in DNA repair, stress resistance and longevity [18–20]. Mammalian Sirt1 NAD^+-dependent protein deacetylase, the closest homolog of Sir2, regulates cell cycle, premature senescence, apoptosis and metabolism by interacting with a number of molecules, including p53, PML, Foxo, Ku70 and PPAR-γ [21–26]. A previous study has shown that Sirt1 antagonizes p53-mediated premature senescence in mouse embryo fibroblasts [25]. Recently, we have demonstrated that Sirt1 inhibition induces premature senescence-like growth arrest in human cancer cells [28].

In the present study, to test the hypothesis that Sirt1 plays a role in endothelial premature senescence and dysfunction in vitro, we evaluated the effects of inhibition and overexpression of Sirt1. Here, we demonstrate in human vascular endothelial cells that inhibition of Sirt1-induced premature senescence-like phenotype in parallel with acetylation of p53, and that Sirt1 overexpression reverted premature senescence induced by hydrogen peroxide.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVEC) were purchased from CAMBREX (Walkersville, MD) and maintained in endothelial growth medium (EGM-2, EGM-2 singleQuots, CAMBREX). Population doubling levels (PDL) were calculated as described previously [29], and all experiments were performed at PDL of 8–9, unless otherwise stated.

2.2. Sirt1 inhibition and overexpression

Exponentially proliferating cells were washed three times with growth medium and exposed for 24 h to the indicated concentrations of sirtinol (Calbiochem, San Diego, CA), a cell permeable 2-hydroxy-1-napthaldehyde derivative and a chemical inhibitor of NAD^+-dependent protein deacetylases of sirtuins. Trichostatin A (TSA) was purchased from Sigma (St. Louise, MO). After exposure, the cells were washed three times with inhibitor-free medium and cultured for up to additional 9 days. Proliferating cells were transfected with 200 pmol/L siRNA for Sirt1 (GAT GAA GTT GAC CTC CTC A [22] and TGA AGT GCC TCA GAT ATT A [28]), Sirt2 (sc-40988, Santa Cruz Biotechnology, Inc., CA), or control siRNA (Dharmacom, Chicago, IL) using siIMPORTER (Upstate, Charlottesville, VA). Sirt1 was overexpressed by transfection with pIRES-Sirt1, which was kindly provided by Dr. R.A. Weinberg [26,27], using jetPEI-HUVEC (Polyplustransfection, Illkirch, France) according to the manufacturer's instruction. Three days after the transfection, HUVEC were treated with 100 μmol/L hydrogen peroxide (Sigma) for 1 h and then washed three times with medium and were cultured for up to 10 days. Inhibition of p53 was performed by transfection of 4 μmol/L non-targeted oligonucleotide (5′-GGAGCCAGGGGGGAGGG-3′) or p53 anti-sense oligonucleotide (5′-CCCTGCTCCCCCTGGCTCC-3′).

2.3. Senescence-associated β-galactosidase (SA-β-gal) Staining

At 10 days after the treatment, the proportion of SA-β-gal-positive cells was determined as described by Dimri et al. [30].

2.4. BrdU incorporation assay

BrdU incorporation was analyzed using a commercially available kit (Roche, Indianapolis).

2.5. NOS activity assay

NOS activity of endothelial cells was determined using a NOS assay kit (Calbiochem) according to the manufacturer's instructions.

2.6. Immunoblotting

Cells were lysed on ice for 1 h in buffer (50 mmol/L Tris-HCl, pH 7.6, 150 mmol/L NaCl, 1%NP-40, 0.1% SDS, 1 mmol/L dithiothreitol, 1 mmol/L sodium vanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin and 10 mmol/L sodium fluoride). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking, the filters were incubated with the following antibodies; anti-p53, anti-acetyl-histone H3 (Lys14), anti-phospho-p44/42 MAPK (Thr202/Tyr204), anti-p44/42 MAPK, anti-phospho-p38 MAPK (Thr180/Tyr182), anti-p38, anti-phospho-JNK (Thr183/Tyr185), anti-JNK (Cell Signaling, Danvers, MA), anti-PAI-1 (Molecular Innovations, Southfield, MI), anti-eNOS (BD Transduction Laboratories, San Jose, CA), anti-Sirt1 (rabbit monoclonal IgG, recognizes human and mouse Sirt1), anti-acetyl-p53 (Lys373/382 and Lys320), anti-acetyl-histone H4 (Lys16) (Upstate), anti-Sirt2 (Santa Cruz) and anti-β-actin (Sigma). After washing and incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Amersham, Piscataway, NJ) for 1 h, the antigen–antibody complexes were visualized by an enhanced chemiluminescence system (Amersham).

2.7. Data analysis

Values are shown as mean±SEM in the text and figures. Differences between the groups were analyzed using one-way analysis of variance, followed by Bonferroni test. Probability values less than 0.05 were considered significant.

3. Results

3.1. Inhibition of Sirt1 induces premature senescence-like phenotype in HUVEC

To investigate whether Sirt1 modulates stress-induced premature senescence-like phenotype in HUVEC, we first examined the effect of Sirt1 inhibition. Sirt1 was inhibited by sirtinol, a specific chemical inhibitor of sirtuins, and Sirt1 siRNA. Sirtinol increased the acetylation of histone H3 (Lys14) and H4 (Lys16), endogenous substrates for Sirt1 (Fig. 1A), as expected. However, the protein expression of Sirt1 was not affected. Knockdown of Sirt1 with siRNA was confirmed by Western blotting (Fig. 1D). Treatment with sirtinol or Sirt1 siRNA induced sustained senescence-like growth arrest for up to 10 days (Fig. 1B). BrdU incorporation was also inhibited by sirtinol or Sirt1 siRNA at 10 days after treatment (Fig. 1C), indicating that the premature senescence-like phenotype and decreased cell number of HUVEC were accompanied by the attenuated DNA synthesis. We examined the effects of sirtinol or Sirt1 siRNA treatment on SA-β-gal activity, a characteristic feature of senescence-related growth arrest. Sirtinol or Sirt1 siRNA significantly increased SA-β-gal activity compared with untreated cells or control siRNA at 10 days after treatment: 64% of sirtinol (60 μmol/L)-treated HUVEC were SA-β-gal-positive, whereas only 17% of vehicle-treated cells were positive (Figs. 2A and B). Sirtinol or Sirt1 siRNA increased SA-β-gal activity in a time-dependent manner (data not shown). These cells also exhibited senescence-like morphological changes, that is, enlarged and flattened shapes (Fig. 2A). Next, because sirtinol can inhibit other NAD^+-dependent protein deacetylases of sirtuin family, especially Sirt2 [32], we examined the effect of Sirt2 inhibition by Sirt2 siRNA on senescence-like phenotype at 10 days after treatment. Sirt2 siRNA successfully eliminated the protein expression of Sirt2 (Fig. 1D) but did not affect the rate of senescence-associated β-galactosidase-positive cells (Fig. 2B).

3.2. Senescent-like phenotype resulting from Sirt1 inhibition is mediated by p53

p53 plays a pivotal role in cellular senescence [11]. Previous studies have shown that Sirt1 inhibition promotes stress-induced apoptosis by enhancing p53 acetylation [21,26], while ectopically expressed Sirt1 blocks p53-mediated premature senescence in mouse embryo fibroblasts [25]. Therefore, we examined whether inhibition of Sirt1 affects p53 in HUVECs. Consistent with pervious studies [21,25,26], Sirt1 inhibition by sirtinol or Sirt1 siRNA increased the acetylation of p53 after 3 days and the expression of p53 after 5 days (Figs. 3A and C). We also treated HUVEC with TSA, an inhibitor of classes I and II HDACs. Acetylation of p53 was increased at Lys373/382 and Lys320 in HUVECs treated with sirtinol but

