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NK-104, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, reduces osteopontin expression by rat aortic smooth muscle cells

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1 It has been suggested that osteopontin promotes the development of atherosclerosis, especially under diabetic conditions.

2 In the present study, we found that NK-104, a new potent synthetic inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, reduced osteopontin expression both at protein and mRNA levels in cultured rat aortic smooth muscle cells.

3 The inhibitory effect of NK-104 was almost completely reversed by mevalonate, suggesting that mevalonate or its metabolites play important roles in the regulation of osteopontin expression.

4 Furthermore, oral administration of NK-104 (3 mg kg^{-1} day^{-1} for 7 days) effectively suppressed abnormally upregulated expression of osteopontin mRNA in the aorta and kidney of streptozotocin-induced diabetic rats.

5 These data support a notion that NK-104 is a suitable drug for the treatment of diabetic patients with hypercholesterolaemia.

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Abbreviations: FPP, farnesylpyrophosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GGPP, geranylgeranyl-pyrophosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low density lipoprotein; OPN, osteopontin; SMC, smooth muscle cell; STZ, streptozotocin

Introduction

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) can achieve relatively large reductions in plasma cholesterol levels (Havel & Rapaport, 1995) and represent an established class of drugs for the treatment of hypercholesterolaemia. Several clinical trials (Scandinavian Simvastatin Survival Study Group, 1994; Shepherd *et al*., 1995; Sacks *et al*., 1996; Downs *et al*., 1998; Long-Term Intervention with Pravastatin in Ischemic Disease (LIPID) Study Group, 1998) have demonstrated that statins can ameliorate atherosclerosis, and reduce cardiovascular-related morbidity and mortality. The preventive effect of statins in coronary artery disease was observed also in patients with diabetes mellitus (Macdonald *et al*., 1998). It has been suggested that statins, apart from their lipid lowering properties, also cause direct anti-atherosclerotic effects on the arterial wall such as modifications of endothelial function, inflammatory responses, plaque stability and thrombus formation (Rosenson & Tangney, 1998), that could prevent significant cardiovascular disease.

Osteopontin (OPN) is a multifunctional phosphoprotein secreted by many cell types, such as osteoclasts, lymphocytes, macrophages, epithelial cells and vascular smooth muscle cells (SMCs) (Butler, 1989). It has been reported that OPN protein and mRNA were expressed in neointima as well as calcified atheromatous plaque (Shanahan *et al*., 1994). A neutralizing antibody against OPN inhibited rat carotid neointimal formation after endothelial denudation (Liaw *et al*., 1997). Recently, we found upregulation of OPN expression in human as well as rat diabetic vascular walls (Takemoto *et al*., 2000). It was also noted that high glucose concentrations stimulated OPN expression *via* a protein kinase C-dependent pathway as well as the hexosamine pathway in cultured rat aortic SMCs (Takemoto *et al*., 1999b). Furthermore, OPN was found to stimulate migration and enhance platelet-derived growth factor-mediated DNA synthesis of cultured rat aortic SMCs (Takemoto *et al*., 2000). Based on these data, we have suggested that OPN plays a role in accelerated atherogenesis in diabetes mellitus.

In the present study, we examined the effect of NK-104, a new potent synthetic inhibitor of HMG-CoA reductase (Aoki *et al*., 1997), on OPN expression not only in cultured rat aortic SMCs but also in the aorta and kidney of diabetic rats, in an attempt to elucidate a possible beneficial effect of the drug for the prevention of diabetic vascular complications.

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Methods

Cell culture

Rat aortic SMCs were isolated by the explant method from adult male Wistar rats and the primary culture and subculture were carried out as described (Morisaki *et al*., 1994). Cells at the 4th to 9th passages were used for the present study. Confluent SMCs in 6-cm dishes were serum-starved for 24 h. Then the medium was replaced with another serum-free medium containing different concentrations of low density lipoprotein (LDL) (0–200 μg protein ml^{-1}), NK-104 (0–8 μM), mevalonate (0–100 μM), geranylgeranylpyrophosphate (GGPP) (0–15 μM) and farnesylpyrophosphate (FPP) (0–30 μM), and the incubation continued at 37°C for 48 h. After removal of the conditioned medium which was subjected to Western blotting, the cells were processed for Northern blotting. No significant differences were found in the number of cells for each condition counted after removal of the conditioned medium (data not shown).

