

Fig. 2. Pi suppresses Gas6 and Axl expression, and rhGas6 inhibits caspase-dependent apoptosis and calcification. HASMC were cultured in the presence or absence of 2.6 mM Pi for 12 h. Cell lysates were collected and subjected to SDS-PAGE followed by immunoblotting with antibodies to Gas6, Axl or β -tubulin (A). After pretreatment with the indicated concentrations of rhGas6, apoptosis was induced by 2.6 mM Pi. All values are presented as mean \pm S.E.M. ($n=3$). * $P<0.05$ vs. 2.6 mM Pi, rhGas6 (-) by Fisher's test (B). HASMC were pretreated with rhGas6 (400 ng/ml) for 1 h, then cultured with 2.6 mM Pi for 12 h. Cell lysates were immunoblotted with an antibody that recognizes caspase-3 (35 kDa) and the cleaved forms of caspase-3 (17 and 19 kDa) (C). For measurement of Ca deposition, HASMC were cultured with the indicated concentrations of rhGas6 in the presence of 2.6 mM Pi for 6 days. All values are presented as mean \pm S.E.M. ($n=6$). * $P<0.05$ by Fisher's test (D). Experiments were performed with at least three different cell populations.

3.3. Downregulation of phospho-Akt participates in Pi-induced apoptosis

Since in NIH-3T3 fibroblasts, the antiapoptotic effect of Gas6-Axl interaction has been shown to be mediated by Akt phosphorylation (Goruppi et al., 1999), we examined whether Akt participates in the signaling of downregulation of the Gas6-Axl interaction during Pi-induced apoptosis. In the presence of 2.6 mM Pi, Akt phosphorylation was downregulated in a time-dependent manner, whereas the expression of total Akt was not changed (Fig. 3A). In addition, rhGas6 abrogated the Pi-induced decrease in Akt phosphorylation, implying that subsequent downregulation of Akt phosphorylation is the pathway of Pi-induced apoptosis (Fig. 3B).

Because Akt phosphorylation is regulated by PI3K, we examined the effect of wortmannin, a specific PI3K inhibitor, on rhGas6-mediated phosphorylation of Akt. As shown in Fig. 3B, wortmannin abrogated the rhGas6-induced phosphorylation of

Akt and further eliminated the inhibitory effect of rhGas6 on Pi-induced apoptosis and calcification (Fig. 3C, D). These results indicate that the preventive effect of rhGas6 on Pi-induced apoptosis and calcification was mediated by the PI3K-Akt pathway.

3.4. Pi suppresses Bcl2 phosphorylation and activates Bad

To establish the downstream components of Pi-induced apoptosis, two key apoptosis-regulating proteins, Bcl2 and Bad, were analyzed. During apoptosis, phosphorylation of Bcl2 (active form) and Bad (inactive form) was markedly reduced by 2.6 mM Pi in a time-dependent manner. The expression level of their total protein was not changed in this period (Fig. 4A, B). By supplementation of the medium with rhGas6, the decrease in phosphorylation of Bcl2 and Bad by Pi was reversed to almost the basal level (Fig. 4C, D). These results indicate that Pi promotes apoptosis by inactivating Bcl2 and activating Bad via a Gas6-dependent pathway.