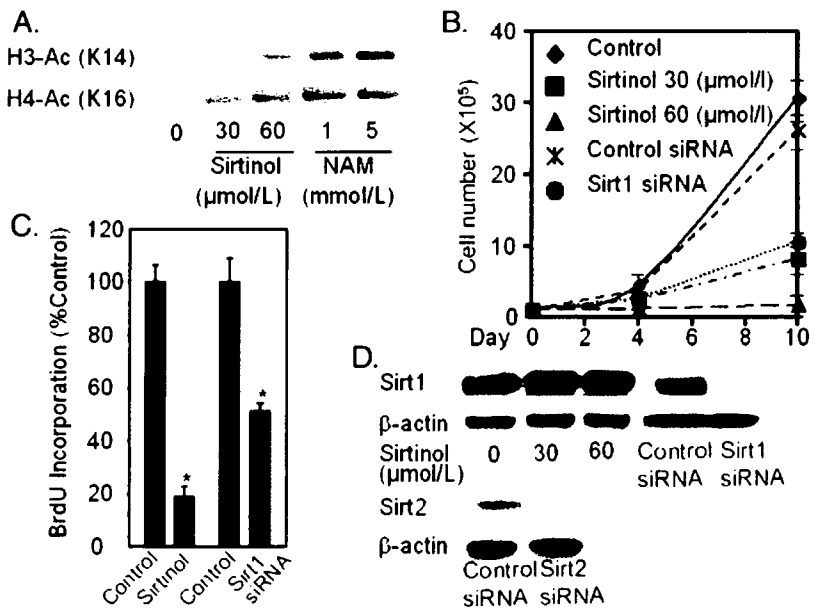

Fig. 1. Effects of Sirt1 inhibition on Sirt1 expression, acetylation of histone H3, H4 and cell growth in HUVEC. (A) Western blot analysis for acetylated histone H3 at lysine 14 (K14) and histone H4 at lysine 16 (K16) at 24 h after treatment with sirtinol (30 and 60 μmol L) or nicotinamide (1 and 5 mmol L), a physiological Sirt1 inhibitor. (B) The effects of sirtinol or Sirt1 siRNA on cell growth for 10 days. (C) BrdU incorporation analysis at 10 days after treatment with sirtinol or Sirt1 siRNA. (D) Western blot analysis for Sirt1 expression at 24 h after treatment with sirtinol or at 72 h after transfection of Sirt1 siRNA. Western blot analysis for Sirt2 expression at 3 days after transfection of Sirt2 siRNA. β-Actin served as a loading control.

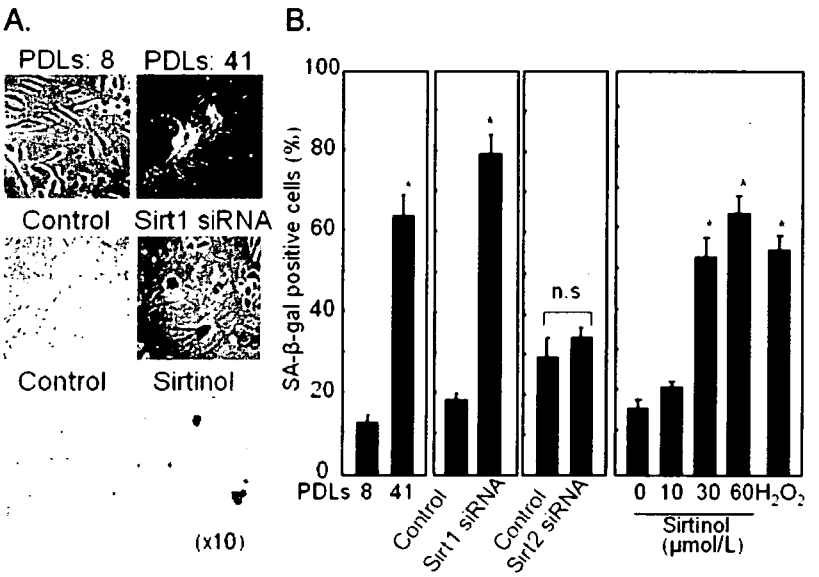

Fig. 2. Effects of Sirt1 inhibition on senescence-associated β-galactosidase. (A) Photomicrographs of HUVEC stained for SA-β-gal. Population doubling levels (PDLs) for the young and senescent cells are indicated, and sirtinol (60 μmol/L)-, Sirt1 siRNA-treated cells were at PDL 8. (B) SA-β-gal-positive cells at 10 days after sirtinol or Sirt1 or Sirt2 siRNA treatment. HUVEC treated with hydrogen peroxide (H_2O_2; 100 μmol/L) for 1 h and the cells at PDL 8 served as positive controls for SA-β-gal staining. $n=3$ for each group. *$p<0.05$ vs. control.

not with TSA, suggesting that endogenous Sirt1 plays an important role in deacetylation of p53 in HUVECs (Fig. 3D). We further examined whether the effects of Sirt1 inhibition were mediated by p53. We found that blockade of p53 by antisense oligonucleotides reversed the effects of Sirt1 inhibition on senescence associated β-galactosidase activity (Fig. 3E) and senescence-like morphological changes (data not shown) at 10 days after Sirt1 inhibition.

Next, we examined the activation status of mitogen-activated protein kinases (MAPKs) in response to epidermal growth factor (EGF). When untreated with sirtinol or Sirt1 siRNA, upon exposure to EGF, robust phosphorylation (activation) of extracellular-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK was observed in HUVEC. By contrast, in sirtinol- or Sirt1 siRNA-treated cells, EGF-stimulated phosphorylation of ERK, JNK and p38 MAPK was markedly attenuated (Fig. 3F). The protein expression of ERK, JNK and p38 MAPK did not differ between sirtinol- or Sirt1 siRNA-treated and untreated cells.

3.3. Sirt1 inhibition promotes endothelial dysfunction

To investigate further the role of Sirt1 in premature senescence and endothelial function, we examined PAI-1 expression, eNOS expression and NOS activity in HUVEC. Sirt1 inhibition by sirtinol or Sirt1 siRNA increased PAI-1 expression by three-fold and decreased eNOS expression and NOS activity (Figs. 4A, B and D). These changes were similar to those observed in HUVEC that underwent hydrogen peroxide-induced premature senescence and also replicative senescence at 41 PDL (Figs. 4A, C and D). The protein expression of PAI-1 and eNOS were increased and decreased, respectively, at 3 days after the addition of sirtinol or Sirt1 siRNA and further increased and reduced at 10 days after sirtinol or Sirt1 siRNA treatment (Fig. 5A). Likewise, the activity of NOS was reduced at 3 and 10 days after sirtinol or Sirt1 siRNA addition in a time-dependent manner (Fig. 5B). These results indicate that Sirt1 inhibition-induced premature senescence-like phenotype was associated with endothelial dysfunction.

To investigate the relationship between p53 and these protein expressions, we inhibited p53 by anti-sense oligonucleotide for p53. Anti-sense p53 reduced the expression of p53 and prevented induction of PAI-1 expression by Sirt1 inhibition, as compared with non-targeting oligonucleotide, although reduced eNOS expression was not reverted (Fig. 5C).

3.4. Overexpression of Sirt1 blocks oxidative stress-induced premature senescence

Next, we examined whether Sirt1 overexpression can protect HUVEC from oxidative stress-induced premature senescence. As shown previously [3,31], hydrogen peroxide induced premature senescence (Fig. 2B). Overexpression of Sirt1 significantly reduced the number of cells positive for SA-β-gal and inhibited senescence-associated morphological changes (Figs. 6A and B). Moreover, overexpression of Sirt1 reversed the changes in eNOS and PAI-1 expression (Fig. 6C) and blocked hydrogen peroxide-induced growth arrest (Fig. 6D). By contrast, when untreated with hydrogen peroxide, overexpression of Sirt1 did not alter cell growth compared with empty vector. In HUVEC transfected with Sirt1, Sirt1 protein expression was increased up to 5 days after treatment with hydrogen peroxide, and then started declining and returned to