Animal experiments

Male Wistar rats weighing approximately 200 g were administered either 50 mg kg^{-1} streptozotocin (STZ, Sigma) or normal saline into the tail vein as described previously (Kanzaki *et al*., 1987). On the same day of STZ injection, administration of NK-104 was initiated. Three mg kg^{-1} of NK-104 suspended in 0.5% carboxymethylcellulose solution or the vehicle alone were orally administered to the rats once a day before feeding for 7 days. Thereafter the rats were killed by anaesthetic overdose with sodium 5-ethyl-5-(1-methylbutyl)barbiturate, the aortas and kidneys were excised, and processed for Northern blotting. At the end of the treatment, body weight and plasma glucose concentration were 383.3±12.0 g and 8.0±0.4 mM for control rats (mean±s.e.mean, n=3), 373.3±8.8 g and 7.8±0.8 mM for control/NK-104-administered rats (n=3), 325.0±8.6 g and 31.6±1.6 mM for STZ-injected rats (n=3), and 322.5±6.3 g and 30.1±1.0 mM for STZ-injected/NK-104-administered rats (n=3), respectively. The administration of NK-104 did not appreciably affect blood glucose level, food intake and body weight of the rats. The animal experiments were carried out with the approval of the Animal Ethics Committee in Chiba University.

Western blotting

OPN content of the conditioned medium was estimated by Western blotting essentially as described previously (Takemoto *et al*., 1999b). Briefly, DE52 gel-precipitable materials in the medium were subjected to SDS-10% polyacrylamide gel electrophoresis, and were blotted to nitrocellulose membranes. The blots were blocked and incubated with an anti-OPN antibody (1 : 1000 dilution). After washing, the blots were further incubated with peroxidase-conjugated anti-mouse immunoglobulins (1 : 5000 dilution). After washing, sites of antibody binding were visualized using the ECL Western blotting detection system (Amersham).

Northern blotting

The expression of OPN mRNA was analysed by Northern blotting. Total RNA was prepared from cultured cells or tissues using ISOGEN (Nippon Gene, Tokyo). Northern hybridization was performed essentially as described (Takemoto *et al*., 1999b) using a ^{32}P-labelled OPN cDNA probe. Hybridization was also performed with a ^{32}P-labelled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe, in order to assess the amount of RNA loaded in each lane. Densitometric analyses of fluorograms and autoradiograms were performed using the imaging scanner (EPSON ES 8000) with the NIH Image 1.44 software.

Statistical analysis

Data are expressed as mean±s.e.mean. The significance of differences was evaluated by Student's t-test.

Reagents

Rat OPN cDNA was provided by Dr Mark Thiede (Pfizer, Groton, CT, U.S.A.). Microgranular anion exchanger, DE52, was purchased from Whatman (Maidstone, U.K.). The mouse monoclonal anti-rat OPN antibody (MPIIIB10) was from American Research Products (Belmont, MA, U.S.A.). Peroxidase-conjugated sheep anti-mouse immunoglobulins was from Amersham Pharmacia Biotech. Mevalonate, GGPP, and FPP were from Sigma Chemical Co. Liposomes containing GGPP or FPP were prepared before adding to culture medium because the experimental use of these compounds is limited by their membrane impermeability and sensitivity to thiol reagents present in the medium (Nishimura *et al*., 1999). NK-104 ((+)-monocalcium bis{(3*R*, 5*S*, 6*E*)-7-[2-cyclopropyl-4-(4-fluorophenyl)-3-quinolyl]-3,5-dihydroxy-6-heptenoate}, CAS 147526-32-7) was organically synthesized at Nissan Chemical Industries, Ltd. (Tokyo). Human LDL was isolated from fresh human blood by sequential ultracentrifugation by the method of Havel *et al*. (1955).

Results

Effect of LDL on OPN expression in cultured rat aortic SMCs

We have previously found a significant negative correlation between plasma levels of OPN and LDL cholesterol in healthy subjects (Takemoto *et al*., 1999a). The finding prompted us to examine the *in vitro* effect of LDL on OPN expression using cultured cells. Rat aortic SMCs were incubated with different concentrations of LDL at 37°C for 48 h. After incubation, OPN secretion from the cells as well as OPN mRNA level in the cells were determined. As shown in Figure 1, LDL dose-dependently decreased both the OPN secretion (Figure 1a) and the mRNA level (Figure 1b).