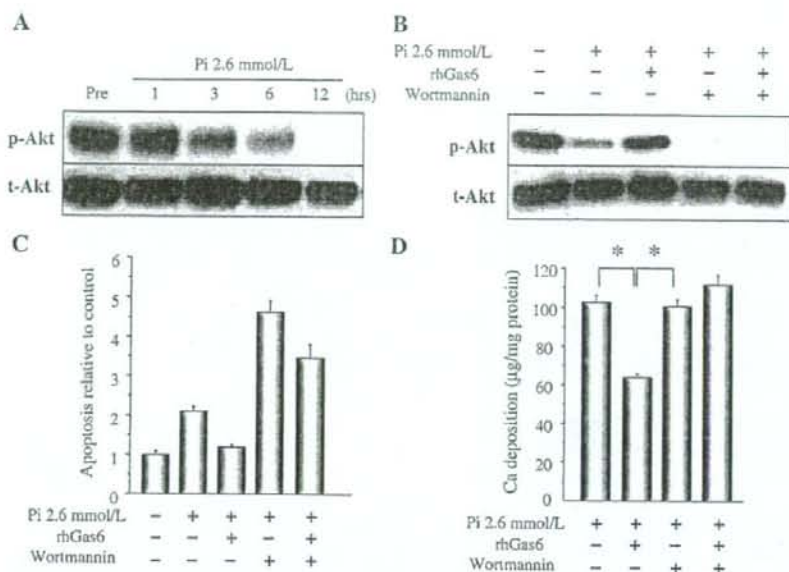


Fig. 3. Pi decreases Akt phosphorylation, and wortmannin abrogates the inhibitory effect of rhGas6 on Akt phosphorylation, apoptosis and calcification. HASMC were cultured in the presence of 2.6 mM Pi for the indicated periods. Cell lysates were immunoblotted with anti-phospho-Akt (p-Akt) antibody and total Akt (t-Akt) antibody (A). HASMC were pretreated with rhGas6 (400 ng/ml), wortmannin (1 µM), or both for 1 h, and then treated with 2.6 mM Pi for 12 h. Cell lysates were immunoblotted with p-Akt and t-Akt antibody (B). After pretreatment with rhGas6 (400 ng/ml) and wortmannin (1 µM), apoptosis was induced by 2.6 mM Pi. All values are presented as mean ± S.E.M. ($n=3$). * $P<0.05$ vs. 2.6 mM Pi, rhGas6 (-), wortmannin (-) by Fisher's test (C). HASMC were cultured with rhGas6 (400 ng/ml) and with or without wortmannin (1 µM) in the presence of 2.6 mM Pi for 6 days. Ca content was measured and normalized by cell protein content. All values are presented as mean ± S.E.M. ($n=6$). * $P<0.05$ by Fisher's test (D).

3.5. Gas6-mediated survival pathway is the target of statins' effect on apoptosis

To investigate whether the antiapoptotic effect of statins is associated with the Gas6-mediated survival pathway, first, we examined the effect of statins on the expression of Gas6 and Axl. As shown in Fig. 5A and B, both fluvastatin and pravastatin restored the expression of Gas6 and Axl, which was downregulated by 2.6 mM Pi. Because we have shown that the Gas6-mediated survival pathway is Akt-dependent, the effect of statins on Akt phosphorylation was examined. The Pi-induced decrease in Akt phosphorylation was restored by both statins, while total Akt expression was not changed. In addition, we found that both statins stimulated phosphorylation of Bcl2 and

Bad. Because we have shown that the Gas6-mediated survival pathway is Akt-dependent, the effect of statins on Akt phosphorylation was examined. The Pi-induced decrease in Akt phosphorylation was restored by both statins, while total Akt expression was not changed. In addition, we found that both statins stimulated phosphorylation of Bcl2 and

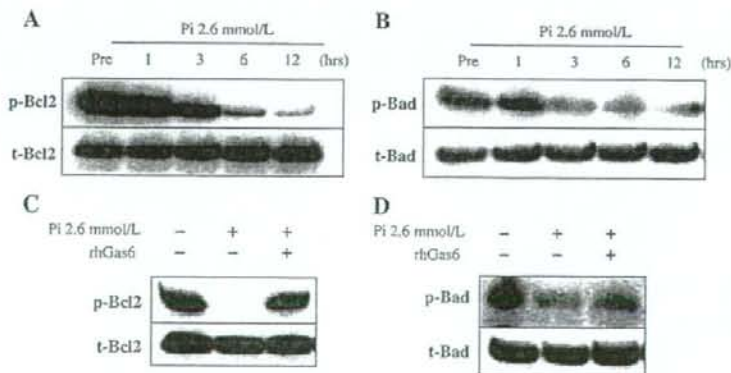


Fig. 4. RhGas6 restores Pi-induced decrease in phosphorylation of Bcl2 and Bad. HASMC were exposed to 2.6 mM Pi for the indicated periods, and cell lysates were subjected to immunoblotting with anti-phospho-Bcl2 (p-Bcl2) antibody and total Bcl2 (t-Bcl2) antibody (A), or with anti-phospho-Bad (p-Bad) antibody and total Bad (t-Bad) antibody (B). HASMC were pretreated with rhGas6 (400 ng/ml) for 1 h, and then treated with 2.6 mM Pi for 12 h. Cell lysates were subjected to immunoblotting with p-Bcl2 and t-Bcl2 antibody (C), or with p-Bad and t-Bad antibody (D).