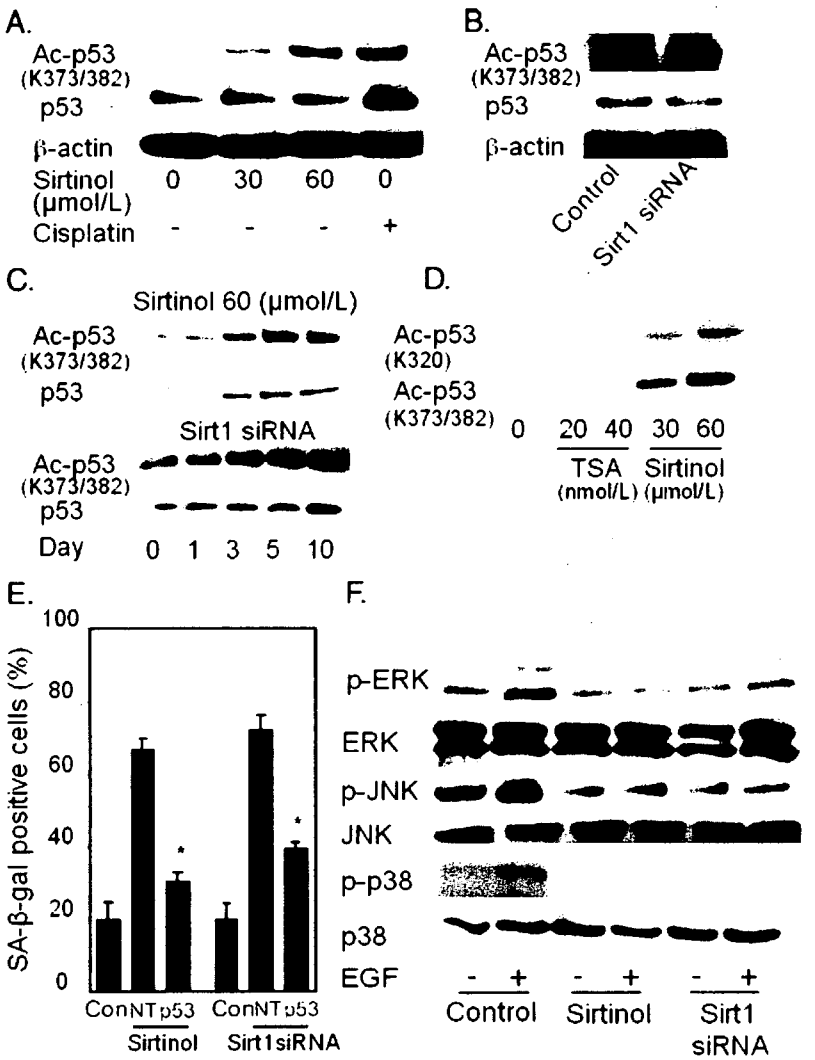

Fig. 3. Effects of Sirt1 inhibition on p53 and EGF-stimulated phosphorylation of MAPKs in HUVEC. (A, B) Acetylation of p53 (Ac-p53) at lysine 373/382 (K373/382) and total protein level of p53 were evaluated by Western blotting analysis at 24 h after treatment with sirtinol (A) and at 72 h after transfection with Sirt1 siRNA (B). Treatment with cisplatin (100 μmol/L) for 4 h served as a positive control. β-Actin served as a loading control. (C) Acetylation and total protein levels of p53 for 10 days after treatment with sirtinol or Sirt1 siRNA. (D) Acetylation of p53 at lysine 372/383 and lysine 320 (K372/383 and K320) was evaluated by Western blot analysis at 24 h after treatment with sirtinol (30 and 60 μmol/L) or Trichostatin A (TSA, 20 and 40 nmol/L). (E) Effect of transfection of non-targeted oligonucleotide (NT, 4.0 μmol/L, 5′-GGAGCCAGGGGGGAGGG-3′) or p53 anti-sense (p53, 4 μmol/L, 5′-CCCTGCTCCCCCTGGCTCC-3′) on SA-β-gal activity in sirtinol- or Sirt1 siRNA-treated cells. $n=3$ for each group. $^{*}p<0.05$ vs. NT control. (F) EGF-stimulated phosphorylation of MAPKs in sirtinol- or Sirt1 siRNA-treated cells. At 10 days after treatment with sirtinol or Sirt1 siRNA, following overnight serum starvation, the cells were exposed to EGF (50 ng/ml) for 20 min.

normal level at 10 days after hydrogen peroxide treatment (Fig. 6E).

4. Discussion

We found that Sirt1 inhibition by a specific chemical inhibitor of sirtuins, sirtinol or gene knockdown by Sirt1 siRNA induced premature senescence-like phenotype, as judged by representative markers for premature senescence, SA-β-gal activity, enlarged and flattened cell morphology, sustained growth arrest, increased PAI-1 expression and suppressed activation of MAPKs in response to a growth factor, EGF. Another chemical inhibitor of sirtuins, splitomicin, also elicited similar effects in HUVEC (data not shown). Sirt1 inhibition-induced premature senescence-like growth arrest was associated with acetylation of p53. These results are consistent with previous studies by others and us showing that: (1) over-expression of Sirt1 antagonizes premature senescence through deacetylation of p53 in mouse fibroblasts [25]; and (2) sirtinol induces premature senescence-like phenotype in human cancer cells [28]. Sirtinol is a cell-permeable 2-hydroxy-1-napthaldehyde derivative that acts as a specific and direct inhibitor of all NAD^{+}-dependent protein deacetylases of sirtuin family. Therefore, our results brought by sirtinol might involve the possible influence of other sirtuins, especially Sirt2 [32]. In this regard, we also examined the role of Sirt2 in premature senescence-like

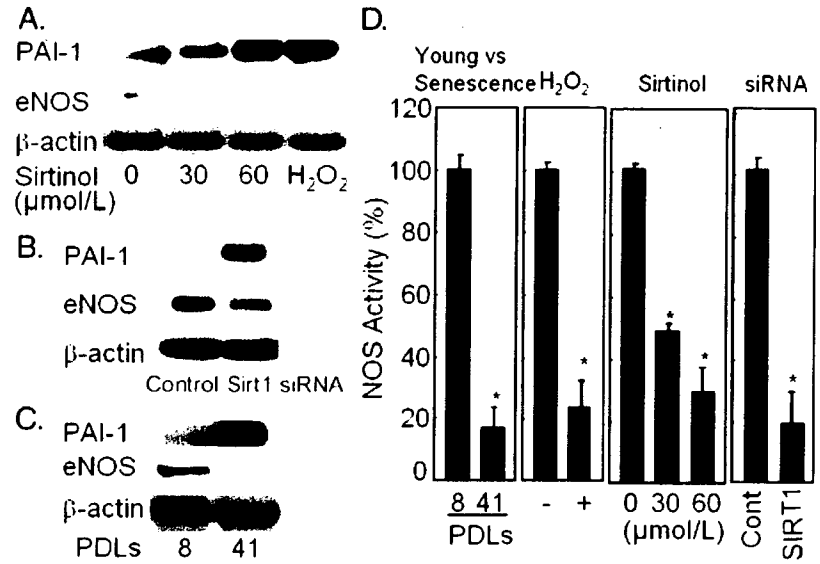

Fig. 4. Effects of Sirt1 inhibition on PAI-1 and eNOS expression in HUVEC. (A, B) PAI-1 and eNOS expressions were evaluated by Western blotting at 10 days after treatment with sirtinol (A) or Sirt1 siRNA (B). Treatment with hydrogen peroxide (H_2O_2, 100 μmol/L) for 1 h served as a positive control for the phenotype of premature senescence. (C) The expression of PAI-1 and eNOS in young (PDL 8) and senescent (PDL 41) HUVEC. (D) The effects of sirtinol or Sirt1 siRNA treatment on NOS activity. HUVEC at PDL 8 and the cells treated with hydrogen peroxide served as positive controls. $n=3$ for each group. $^*p<0.05$ vs. control.

phenotype in HUVEC. Little influence of gene knockdown by Sirt2 siRNA suggests that Sirt2 does not play a major role in our model of premature senescence, even though Sirt2 may be a target enzyme of sirtinol. However, there are 7 mammalian sirtuins and several might be involved in human endothelial senescence. Further studies are needed to address this point.

Moreover, overexpression of Sirt1 prevented premature senescence induced by hydrogen peroxide in HUVEC. This finding is in accord with a previous report that overexpression of Sirt1 enhanced stress resistance against hydrogen peroxide through deacetylation of p53 in human mesangial cells [33]. Taken together, these findings suggest that Sirt1 may play an important role in stress resistance and the modulation of stress-induced premature senescence (SIPS), at least in part, through deacetylation of p53.

Sirtinol- or Sirt1 siRNA-induced premature senescence was accompanied by attenuated response to EGF in terms of activation of MAPKs signaling. A hallmark feature of senescent

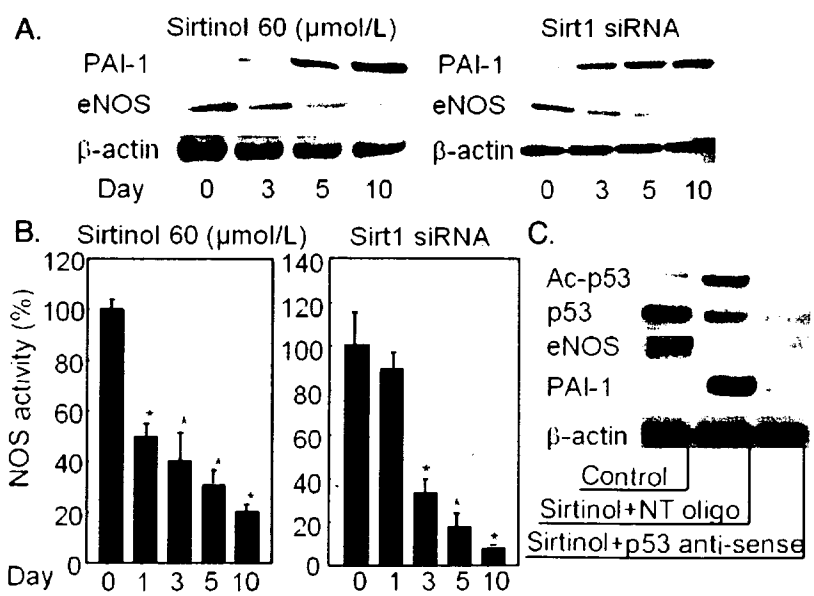

Fig. 5. Time-dependent alterations in PAI-1 and eNOS expression by inhibition of Sirt1 in HUVEC. (A, B) PAI-1 and eNOS expression (A) and NOS activity (B) were evaluated after treatment with sirtinol or Sirt1 siRNA. (C) The effect of transfection of non-targeted oligonucleotide (NT oligo) and p53 antisense (4.0 μmol/L) on PAI-1 and eNOS expression in sirtinol-treated cells. $^*p<0.05$ vs. control.