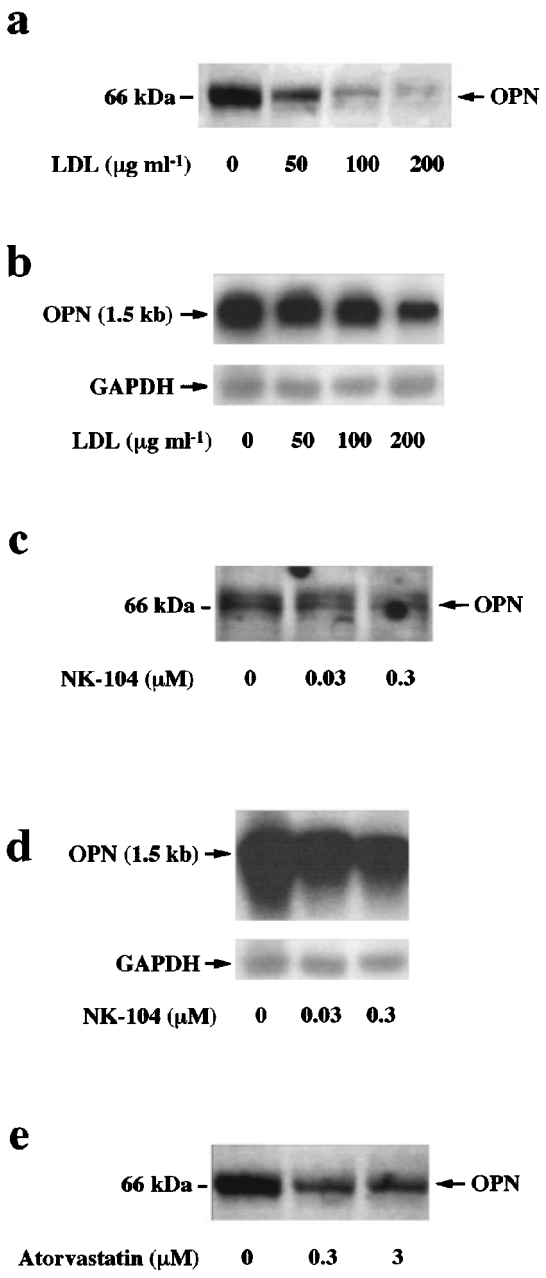

Figure 1 Effects of LDL and NK-104 on OPN expression in cultured rat aortic SMCs. After serum starvation for 24 h, medium was replaced with another serum-free medium containing the indicated concentrations of LDL (a,b), NK-104 (c,d), or atorvastatin (e), and incubation continued at 37°C for 48 h. After incubation, the conditioned medium and the cells were processed for Western blotting (a,c,e) and Northern blotting (b,d), respectively. (a,c,e) OPN was semipurified from the medium using DE52 gel and subjected to SDS-polyacrylamide gel electrophoresis. The materials were transferred to nitrocellulose membranes, and the membranes were immunoblotted with the anti-OPN antibody. (b,d) Total RNA was isolated from the cells, separated by agarose gel electrophoresis, and transferred to nylon membranes. The membranes were hybridized with ^{32}P-labelled rat OPN cDNA probe or rat GAPDH cDNA probe, and signals were detected by autoradiography. The blots shown in this figure are representative of three independent experiments providing essentially similar results.

NK-104 reduces OPN expression in cultured rat aortic SMCs

It is well known that LDL supplies cholesterol to cells and, thereby, suppresses cellular cholesterol biosynthesis. Therefore it seemed possible that the decreased cholesterol biosynthesis is a causative element of the observed inhibitory effect of LDL on OPN expression. To prove the possibility, another method to suppress cellular cholesterol biosynthesis, namely, treatment of cells with NK-104, a potent HMG-CoA reductase inhibitor, was employed and its effect on OPN expression was examined. As shown in Figure 1, NK-104 was found to dose-dependently decrease OPN expression in cultured rat aortic SMCs both at protein (Figure 1c) and mRNA (Figure 1d) levels, as expected. Another HMG-CoA reductase inhibitor, atorvastatin, was also found to dose-dependently decrease the OPN expression (Figure 1e), supporting an interpretation that the inhibitory effect of these drugs is attributable to their ability to inhibit HMG-CoA reductase.