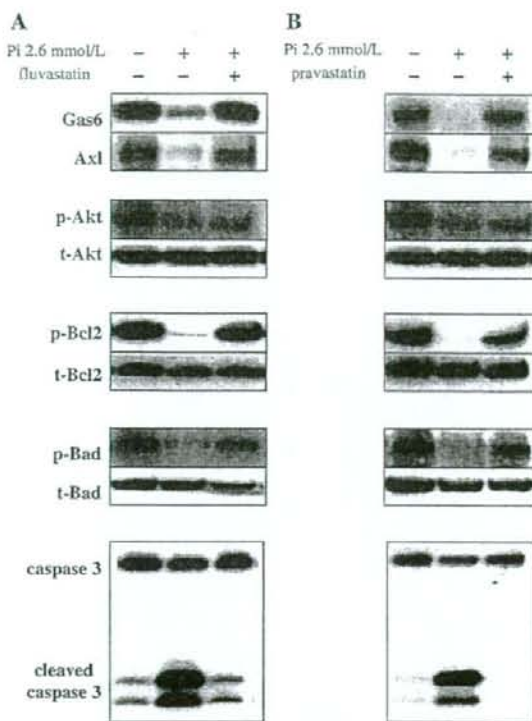


Fig. 5. Antiapoptotic effect of statins is associated with upregulation of Gas6-Axl survival pathway. After pretreatment with 0.1 μ M fluvastatin (A) and 50 μ M pravastatin (B) for 12 h, apoptosis was induced by 2.6 mM Pi. After 12 h, cell lysates were collected and subjected to SDS-PAGE followed by immunoblotting with antibodies that recognize Gas6 and Axl, with phospho-specific Akt (p-Akt) and total Akt (t-Akt) antibody, with phospho-specific Bcl2 (p-Bcl2) and total Bcl2 (t-Bcl2) antibody, or with phospho-specific Bad (p-Bad) and total Bad (t-Bad) antibody. Cell lysates were immunoblotted with an antibody that recognizes uncleaved caspase-3 (35 kDa) and the cleaved forms of caspase-3 (17 and 19 kDa).

Bad, with total expression unchanged. Pi-induced caspase 3 activation was also prevented by both statins. Taken together, these findings suggest that the inhibitory effect of statins on Pi-induced apoptosis is mediated by restoration of the Gas6-mediated survival pathway; PI3K-induced Akt phosphorylation, Bcl2 activation, Bad inactivation, and caspase 3 inactivation.

4. Discussion

In the present study, we found that both lipophilic fluvastatin and hydrophilic pravastatin protected against Pi-induced apoptosis and calcification in HASMC, as we found with atorvastatin previously. With regard to the different potency of statins, we found that the inhibitory effect of pravastatin was inferior to those of fluvastatin and atorvastatin, which exerted similar effects on calcification and apoptosis. This might relate to our previous finding that the inhibition of calcification by statins

was not dependent on the mevalonate pathway (Son et al., 2006). Consequently, the inhibitory effect on calcification was not parallel to the cholesterol-lowering effect. We speculate that the difference between statins was derived from their affinity to *vascular smooth muscle cells* (VSMC), that is, lipophilic statins have stronger effects on VSMC calcification than hydrophilic statins.

The antiapoptotic effect of statins was induced by restoration of the Gas6-mediated survival pathway: PI3K-induced Akt phosphorylation, Bcl2 and Bad phosphorylation, and caspase 3 inactivation. Gas6 plays a crucial role in the effect of statins on Pi-induced apoptosis. Gas6, a secreted vitamin K-dependent protein, binds to the receptors of the mammalian Axl protein-tyrosine kinase family; Axl, Sky, and Mer, with different affinities (Nagata et al., 1996). Gas6 and Axl have been shown to localize in the neointima of the artery after balloon injury, in which they presumably modulate several cell functions such as differentiation, adhesion, migration, proliferation, and survival in a cell-specific manner (Melaragno et al., 1998). The Gas6-Axl interaction is also shown to upregulate scavenger receptor A expression in VSMC (Ming et al., 2001), and facilitates the clearance of apoptotic cells by macrophages (Ishimoto et al., 2000). Of the above functions, protection against apoptotic cell death has been most studied (Goruppi et al., 1996; Healy et al., 2001; Lee et al., 2002; Nakano et al., 1996). Consistently, the expression of Gas6 and Axl was downregulated by Pi, leading to apoptosis and subsequent calcification.