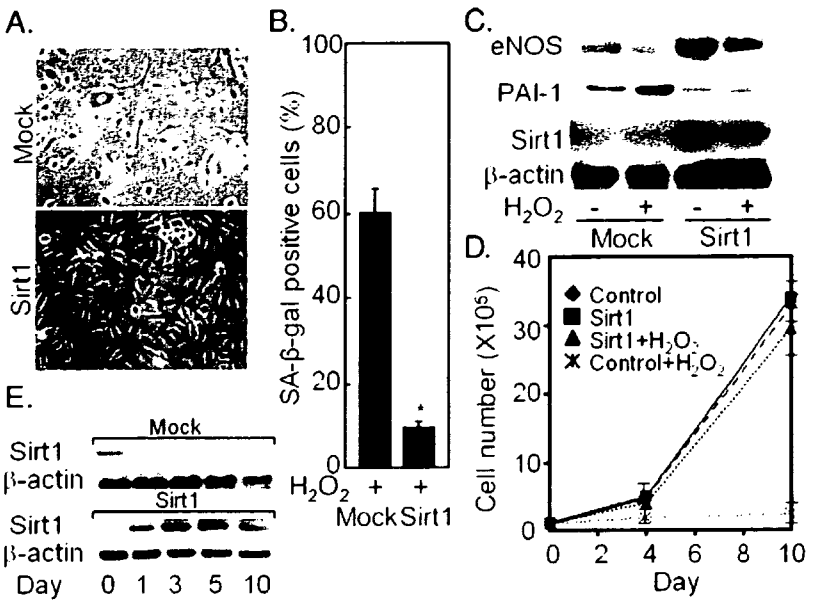

Fig. 6. Effects of ectopically expressed Sirt1 on hydrogen peroxide-induced premature senescence in HUVEC. (A–C) The effects of Sirt1 overexpression on cell morphology (A), SA-β-gal staining (B) and the expression of PAI-1 and eNOS (C) in HUVEC treated with and without hydrogen peroxide (H_2O_2, 100 μmol/L) for 1 h. The effects of Sirt1 overexpression on SA-β-gal-positive cells in hydrogen peroxide-treated HUVEC. *$p < 0.05$ vs. Mock. (D) The effects of Sirt1 overexpression on cell growth. $n = 3$ for each group. (E) Sirt1 protein expression in HUVEC transfected with empty vector and pIRES-Sirt1 during the 10-day culture.

cells is unresponsiveness to cell growth stimuli in induction of c-*fos* as well as cell proliferation. Our results are in agreement with previous studies that growth factor-stimulated activation of the ERK pathway is impaired in senescent human diploid fibroblasts [34].

Endothelial dysfunction has been implicated in the development of cardiovascular diseases, including hypertension and atherosclerosis [35]. Nevertheless, molecular mechanisms responsible for endothelial dysfunction remain to be determined. Recently, SIPS of endothelial cells has been proposed as a contributor to endothelial dysfunction [36,37]. We found that Sirt1 inhibition increased PAI-1 expression and decreased eNOS expression and NOS activity in HUVEC, and that overexpression of Sirt1 reverted hydrogen oxide-induced alterations in these proteins. Collectively, these findings suggest that Sirt1 may protect from endothelial dysfunction by inhibiting premature senescence and thereby contribute to maintenance of vascular homeostasis. One can reasonably speculate therefore that Sirt1 may exert salutary actions against cardiovascular diseases (e.g., hypertension, atherosclerosis) by preventing SIPS-associated endothelial dysfunction. However, further studies will be required to clarify this point.

p53 is a major substrate for Sirt1, and that deacetylation of p53 regulates cell cycle, cellular senescence and stress resistance in various cell types, including cardiac myocytes [38]. Consistent with previous studies [21,25,26], Sirt1 inhibition by sirtinol or Sirt1 siRNA increased the acetylation of p53 after 3 days and the expression of p53 after 5 days. This result is consistent with the finding by van der Veer et al. [39] that nicotinamide phosphoribosyltransferase-induced and Sirt1-mediated p53 deacetylation was associated with the decreased expression of p53 in vascular smooth muscle cells. A recent study has revealed that PAI-1 is a direct downstream target gene of p53 and a major player in the induction of cellular senescence [40]. In agreement with this, we found that inhibition of p53 reversed SA-β-gal activity and PAI-1 expression that were induced by Sirt1 inhibition. These results indicate that the acetylation of p53 may play a role in Sirt1 inhibition-induced premature senescence-like phenotype in HUVEC.

Mitosis-related telomere shortening is the critical event for replicative senescence. However, telomere attrition is dispensable for SIPS, which can be induced in a few days. A previous study has shown that changes in telomere length or in telomerase activity were not detected when SIPS was induced by oxidative stress in human endothelial cells [41]. Moreover, a recent study has demonstrated that telomere extension by overexpression of human telomerase does not affect SIPS [2], although it rescues replicative senescence [42]. These data clearly indicate that in contrast to replicative senescence, telomere erosion does not have an important role in the induction of SIPS.

Previous studies have shown senescence-like phenotype in endothelial cells in atherosclerotic lesion in humans and rodents [6,37], as discussed above. Oxidative stress and other stressors have been implicated in the pathogenesis of endothelial dysfunction, hypertension and atherosclerosis [35]. In contrast, there is no direct in vivo evidence indicating excessive replication of endothelial cells, which in turn causes replicative senescence, in cardiovascular diseases. In aggregate, it is reasonably conceivable that SIPS rather than genuine replicative senescence may be a major contributor to senescence-like phenotype of endothelial cells in cardiovascular diseases. Therefore, we focused on the role of Sirt1 in SIPS-like phenotype in endothelial cells in the present study.

In summary, our data showed that Sirt1 inhibition increased p53 acetylation and induced SIPS-like phenotype in HUVEC in parallel with increased PAI-1 and decreased eNOS expression, and that Sirt1 overexpression reversed the SIPS induced by oxidative stress. These results suggest that Sirt1 may exert protective effects against endothelial dysfunction by preventing premature senescence and deranged expression of PAI-1 and eNOS in endothelial cells.

4.1. Perspectives

Prevention and reversal of endothelial dysfunction, a major pathogenic factor for cardiovascular diseases, has been an issue of intense investigation for a number of years. Recently, SIPS has been proposed as a contributor to endothelial dysfunction. Nonetheless, it remains largely unknown how stressors such as oxidative stress induces SIPS in endothelial cells. Our data suggest an important role for Sirt1 in the regulation of SIPS. Collectively, these findings highlight Sirt1 as a possible molecular target to prevent and/or treat cardiovascular diseases, including hypertension and atherosclerosis by protecting endothelial cells from SIPS-involved dysfunction. This possibility deserves further investigation.

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Original Article

Low Testosterone Level Is an Independent Determinant of Endothelial Dysfunction in Men

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We investigated whether a low plasma testosterone level is related to endothelial dysfunction in men with coronary risk factors. One hundred and eighty-seven consecutive male outpatients (mean age±SD: 47±15 years) who underwent measurement of flow-mediated vasodilation (FMD) of the brachial artery using ultrasonography were enrolled. The relationship between plasma hormones and FMD was analyzed. Total and free testosterone and dehydroepiandrosterone-sulfate (DHEA-S) were significantly correlated with %FMD (r=0.261, 0.354 and 0.295, respectively; p<0.001), while estradiol and cortisol were not. %FMD in the highest quartile of free testosterone was 1.7-fold higher than that in the lowest quartile. Multiple regression analysis revealed that total and free testosterone were related to %FMD independent of age, body mass index, hypertension, hyperlipidemia, diabetes mellitus and smoking (β=0.198 and 0.247, respectively; p<0.01), and were independent of age, body mass index, systolic blood pressure, total cholesterol, high-density lipoprotein cholesterol, fasting plasma glucose, smoking and nitroglycerin-induced dilation (β=0.196 and 0.227, respectively; p<0.01). DHEA-S was not significantly related to %FMD in multivariate analysis. In conclusion, a low plasma testosterone level was associated with endothelial dysfunction in men independent of other risk factors, suggesting a protective effect of endogenous testosterone on the endothelium. (*Hypertens Res* 2007; 30: 1029–1034)

Key Words: **androgen, sex hormone, vasodilation, endothelium, risk factor**

Introduction

Androgen levels decline with advancing age in men (*1, 2*). Decreases in hormonal activity have been considered physiologic, but are often associated with the pathological process of aging, which includes such effects as erectile dysfunction, osteopenia, sarcopenia, depressed mood and cognitive impairment (*1, 3*). Also, not all but many recent observational studies have shown that a low plasma testosterone level is associated with advanced atherosclerosis (*4, 5*), and a higher incidence of cardiovascular disease (*6*), suggesting that endogenous testosterone may protect against the development of cardiovascular disease in men. The inverse correlations between testosterone and coronary risk factors such as obesity (*4, 7*) and high blood pressure (*8, 9*), plasma lipids (*4, 7, 8*), and plasma glucose (*7, 10*) may provide insight into the mechanism of the effect of testosterone on cardiovascular disease. Furthermore, anti-ischemic (*11, 12*) and endothelium-dependent vasodilating (*13, 14*) effects of testosterone supplementation have been reported. These findings led us to hypothesize that men with a low plasma testosterone level would have impaired vasomotor function.