Mevalonate and GGPP, but not FPP, prevent NK-104-induced inhibition of OPN expression in cultured rat aortic SMCs

HMG-CoA reductase, which converts HMG-CoA to mevalonate, is the rate-limiting enzyme of the mevalonate pathway. This pathway is important for the biosynthesis of isoprenoids such as GGPP and FPP, as well as cholesterol. As shown in Figure 2a, NK-104-induced inhibition of OPN expression was almost completely recovered by addition of mevalonate, indicating that the observed inhibition of OPN expression was due to decreased mevalonate production occurring because of HMG-CoA reductase inhibition. The data suggest that mevalonate itself and/or some of its metabolites are required for OPN expression in cultured rat aortic SMCs. Therefore, we examined the effect of GGPP and FPP on the inhibitory effect of NK-104. As shown in Figure 2c,e, NK-104-induced inhibition of OPN expression was almost completely recovered by addition of GGPP, but was not recovered by addition of FPP. Essentially similar results were obtained by Northern blotting performed in parallel with Western blotting (Figure 2b,d,f).

Effect of oral administration of NK-104 on OPN expression in aorta and kidney of STZ-induced diabetic rats

We and others have reported the upregulation of OPN expression in the aorta (Towler *et al*., 1998; Takemoto *et al*., 2000) and renal cortex (Fischer *et al*., 1998) of diabetic rats. Therefore, we next examined the *in vivo* effect of NK-104 on OPN expression by administering the drug to STZ-induced diabetic rats. As shown in Figure 3a, Northern blot analysis revealed that OPN expression in the aorta as well as kidney of STZ-induced diabetic rats was significantly higher than that of control rats, as expected. Oral administration of NK-104 to control rats did not appreciably affect the expression level of OPN (Figure 3b). On the other hand, as shown in Figure 3c, NK-104 administration significantly decreased the expression level of OPN in the aorta and kidney of STZ-induced diabetic rats.