Several intracellular signaling pathways mediated by Gas6-Axl interaction have been shown previously (Goruppi et al., 1999; Lee et al., 2002; Ming et al., 2001). Akt, which is necessary for Gas6-dependent survival, is a critical downstream effector of the PI3K-dependent antiapoptotic pathway. In VSMC, it has been reported that the PI3K-Akt pathway mediates Gas6 induction of scavenger receptor A (Ming et al., 2001). Consistent with these reports, our study provides evidence that the PI3K-Akt pathway is a target of Gas6-Axl interaction, and downregulation of Akt phosphorylation is associated with Pi-induced apoptosis and calcification. Moreover, it is known that PI3K-Akt affects the cell death program through the Bcl2 family of proteins. This protein family is a critical regulator of apoptosis in a variety of cell types, and the balance of antiapoptotic members, such as Bcl2, versus proapoptotic mediators, such as Bad, determines cell fate (Reed, 1997). Bcl2, whose phosphorylation is required for its antiapoptotic activity (Ruvolo et al., 2001), inhibits programmed cell death by several mechanisms: It binds to caspase CED-4 (Apaf-1) and prevents the cell execution cascade; Bcl2 alters mitochondrial membrane potential and inhibits the release of cytochrome c. On the other hand, Bad plays a proapoptotic role in its dephosphorylated form by binding to Bcl2 and reversing its antiapoptotic effect; phosphorylation of Bad results in its cytosolic sequestration by 14-3-3 and hampers its binding to Bcl2 (Zha et al., 1996). It was also reported that Bad is directly phosphorylated by PI3K-Akt (del Peso et al., 1997). In the present study, Bcl2 was inactivated and Bad was activated (both proteins were dephosphorylated) by Pi, directing the cells to apoptosis, and rhGas6 restored phosphorylation of Bcl2 and Bad. During apoptosis, one of the final biochemical events leading to programmed

cell death is activation of the caspase cascade. Activation of caspase 3 is required for internucleosomal DNA degradation (Woo et al., 1998), and caspase inhibition prevents the release of apoptotic bodies from cells (Zhang et al., 1999). In the present study, supplementation of the medium with rhGas6 prevented Pi-induced caspase 3 activation. These results clearly show that Pi downregulates Gas6-Axl, decreases PI3K-mediated Akt phosphorylation, inactivates Bcl2, activates Bad, and activates caspase 3, leading to apoptosis.

The present study demonstrated that statins restored the Gas6-mediated survival pathway. Consistent with these results, Akt phosphorylation has been reported to be an antiapoptotic mechanism of statins: pravastatin inhibited hypoxia-induced apoptosis through activation of Akt in cardiomyocytes (Bergmann et al., 2004), and simvastatin and pravastatin enhanced phosphorylation of Akt and promoted angiogenesis in endothelial cells (Kureishi et al., 2000). Recently, it was reported that statins inhibit caspase 3 activation driven by protein kinase C inhibitors in the process of apoptosis, suggesting that caspase 3 is also under the control of statins during apoptosis (Tanaka et al., 2004).

In this study, we performed experiments under both short-term (within 24 h) and long-term (up to 10 days) conditions. In general, short-term experiments are able to examine acute cell behavior, such as signaling and transcription. However, because obvious HASMC calcification takes at least 3 days, we also performed long-term experiments. Downregulation of Gas6, Axl expression and reduced phosphorylation of Akt, Bcl2, and Bad, and a beneficial effect of statins were consistently found in the long-term condition. This confirms that the Gas6-Axl survival signal is the key mechanism for Pi-induced calcification.