To test this hypothesis, we conducted a cross-sectional sur-

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Table 1. Characteristics of Study Subjects (N=187)

Age (years)	47±15	[20–79]
Body mass index (kg/m^2)	25.7±4.6	[19.0–47.2]
Risk factors		
Hypertension (n (%))	69 (37)	
Hyperlipidemia (n (%))	82 (44)	
Diabetes mellitus (n (%))	37 (20)	
Current smoker (n (%))	87 (46)	
Hemodynamic and vascular measurements		
Systolic blood pressure (mmHg)	126±16	[98–185]
Diastolic blood pressure (mmHg)	76±13	[53–128]
%FMD	5.4±3.7	[0.0–20.2]
%NTG	13.6±5.0	[1.6–27.2]
Carotid IMT (mm)	0.96±0.36	[0.3–1.4]
Blood chemistry and hormones		
Total cholesterol (mmol/L)	5.23±1.00	[3.06–8.70]
HDL cholesterol (mmol/L)	1.28±0.42	[0.67–3.42]
Triglycerides (mmol/L)	1.73±1.40	[0.36–9.94]
Fasting plasma glucose (mmol/L)	5.78±1.10	[4.21–12.54]
Hemoglobin A1c (%)	5.5±1.3	[3.9–10.4]
Total testosterone (nmol/L)	17.4±5.7	[4.6–33.6]
Free testosterone (pmo/L)	61.0±22.5	[18.7–166.8]
DHEA-S (μmol/L)	4.78±2.51	[0.56–11.96]
Estradiol (pmol/L)	120±31	[50–216]
Cortisol (nmol/L)	375±133	[83–742]

Values except risk factors are expressed as the mean±SD [range]. %FMD, percent flow-mediated dilation of brachial artery; %NTG, percent nitroglycerin-induced dilation of brachial artery; IMT, intima-media thickness of common carotid artery; HDL, high-density lipoprotein; DHEA-S, dehydroepiandrosterone-sulfate.

vey of 187 men by examining flow-mediated dilation of the brachial artery (%FMD) and plasma sex hormones, and showed that a low testosterone level was associated with endothelial dysfunction.

Methods

Subjects

One hundred and eighty-seven consecutive male outpatients of our department, who underwent examination of vasomotor function of the brachial artery and intima-media thickness (IMT) of the carotid artery in our department, were enrolled. The subjects were referred to our department to check for cardiovascular disease or risks. All of them were in chronic stable condition. A history was taken, and physical examination and laboratory tests were performed in all subjects. Subjects with a history of cardiovascular disease, including stroke, coronary heart disease, congestive heart failure or peripheral arterial disease, malignancy, overt endocrine disease or use of

Table 2. Pearson's Correlation Coefficients between Age, Vascular Measurements and Plasma Hormones

	Age	%FMD	Carotid IMT
Total testosterone	0.057	0.261†	0.003
Free testosterone	−0.288†	0.354†	−0.259†
DHEA-S	−0.604†	0.295†	−0.356†
Estradiol	0.155*	−0.062	0.234*
Cortisol	−0.047	0.081	−0.082

%FMD, percent flow-mediated dilation of brachial artery; IMT, intima-media thickness of common carotid artery; DHEA, dehydroepiandrosterone-sulfate. †$p<0.001$, *$p<0.05$

steroid hormones were excluded, because these conditions may have a serious influence on both plasma sex hormones and endothelial function. Subjects who showed a carotid IMT >1.5 mm were also excluded, because such subjects might have significant subclinical atherosclerosis. The characteristics of the study subjects are shown in Table 1.

Seventy-six percent of the subjects had one or more of the classical coronary risk factors, such as hypertension, hyperlipidemia, diabetes mellitus or current smoking. Hypertension, hyperlipidemia and diabetes mellitus were defined according to the diagnostic criteria (*15–17*) or if the subjects were taking any medications for these diseases. Ninety-five percent of the hypertensive subjects were treated: 77% with calcium antagonists, 18% with angiotensin-converting enzyme inhibitors, 12% with diuretics and 7% with β-blockers. Seventy-seven percent of the hyperlipidemic subjects were treated with statins, and 81% of the diabetic subjects were treated with oral hypoglycemic agents. None of the study subjects were taking nitrates. Each subject gave written informed consent before enrollment in this study. The study protocol was approved by the ethics committee of the Graduate School of Medicine, The University of Tokyo.

Vascular Measurement

Vasomotor function of the brachial artery was evaluated using an ultrasound machine according to the method described previously (*18*). Briefly, endothelium-dependent %FMD was measured as the maximal percent change of the vessel diameter after reactive hyperemia. Subsequently, endothelium-independent nitroglycerin-induced vasodilation (%NTG) was measured as the maximal percent change of the vessel diameter after sublingual administration of nitroglycerin spray (0.3 mg; Toa Eiyo Co., Tokyo, Japan). Carotid IMT was evaluated using an ultrasound machine as described previously (*18*). The same examiner performed the measurements of FMD throughout this study. The subjects were examined in the morning after a 14-h overnight fast, and reclined on the bed for 15 min in a quiet, temperature-controlled (22–24 C) room before measurements.

Table 3. Age-Adjusted Regression Coefficients between Vascular Measurements and Plasma Hormones

	%FMD	Carotid IMT
Total testosterone	0.282†	−0.050
Free testosterone	0.324†	−0.090
DHEA-S	0.262†	0.036
Estradiol	−0.005	0.139
Cortisol	0.071	−0.053

Standardized regression coefficients by multiple regression analyses with %FMD or carotid IMT as a dependent variable and age and each of the hormones as independent variables are shown. †$p<0.001$. %FMD, percent flow-mediated dilation of brachial artery; IMT, intima-media thickness of common carotid artery; DHEA, dehydroepiandrosterone-sulfate.

Table 4. Regression Coefficients between %FMD and Plasma Hormones Adjusted for Coronary Risk Factors

	Model 1	Model 2	Model 3	Model 4
Total testosterone	0.198§	0.210§	0.216§	0.196§
Free testosterone	0.247§	0.266§	0.255§	0.227§
DHEA-S	0.091	0.150	0.175	0.170
Estradiol	0.033	0.024	0.061	−0.001
Cortisol	0.012	−0.001	−0.004	−0.073

Standardized regression coefficients by multiple regression analyses with %FMD as a dependent variable and coronary risk factors (covariates used in each analysis are listed below) and each of the hormones as independent variables are shown. §$p<0.01$. Model 1: age, body mass index, hypertension, hyperlipidemia, diabetes mellitus, and current smoking. Model 2: age, body mass index, systolic blood pressure, total cholesterol, HDL cholesterol, fasting plasma glucose and current smoking. Model 3: Model 2 plus carotid intima-media thickness. Model 4: Model 2 plus percent nitroglycerin-induced dilation of brachial artery. %FMD, percent flow-mediated dilation of brachial artery; DHEA-S, dehydroepiandrosterone-sulfate; HDL, high-density lipoprotein.

Plasma Hormones

Blood sampling was performed in the morning of the vascular measurement after a 14-h overnight fast, to measure plasma hormones and other chemical parameters. Plasma total and free testosterone, dehydroepiandrosterone-sulfate (DHEA-S), estradiol and cortisol concentrations were determined using sensitive radioimmunoassays by a commercial laboratory (SRL Inc., Tokyo, Japan). The intra-assay coefficients of variation for these measurements were less than 5%.