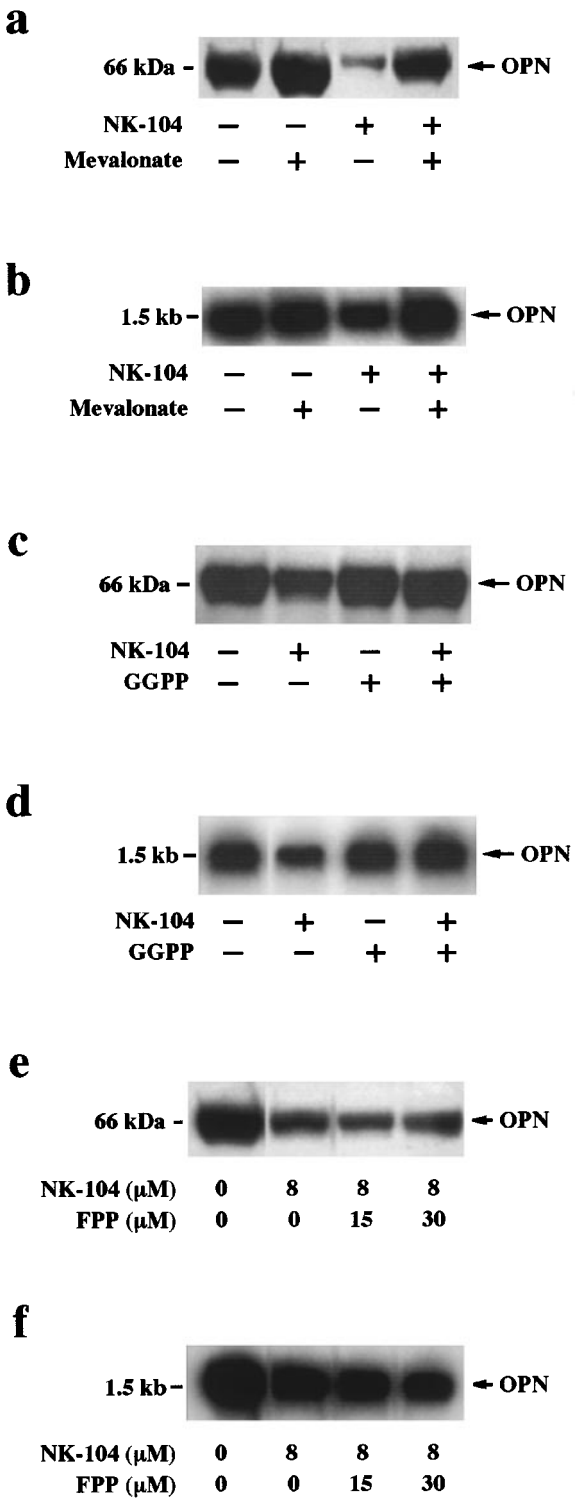

Figure 2 Mevalonate and GGPP, but not FPP, prevent NK-104-induced inhibition of OPN expression in cultured rat aortic SMCs. After serum starvation for 24 h, medium was replaced with another serum-free medium and cells were incubated with or without 8 μM NK-104 at 37°C for 48 h in the presence or absence of 100 μM mevalonate (a,b), 15 μM GGPP (c,d) and 15–30 μM FPP (e,f). After incubation, the conditioned medium and the cells were processed for Western blotting (a,c,e) and Northern blotting (b,d,f), respectively, as described in the legend to Figure 1. The blots shown in this figure are representative of three independent experiments providing essentially similar results.

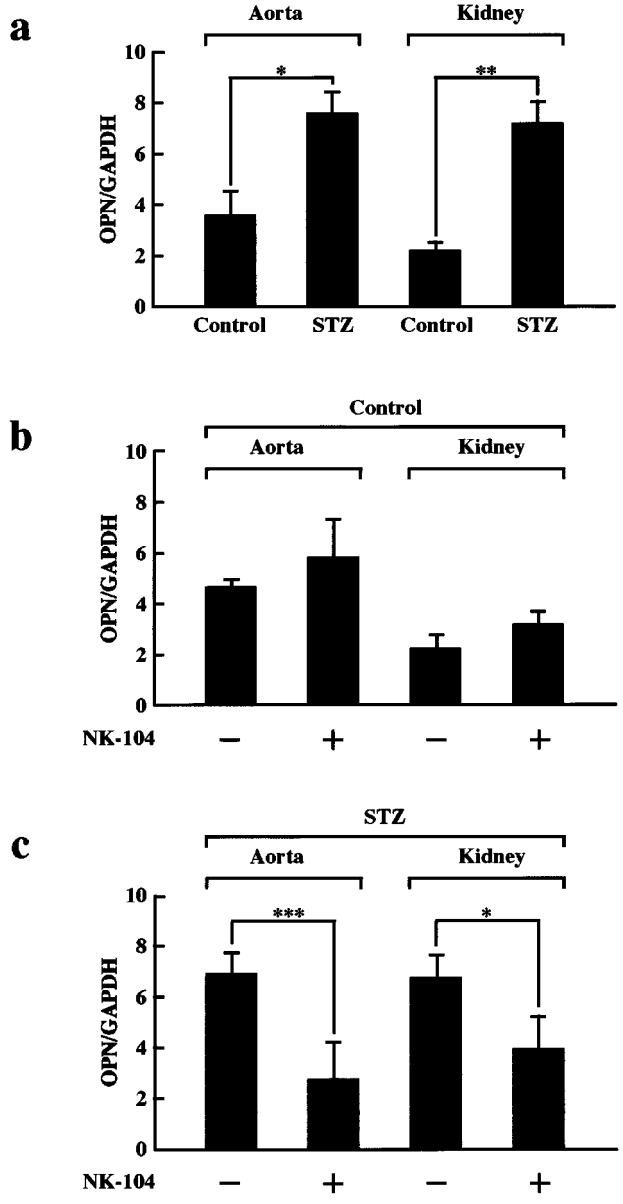

Figure 3 Effect of oral administration of NK-104 on OPN expression in aorta and kidney of STZ-induced diabetic rats. NK-104 (3 mg kg^{-1}) was orally administered once a day to STZ-induced diabetic rats ($n=3$) and control rats ($n=3$). After 7 days of administration, the aortas and kidneys were excised and processed for Northern blotting as described in the legend to Figure 1. The level of OPN transcript was estimated by the ratio of OPN signal to GAPDH signal on autoradiograms measured with the image analyzer. Data are mean ± s.e.mean of triplicate determinations. *$P<0.05$; **$P<0.005$; ***$P<0.01$.