It is concluded that statins inhibit Pi-induced apoptosis via the Gas6/Axl-PI3K-Akt signal pathway, which has a crucial role in the prevention of HASMC calcification. This study adds further evidence of the pleiotropic effects of statins, suggesting a therapeutic strategy for the prevention of vascular calcification.

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ORIGINAL ARTICLE

Multiple consultations and polypharmacy of patients attending geriatric outpatient units of university hospitals

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Background: Multiple consultations in older patients may increase the chance of overlapping prescriptions or inappropriate drug prescribing.

Methods: We carried out a survey investigating the status of multiple consultations and the polypharmacy of patients attending geriatric outpatient units of five university hospitals.

Results: The patients who received multiple consultations did not have a different number of diagnosed disorders and drugs prescribed by geriatricians compared with the patients who received a single consultation.

Conclusions: No significant difference in diagnostic and prescribing profiles between the patients with referrals and those without, together with the relatively smaller incidence of inappropriate prescriptions by referrals to non-geriatric specialists, suggest that multiple consultations per se may not necessarily increase the risk for adverse drug events in clinical settings.

Keywords: adverse drug reactions (ADR), multiple consultations, polypharmacy, university hospitals.

Introduction

Because of comorbidity and the presence of various clinical manifestations, elderly patients are often characterized by their multiple consultations across specialties. Under the existing health care system in Japan, free access to specialists is granted to all patients even though consultations to specialists are encouraged only through primary care physicians' referrals, without which patients have to pay an extra fee for specialist

consultations. Multiple consultations in older patients may not be desirable in terms of preventing inappropriate drug prescribing. They may increase the chance of overlapping prescriptions or unexpected drug interactions caused by polypharmacy, leading to an elevated risk of adverse drug reactions (ADR) or poor compliance to pharmacotherapeutics. Despite suggestions that ADR in older patients are commonly observed^{1,2} and can become a cause of hospital admission,^{3–9} inappropriate drug prescribing has been reported in various care settings for older adults.¹⁰ In terms of optimal drug therapy for older patients, physicians must always take into consideration the unique aspects of age-related changes in pharmacokinetics/pharmacodynamics and the potential harm of prescribing inappropriate medication.¹¹ Since the Beers criteria for determining potentially inappropriate medication use by the elderly and its

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revised version,^{12,13} much attention has been paid to the potential harm of prescribing drugs inappropriate for older adults, but awareness of this problem is mainly shared within geriatric specialists, and there has been insufficient outreach on this problem across specialties. Therefore, in terms of referring older patients to non-geriatric specialists, not all specialists may be aware of possible ADR in considering pharmacotherapy. University hospitals in particular have many clinical departments and sections, and the clinical environment thus encourages the referral of geriatric outpatients to other specialists, which may increase the risk of inappropriate drug prescribing. In this study, we carried out a survey investigating the status of multiple consultations and the polypharmacy of patients being treated at geriatric outpatient units of five university hospitals.

Methods

Subjects

We randomly sampled 660 patients who had been attending geriatric outpatient units of five university hospitals (Kanazawa Medical University Hospital, Kyoto University Hospital, Nagoya University Hospital, University of Tokyo Hospital, Kyorin University Hospital) from October 2003 to December 2003, and surveyed the patients' clinical background (age, identified diagnoses), prescribed drugs and consultations to other specialists within the university hospitals by chart reviews. Differences in continuous variables among the five institutions were determined by a one-way analysis of variance (ANOVA). Correlation coefficients between each of the variables were calculated by Pearson's method. The patients were divided into two groups, one group in which patients had received multiple consultations and one group in which they had not, and differences in the variables between the two groups were tested using the Student's *T*-test. Values of $P < 0.05$ were considered to indicate statistical significance. Inappropriate drug prescribing was identified based on the 2002 Beers criteria,¹³ an updated version of the original Beers criteria,¹² which is an explicit criteria for determining potentially inappropriate medication use by the elderly. The origi-

nal Beers criteria constructs guidelines on the inappropriate use of medications based on consensus from a panel of six nationally recognized experts in the US on the appropriate use of medication in the elderly. The updated Beers criteria review covered two types of statements: (i) 48 individual medications or medication classes that should generally be avoided in persons 65 years or older because they are either ineffective or they pose unnecessarily high risk for older persons and a safer alternative is available; and (ii) 20 diseases/conditions and medications that should not be used in older persons known to have specific medical conditions.