Data Analysis

The values are expressed as the means±SD in the text. Pearson's simple correlation coefficients between age, vascular measurements and plasma hormones were determined. Standardized regression coefficients from multiple regression analysis of vascular measurements in relation to age, coronary risk factors and plasma hormones were determined. Differences between the groups were analyzed using one-factor ANOVA, followed by Newman-Keuls' test. A value of $p<0.05$ was considered statistically significant.

Results

Changes in Plasma Hormones and Vascular Measurements According to Age and Coronary Risk Factors

Plasma levels of free testosterone and DHEA-S declined with age, while those of total testosterone and cortisol did not significantly change (Table 2). Conversely, estradiol showed a weak but significant positive correlation with age. %FMD decreased ($r=-0.365$, $p<0.001$) and carotid IMT increased ($r=0.546$, $p<0.001$) with advancing age.

The subjects with hypertension, hyperlipidemia or diabetes mellitus showed impaired %FMD compared to those without these diseases (hypertension, 3.8±2.4 *vs.* 6.3±4.0; hyperlipidemia, 4.4±3.4 *vs.* 6.1±3.7; diabetes mellitus, 3.1±2.4 *vs.* 5.9±3.7; $p<0.01$ for each). %FMD in the patients taking antihypertensive agents, statins or hypoglycemic agents was comparable to or smaller than that in the patients without medical agents (hypertension, 3.8±2.5 *vs.* 4.6±1.3, n.s.; hyperlipidemia, 3.9±2.7 *vs.* 6.3±4.7, $p<0.05$; diabetes mellitus, 2.6±2.0 *vs.* 4.9±3.0, $p<0.05$), suggesting that the favorable effects of medical treatment on endothelial function, if present, might have been lost in patients with a long history of coronary risk factors. In contrast, no significant associations were found between any of the plasma hormones and either coronary risk factors or medications.

Relationship between Plasma Hormones and Vascular Measurements

First, simple correlation coefficients between plasma hormones and vascular measurements were determined. As shown in Table 2, %FMD was positively correlated with total testosterone, free testosterone and DHEA-S. Carotid IMT was negatively correlated with free testosterone and DHEA-S, and was positively correlated with estradiol. There was no significant correlation between cortisol and vascular measurements.

Next, age-adjusted regression coefficients were determined, because age was correlated with both hormones and vascular measurements, as mentioned above. The results showed that none of the hormones was significantly related to carotid IMT, and estradiol was not related to either of the vascular measurements (Table 3). In contrast, total testosterone, free testosterone and DHEA-S were significantly related to %FMD, independent of age.

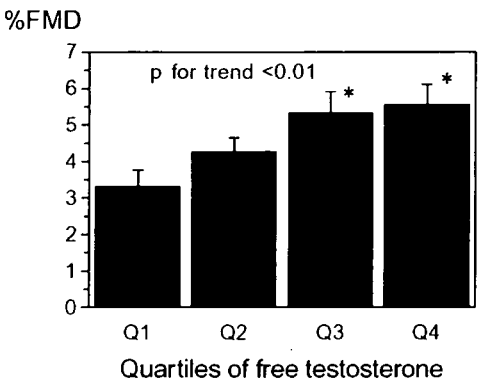

Fig. 1. *Percent flow-mediated dilation of the brachial artery (%FMD) according to quartiles of plasma free testosterone. Values are expressed as the means±SEM. *$p < 0.05$ vs. Q1.*

Finally, multiple regression analyses were performed to exclude the influence of coronary risk factors on the relationship between hormones and %FMD. As shown in Table 4, total and free testosterone were related to %FMD, independent of age, body mass index, hypertension, hyperlipidemia, diabetes mellitus and current smoking (Model 1), and were independent of age, body mass index, systolic blood pressure, total cholesterol, high-density lipoprotein cholesterol, fasting plasma glucose and current smoking (Model 2). Furthermore, the relationship between total and free testosterone and %FMD was significant after addition of carotid IMT to the model (Model 3), suggesting that the relationship was not attributable to the effect of testosterone on the development of subclinical atherosclerosis. Also, the statistical result was unchanged after %NTG of the brachial artery to the model (Model 4), indicating that testosterone is related to endothelial function independent of arterial compliance. DHEA-S, estradiol and cortisol were not significantly related to %FMD in similar multivariate analyses (Table 4). As shown in Fig. 1, %FMD showed a stepwise increment according to quartiles of free testosterone, and %FMD in the highest quartile of free testosterone was 1.7-fold higher than that in the lowest quartile.

Discussion

In this cross-sectional study, both total and free testosterone levels were positively correlated with %FMD, a surrogate marker of clinical atherosclerosis that reflects endothelial function (*19, 20*). Adjustment for potential confounders such as age, coronary risk factors and %NTG had little influence on the results. These results suggest that testosterone level is an independent determinant of endothelial vasomotor function in men.

A number of studies have shown an association between low testosterone level and cardiovascular disease (*6, 21, 22*) or risk factors (*4, 5, 7–10*), but others have shown no association (*23, 24*) and have reported that a low level of DHEA-S (*6, 25, 26*) or estradiol (*24*) is associated with cardiovascular disease. Also, a positive association between the cortisol:testosterone ratio and the incidence of coronary heart disease has been reported (*27*). Therefore, we added DHEA-S, estradiol and cortisol to the present analysis. However, our results showed that estradiol and cortisol were not related to %FMD. The ratio of cortisol to total testosterone ($r=-0.162$, $p<0.05$) and that of cortisol to free testosterone ($r=-0.194$, $p<0.05$) were significantly related to %FMD in simple regression analyses, but statistical significance was not found in multiple regression analyses (data not shown). DHEA-S was positively correlated with %FMD, but the statistical significance disappeared after adjustment for coronary risk factors. Taking these results together, testosterone was the only steroid hormone that was significantly related to %FMD in the multivariate analyses.

Several studies (*4, 8*) have assayed bioavailable testosterone, non-globulin-bound or free plus albumin-bound testosterone (*28*), whereas others have measured total (*5, 7, 21–24*) or free (*6, 9, 10, 21, 22*) testosterone in the plasma. These differences in assays might influence the results. In this study, we did not analyze bioavailable testosterone, because a direct assay is not available in Japan, and we did not measure the levels of sex hormone binding globulin and albumin, which are needed to estimate the value of bioavailable testosterone. However, both total and free testosterone levels were positively associated with %FMD, although free testosterone showed a stronger impact throughout the statistical analyses. Accordingly, we believe that the assays do not affect our conclusion that testosterone level is an independent determinant of endothelial vasomotor function in men.

The mechanisms by which testosterone regulates vasomotor function should be discussed. Short-term intracoronary administration of testosterone has been reported to elicit vasodilation and increased blood flow in men (*29*) and in animals (*30*). A supra-physiologic dose of testosterone induced relaxation of isolated blood vessels *in vitro* (*31*). These direct vasodilator actions of testosterone observed at higher concentrations seem to be endothelium- and androgen receptor-independent, and to be mediated *via* membrane ion channels of smooth muscle cells (*31*). On the other hand, both acute (*13*) and chronic (*14*) supplementation of testosterone in men enhanced %FMD without affecting the basal diameter of the brachial artery, suggesting an endothelium-dependent vasodilator action of testosterone. We also showed that the relation between %FMD and testosterone was not altered after adjustment for %NTG, further supporting the action of testosterone on endothelial function. Although the existence of androgen receptors in endothelial cells is recognized (*32*), the cellular and molecular mechanism linking testosterone to endothelial release of vasoactive agents such as nitric oxide is uncertain. We recently found that ginsenoside Rb1 stimulated nitric oxide production and endothelial nitric oxide synthase activ-

ity *via* androgen receptors in human aortic endothelial cells (*33*). To date, however, there has been no experimental evidence showing a direct effect of testosterone on endothelial nitric oxide synthesis. Another less likely hypothesis is that estradiol converted from testosterone by aromatase might exhibit vasoreactivity. Although the plasma level of estradiol was not correlated with %FMD in the present study, tissue conversion of testosterone into estradiol might play a role. Further *in vitro* and animal studies will be needed to clarify these issues.

The results of this study do not imply that testosterone has favorable effects in women. In fact, in a preliminary study, we observed that the plasma testosterone level was not related to FMD in postmenopausal women (unpublished observation). It has been reported that testosterone may impair endothelial function in women, and especially in young women with polycystic ovary syndrome (*34*) and women taking high-dose androgens (*35*). Aortic rings obtained from female rats treated with testosterone showed a significant decrease in prostacyclin synthesis (*36*), supporting the idea that testosterone influences vasoconstriction in women. Taken together, these results indicate that the vascular responses to testosterone are clearly different between men and women. Gender differences in the steroid hormone receptor expression in arteries (*37, 38*) might play a mechanistic role.