Discussion

The present study has demonstrated for the first time that treatment of cultured rat aortic SMCs with either LDL or NK-104 reduced OPN expression (Figure 1). The inhibitory effect of NK-104 was almost completely reversed by mevalonate (Figure 2), which suggests that mevalonate or its metabolites play important roles in the regulation of OPN expression. Furthermore, oral administration of NK-104

effectively suppressed abnormally upregulated expression of OPN in the aorta and kidney of STZ-induced diabetic rats (Figure 3). These data raise a possibility that NK-104 possesses a beneficial effect on the prevention of diabetic vascular complications.

Mevalonate acts as an isoprenyl precursor for farnesyl or geranylgeranyl molecules, as well as a precursor for cholesterol. Therefore, exogenous FPP and/or GGPP were expected to counteract the effect of NK-104. As shown in Figure 2, the inhibitory effect of NK-104 on OPN expression was almost completely reversed by GGPP, but not by FPP. GGPP is biosynthetically derived from the single condensation of FPP and isopentenylpyrophosphate. Because isopentenylpyrophosphate could not be synthesized in NK-104-treated cells, FPP could not be converted to GGPP. This means that GGPP can rescue the NK-104-induced inhibition of OPN expression without upstream intermediates of cholesterol biosynthesis. It has been demonstrated that many proteins including small G-proteins such as Ras, Rho and Rac, are modified by isoprenoids. This modification is necessary for proper cellular localization and function of the proteins (Casey *et al*., 1989; Danesi *et al*., 1995). It is thus conceivable that geranylgeranylation of some protein(s) is required for the expression of OPN in cultured rat aortic SMCs.

The human, porcine, and murine OPN promoters have been cloned and show a diverse number of homologous *cis*-acting consensus sequences (Denhardt & Guo, 1993). Several sites have been shown to have functional roles in OPN regulation. An ets-1-like element appears to be important for elevated expression of OPN in transformed cells compared to nontransformed cells (Guo *et al*., 1995). Vitamin D response elements have also been identified and appear to be involved in dihydroxycholecalciferol-induced OPN expression in osteoblasts (Noda *et al*., 1990). The CCAAT box binding factor, CBF, is involved in v-*src* mediated stimulation of OPN in human fibrosarcoma cells (Tezuka *et al*., 1996). In cultured vascular SMCs, Malyankar *et al*. (1999) have reported that upstream stimulatory factor 1 positively regulates OPN expression during phenotype transition from the normal contractile to the injury-induced synthetic phenotype. The regulation of OPN requires a composite site of the OPN promoter that contains an E-box binding sequence (CAGGTG) and a GC-rich region. A possibility remains to be elucidated that some of these transcription factors and their cognitive binding sites are also involved in NK-104-sensitive mechanism of OPN expression, however, requirement of geranylgeranylation for the function of these transcription factors has not been reported so far.

Oral administration of NK-104 significantly reduced OPN expression in the aorta and kidney of STZ-induced diabetic rats, while the drug did not show any effect on control rats (Figure 3). It seems thus likely that the NK-104-sensitive pathway is not required under a steady state for the expression of OPN in the rat aorta and kidney. Although the precise underlying mechanism is not known at present, the preferential inhibitory effect of NK-104 on diabetes-induced upregulation of OPN expression looks desirable from a therapeutic point of view for diabetic complications.

Recently, it has been recognized that statins, besides lowering blood cholesterol, exert broader effects that could ameliorate cardiovascular diseases (Corsini *et al*., 1999). In fact, we have found that NK-104 could suppress neointimal thickening by inhibiting SMC growth and fibronectin production in balloon-injured rabbit carotid artery (Kitahara *et al*., 1998). Our present study also suggests a therapeutic application of statins for the prevention of diabetic vascular complications. Further study is necessary to confirm the remedial effect of NK-104-induced inhibition of OPN expression on accelerated atherogenesis in diabetes mellitus. The statins on the market are not necessarily ideal for use as therapeutic agents on vascular diseases, since they were selected for their capacity to lower serum cholesterol, which requires targeting to HMG-CoA reductase in hepatic cells. Therefore, development of new statins preferentially distributing to vascular tissues is also expected for the establishment of a novel strategy to interfere with the onset of diabetic vascular diseases.

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