Results

The mean age of the subjects sampled was 77 ± 9 (Male: 37%). The clinical profiles of all the study subjects are shown in Table 1. Table 2 compares the mean age, number of diagnosed disorders, and number of drugs prescribed in the patients attending the five geriatric outpatient units. There were no differences in all the parameters examined among the five institutions. Regarding the correlations between the parameters, although correlations of all the pairs showed statistical significance ($P < 0.001$), the correlation between the number of diagnosed disorders and that of prescribed drugs showed a much stronger correlation coefficient ($r = 0.768$) relative to the other pairs (age \times number of diagnosed disorders: $r = 0.246$, age \times number of

Table 1 Clinical profile of the study subjects

Cardiovascular disorders (including hypertension)	406 (61.5%)
Cerebroneurologic disorders	373 (56.5%)
Gastrointestinal disorders	286 (43.3%)
Endocrine and metabolic disorders	264 (40.0%)
Joint and muscle disorders (including osteoporosis)	139 (21.1%)
Pulmonary disorders	64 (9.7%)
Disorders of the genitourinary system	54 (8.2%)

Table 2 Comparison of variables surveyed in five institutions

	Number of cases	Age	Number of disorders	Number of drugs
Total	660	77 ± 9	3.5 ± 1.9	4.4 ± 2.8
Kanazawa	217	77 ± 10	4.1 ± 1.9	4.5 ± 2.5
Kyoto	120	76 ± 6	2.7 ± 1.5	4.1 ± 2.5
Nagoya	120	78 ± 7	3.3 ± 1.8	5.0 ± 3.4
Kyorin	88	74 ± 11	3.0 ± 1.6	3.2 ± 2.3
Tokyo	115	76 ± 8	3.5 ± 2.0	5.0 ± 2.9

All data except number of patients are expressed as mean \pm SD.

prescribed drugs: $r = 0.191$). Regarding multiple consultations, 148 patients (22%) were referred from geriatricians to specialists within the same institution. The distribution of specialist referrals is shown in Figure 1. Patients who received multiple consultations did not have a different number of diagnosed disorders and number of drugs prescribed by geriatricians than the patients who received a single consultation (geriatrician only) (Table 3). Because patients who had multiple consultations were prescribed with a mean of 1.8 ± 2.1 drugs by other specialists, their total number of drugs prescribed was greater than that of the patients who had received a single consultation. As for overlapping prescriptions across specialties, only one case, in which vitamin B12 was prescribed by both the geriatrician and otorhinolaryngologist at the same time, was found in this survey.

Regarding inappropriate drug prescribing based on the Beers criteria,¹³ 98 inappropriate cases (14.8% of all the patients) were prescribed by geriatricians, while 14 cases (9.4% of all the patients with referrals) were prescribed by other specialists. The number of identified inappropriate prescribing of drugs included in the Beers criteria is shown in Table 4.

Discussion

Although the recent dissemination of electronic chart review systems in general hospitals would seem to

enable physicians to find out what medications are being prescribed to their patients, the inaccuracy of computerized medication histories can be suggested given the substantial numbers of omissions for over-the-counter products or a variety of supplements available elsewhere.¹⁴ Under the current health care system in Japan, older patients enjoy free access to medical practitioners at their own discretion. Unless older patients are placed in certain types of care facilities such as nursing homes, where prescribing is sometimes restricted, presumably because of financial reasons, they can easily be at risk for polypharmacy, which has recently been identified as a medication safety issue. It has been reported that the risk for ADR increases as the number of medications a patient takes increases.¹⁵ Although multiple definitions are used in the literature to define polypharmacy, if the most stringent criteria is applied,¹⁶ the medication profiles of the patients being treated at all five institutions in the present survey fall into the category of polypharmacy, in which more than three drugs are prescribed regularly. However, as reported by Arai *et al.*¹⁷ who investigated the incidence of ADR in geriatric inpatients of six university hospitals, the average incidence rate (9.2% of all cases) was lower than would be expected from the average number of medications, which exceeded five. Underestimations or neglect by attending physicians of symptoms related to adverse drug events might account for the discrepancy in the results from a previous report by Prybys *et al.*