This study has some limitations. First, since this was a cross-sectional study, the causal relationship between testosterone and vasomotor function could not be determined. Endothelial dysfunction might be associated with a reduction in blood flow of endocrine organs, leading to decreased hormone production. Longitudinal studies following the subjects might add some information. Secondly, a population bias was possible. The study subjects ranged from young to elderly men with or without coronary risk factors. Consequently, the results might have been different if homogeneous subjects in terms of age and health status had been studied. In our subgroup analyses according to age and coronary risk factors and in multiple regression analyses including drug classes, comparable regression coefficients were obtained between testosterone and %FMD, although the statistical power was weakened (data not shown).

In summary, a low plasma testosterone level was associated with endothelial dysfunction in men independent of other risk factors, suggesting a protective effect of testosterone on the endothelium. This finding provides mechanistic insight into the role of endogenous testosterone in the development of cardiovascular disease in men.

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Circ J 2007; **71:** 1697–1702

Increased Incidence of Coronary In-Stent Restenosis in Type 2 Diabetic Patients is Related to Elevated Serum Malondialdehyde-Modified Low-Density Lipoprotein

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Background Type 2 diabetes mellitus (T2DM) has been reported as a major risk factor for in-stent restenosis (ISR) after intracoronary stenting, although the details of the mechanisms remain undefined. The aim of present study was to investigate the diabetes-related risk factor for ISR.

Methods and Results A total of 131 patients who were implanted with bare metal stent(s) were enrolled in this study. Based on follow-up coronary angiography at 6 months after stenting, the patients were classified according to the presence or absence of ISR. Various coronary risk factors, including serum malondialdehyde-modified low-density lipoprotein (MDA-LDL) levels, were investigated at follow-up angiogram to relate to ISR in patients with or without T2DM. The increased incidence of ISR was observed in diabetic patients, which was significantly related to the increased serum MDA-LDL concentrations. The serum MDA-LDL concentration was positively correlated to glycohemoglobin levels in diabetic patients. In addition, MDA-LDL concentration was not altered after the treatment of ISR.

Conclusions The elevated serum MDA-LDL level is considered to be a potent risk factor for ISR in diabetic patients. MDA-LDL, which might be a consequence of metabolic abnormalities caused by diabetes, may act as a growth factor for neointimal tissues inside the implanted stent. (*Circ J* 2007; **71:** 1697–1702)

Key Words: Angioplasty; Diabetes mellitus; Lipids; Restenosis; Stents

In-stent restenosis (ISR) remains the major limitation to long-term positive outcomes after percutaneous coronary intervention (PCI)[1,2] Pathological analyses indicate that the formation of neointimal tissues in the implanted stent is a major cause of ISR, which is characterized by an inflammatory reaction at the site of balloon-induced vascular injury, the migration and proliferation of vascular smooth muscle cells, and the synthesis of excess matrix. Despite the well-defined nature of the lesion, the pathogenic mechanisms leading to accelerated neointimal hyperplasia remain largely undefined. Multiple studies have examined potential risk factors for ISR and have reported that conventional coronary risk factors such as hypertension, hyperlipidemia, smoking habits, and a family history of coronary artery disease (CAD) are not associated with an increased risk of ISR[2,3] This may be attributable to differences in the pathology of post-stenting restenosis and atheromatous plaque in CAD[4]

In contrast, type 2 diabetes mellitus (T2DM) has been reported as a strong risk factor for ISR, possibly because patients with diabetes display a tendency toward exaggerated intimal hyperplasia inside the stent[3,5,6] Studies in animal models have suggested that the inflammatory and proliferative responses to balloon-induced vascular injury are enhanced in diabetes[7,8] The metabolic alterations that occur as a result of hyperglycemia or hyperinsulinemia can accelerate many of the pathophysiologic processes that lead to restenosis. Diabetes results in endothelial dysfunction and accelerated platelet deposition, both of which increase the propensity to thrombosis. Several growth factors known to promote the restenosis process are overexpressed in the presence of hyperglycemia. Advanced glycosylation promotes inflammatory cell recruitment and smooth muscle cell proliferation[9] Although the accumulating evidence from in vitro and in vivo studies demonstrate the increased risk of ISR in diabetes, the precise mechanism underlying the clinical observations remains uncertain.

Oxidative modification of low-density lipoprotein (LDL) has been demonstrated to play a central role in the initiation and acceleration of atherosclerosis[10] Oxidized LDL exerts several proatherogenic effects, including direct cytotoxicity to endothelial cells, the promotion of increased synthesis and secretion of adhesion molecules, increased monocyte chemotaxis and adhesion, and enhanced foam cell formation in atherosclerotic lesions[11,12] It has been reported that oxidized LDL promotes vascular smooth muscle cell proliferation[13,14] Recently, immunoassays using monoclonal antibodies prepared against oxidized LDL demonstrated that human blood contains oxidized LDL and that higher levels were found in patients with CAD[15] Serum level of

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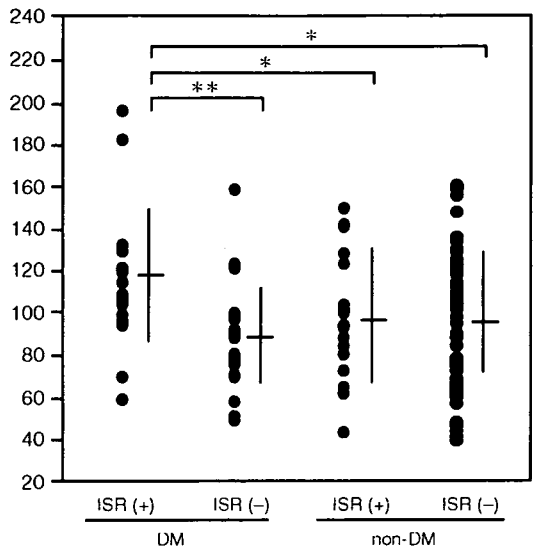

Fig 1. Serum malondialdehyde-modified low-density lipoprotein (MDA-LDL) concentrations in diabetic (DM) and non-diabetic (non-DM) patients with or without in-stent restenosis (ISR). Serum MDA-LDL concentrations were significantly higher in diabetic patients who developed ISR than in patients of the other groups. Data are means ± SD. Statistical analysis was performed with 1-way analysis of variance followed by Fisher's post-hoc test. Significant differences between the groups are indicated. *p<0.05, **p<0.01.

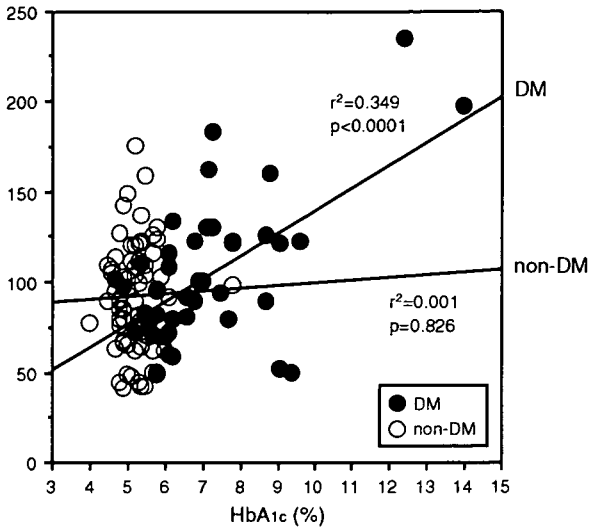

Fig 2. Linear regression analysis and Pearson correlation coefficients for comparisons between the glycohemoglobin (HbA_{1c}) and serum malondialdehyde-modified low-density lipoprotein (MDA-LDL) concentrations in diabetic (DM) and non-diabetic (non-DM) patients. A positive correlation was seen only for diabetic patients ($p<0.0001$, $r^2=0.349$).

oxidized LDL was also increased in diabetic patients who developed vascular complications.[16] A high concentration of circulating oxidized LDL is considered as an independent and significant predictor for future cardiac events in T2DM patients with CAD.[17] Although the nature of the oxidized LDL in the serum of diabetic patients has not been defined, adverse effects of circulating oxidized LDL in diabetes have been strongly suggested. In the present study, we investigated the potential risk factors for ISR, especially in T2DM patients, and we disclose for the first time that an elevated serum level of malondialdehyde-modified LDL (MDA-LDL), a major oxidized LDL, relates to the increase in the incidence of ISR observed in diabetic patients.

Methods

Patient Population

A total of 131 patients (old myocardial infarction, 65; stable effort angina pectoris, 66) who underwent coronary stenting were enrolled in this study. Patients with acute myocardial infarction, unstable angina pectoris, chronic total occluded lesion, renal failure (serum creatinine >2 mg/dl), liver disorder, or malignancy were excluded. All patients were treated with 100 mg/day aspirin and 200 mg/day ticlopidine after stenting. Ticlopidine was given for 1 month after stenting, but aspirin was administered indefinitely. Subacute stent thrombosis did not occur in the enrolled patients. None of the patients in this study received drug-eluting stents, intracoronary brachytherapy, or any other type of investigational local drug therapy or investigational stent implantation.

Coronary Angiographic Evaluation

All patients who received intracoronary stent(s), underwent an angiographic follow-up examination 6 months after stenting. Coronary angiograms were obtained in multiple projections in identical views at baseline, immediately after stent placement, and at follow-up after 6 months, and were stored as digital images. Quantitative angiographic analysis was made by operators unaware of the clinical characteristics of the patients, using the automated edge detection system (CAAS II for Toshiba Infinix; Pie Medical imaging, The Netherlands). The length of the stenosis was estimated with electronic calipers and the contrast-filled catheter tips were used for calibration. The diameters of the proximal and distal reference segments were averaged by the system to yield the reference luminal diameter (RD). The percent diameter stenosis was calculated as the difference between the minimal luminal diameter (MLD) and RD. Binary ISR was defined as >50% diameter stenosis at follow-up.

Measurement of MDA-LDL

Venous blood sampling, including MDA-LDL, was performed in the fasting state upon admission for follow-up angiography. In some diabetic patients, MDA-LDL was measured just before and 30 min after treatment of ISR (ie, re-ballooning of restenosis site). We used an enzyme-linked immunosorbent assay for the detection of MDA-LDL, based on the principles previously reported by Kotani et al.[18] Venous blood samples were collected and the sera were separated within 4 h. Prior to storage at −20°C, the serum samples were mixed with a stabilizing reagent according to the procedure described by Kitano et al.[19] The samples were diluted 2,000-fold in a dilution buffer containing SDS. Duplicate 100-μl aliquots of the diluted sample were then added to the wells of plates coated with monoclonal antibody against MDA-LDL (ML25; Daiich Pure Chemicals, Tokyo, Japan). ML25 has previously been shown to recognize MDA residues. After incubation for 2 h at room temperature, the plates were washed, and β-galactosidase-conjugated monoclonal antibody against apoprotein B (AB16; Daiich Pure Chemicals) was added. It has been shown that the combination of positive immunoreactions with both ML25 and

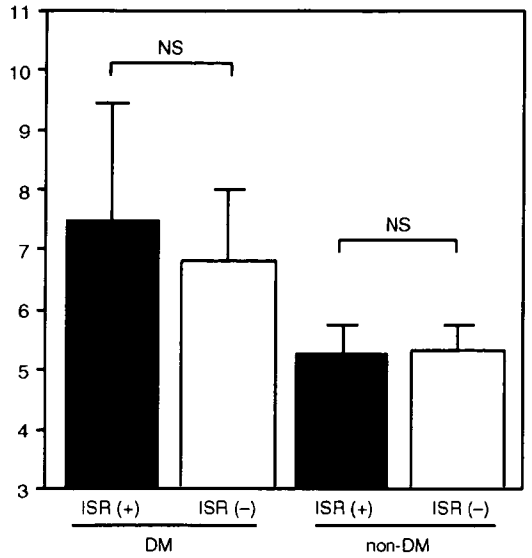

Fig 3. Glycohemoglobin (HbA_{1c}) levels in diabetic (DM) and non-diabetic (non-DM) patients with or without in-stent restenosis (ISR). HbA_{1c} levels were not significant difference between ISR (+) and ISR (−) in both DM and non-DM patients. Data are means ± SD. Statistical analysis was performed with 1-way analysis of variance followed by Fisher's post-hoc test. NS, no significance.

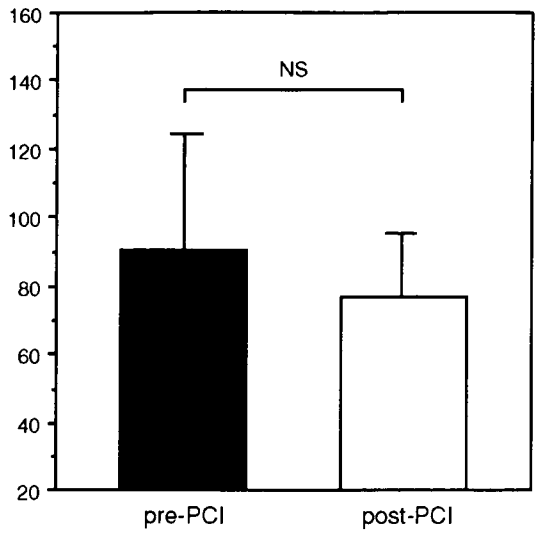

Fig 4. Change in the serum malondialdehyde-modified low-density lipoprotein (MDA-LDL) level before (pre-percutaneous coronary intervention (pre-PCI)) and after (post-PCI) treatment of in-stent restenosis. Serum MDA-LDL was not statistically different after the mechanical crushing of the neointima with a PCI balloon. Data are means ± SD. Statistical analysis was performed with Student's unpaired t-test. NS: no significance.

Table 1 Clinical and Biochemical Data Relating to In-Stent Restenosis in the Enrolled Patients

Characteristic	*Restenosis (+) (n=38)*	*Restenosis (−) (n=93)*	*Univariate p value*	*Multivariate p value*
Age (years)	*69 (10)*	*69 (11)*	*0.981*	*NS*
Sex (M/F)	*32/6*	*60/33*	*0.023**	*NS*
OMI/SAP	*17/21*	*47/46*	*0.547*	*NS*
Body mass index (kg/m²)	*23.3 (2.9)*	*24.5 (4.2)*	*0.119*	*NS*
T2DM	*50%*	*26%*	*0.009***	*0.009***
Hypertension	*47%*	*51%*	*0.694*	*NS*
Smoking	*20%*	*22%*	*0.786*	*NS*
Medication of statin	*32%*	*57%*	*0.014**	*NS*
Fasting plasma glucose (mg/dl)	*109 (30)*	*104 (24)*	*0.344*	*NS*
Glycohemoglobin (%)	*6.3 (1.8)*	*5.7 (1.1)*	*0.037**	*NS*
LDL-cholesterol (mg/dl)	*109 (24)*	*105 (22)*	*0.408*	*NS*
Triglyceride (mg/dl)	*121 (56)*	*117 (55)*	*0.761*	*NS*
HDL-cholesterol (mg/dl)	*43.1 (12.2)*	*45.9 (11.2)*	*0.208*	*NS*
Remnant-like particle (mg/dl)	*4.0 (1.5)*	*4.1 (1.9)*	*0.772*	*NS*
Lipoprotein(a)	*19.2 (14.4)*	*24.6 (21.7)*	*0.200*	*NS*
Uric acid (mg/dl)	*6.1 (1.6)*	*5.8 (1.3)*	*0.199*	*NS*
hs-CRP	*0.179 (0.220)*	*0.138 (0.175)*	*0.298*	*NS*
MDA-LDL (U/L)	*107.0 (34.2)*	*95.4 (29.6)*	*0.058*	*NS*
Stent diameter (mm)	*3.0 (0.4)*	*3.0 (0.4)*	*0.766*	*NS*
Stent length (mm)	*16.6 (4.5)*	*15.5 (3.5)*	*0.109*	*NS*
MSA (mm²)	*6.38 (1.58)*	*6.45 (1.55)*	*0.782*	*NS*

*Data are means (SD) or percentages. Statistical analysis was conducted using a stepwise multiple regression analysis. Values of p<0.05 indicate significant differences. *p<0.01; **p<0.05.*
NS, no significance; OMI, old myocardial infarction; SAP, stable angina pectoris; T2DM, type 2 diabetes mellitus; LDL, low-density lipoprotein; HDL, high-density lipoprotein; hs-CRP, high-sensitivity C-reactive protein; MDA-LDL, malondialdehyde-modified LDL; MSA, minimum stent area immediately after percutaneous coronary intervention.

AB16 recognizes MDA-LDL. After an additional incubation for 1 h at room temperature, the plates were washed, and 100-μl of 10 mmol/L *o*-nitrophenyl-galactopyranoside was added. The reaction was stopped after 2 h by adding 100-μl of 0.2 mol/L sodium carbonate (pH 12). The absorbance at 405 nm and the reference absorbance at 650 nm were determined using a microplate reader (M-Vmax, Molecular Devices, CA). A prepared solution of MDA-LDL was used as a primary standard, in which 15% of the total amino groups were modified. We tentatively defined 1 unit/L MDA-LDL as the absorbance obtained with the primary standard at a concentration of 1 mg/L. A calibration curve was prepared using 500- to 8,000-fold dilutions of a reference serum, the secondary standard, and the amount of MDA-LDL in the samples was calculated from the curve. The reference sera were prepared from pooled sera from