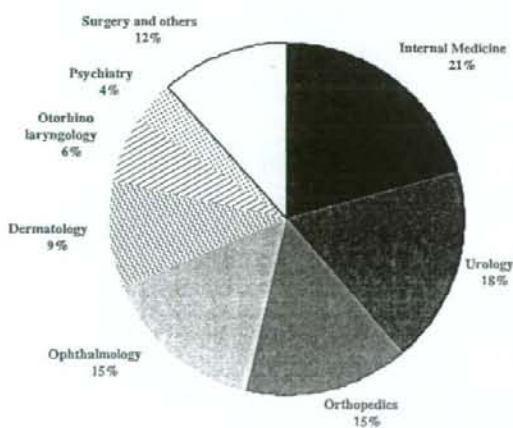


Figure 1 Distribution of specialist referrals.

Table 4 Number of inappropriate prescribing for the drugs listed in the Beers criteria

Prescriber	Geriatricians	Other specialists
Ticlopidine	47	
Mid-long acting benzodiazepines	20	5
Oxybutynine	13	3
Dipyridamole	6	1
Alfacalcidol	5	1
Digoxin	5	
Tricyclic antidepressant	4	1
Disopyramide	3	1
Diclofenac		1
Indometacin		1
Phenobarbital		1

Table 3 Comparison of variables with or without referral to non-geriatric specialists

	Number of cases	Age	Number of disorders	Number of drugs
Referral (-)	511	77 ± 9	3.5 ± 1.9	4.5 ± 2.7
Referral (+)	148 (22%)	76 ± 8	3.3 ± 1.8	4.1 ± 2.9

All data except number of patients are expressed as mean ± SD.

showing that the risk for ADR increases to 58% for five medications.

Apart from cases of referrals within the same institution, physicians do not always monitor prescriptions made by other doctors, and because prescriptions can be changed over time, there is a possibility of medications overlapping or the inappropriate use of drugs. Contrary to our expectations, there was only one overlapping prescription in the present survey. Even though this study surveyed subjects who were attending geriatric outpatient units of university hospitals, where most of the referrals usually take place within the same institutions and medication records are shared across specialties by computerized prescription systems, a survey for tracking the record of consultations outside of each institution (e.g. consultation with local general practitioners) has not been implemented, and thus some overlapping or inappropriate use of drugs might have been overlooked.

As shown in Figure 1, referrals of the patients from the geriatric outpatient unit vary across specialties depending on the needs of each patient. Other than Internal Medicine, the majority of referrals are to specialists, whose expert knowledge and skills are helpful for the management of the common symptoms older patients exhibit (e.g. urinary incontinence, osteoporosis and related fractures, cataract, decubitus ulcers). Considering the circumscribed cases of referrals confirmed in the present survey, older patients attending geriatric outpatient units seem to regard geriatricians as their primary physicians responsible for the overall management of various clinical symptoms. To gain a view of this from the opposite perspective, it would be interesting to survey the status of referrals from other specialists to geriatricians. Regarding the adequacy of medications, geriatricians seem to prescribe more inappropriate drugs for older patients than other specialists in this survey. However such conclusions cannot readily be drawn, given the limited number of referral cases and drugs prescribed by other specialists. Comparing prescribing status to older patients by geriatricians with those by non-geriatric attending physicians under the matched clinical settings would address the question of whether geriatricians are more aware of potential ADR in older patients relative to other general/specialist physicians.

In conclusion, our results showing no significant difference in diagnostic and prescribing profiles between the patients who were referred and those who weren't, together with the relatively smaller incidence of inappropriate prescriptions by referrals to non-geriatric specialists, suggests that multiple consultations per se may

not necessarily increase the risk for ADR in clinical settings.

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Potent free radical scavenger, edaravone, suppresses oxidative stress-induced endothelial damage and early atherosclerosis

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Abstract

Objective: Effects of potent free radical scavenger, edaravone, on oxidative stress-induced endothelial damage and early atherosclerosis were investigated using animal models and cultured cells.

Methods and results: Endothelial apoptosis was induced by 5-min intra-arterial exposure of a rat carotid artery with 0.01 mmol/L H₂O₂. Edaravone treatment (10 mg/kg i.p.) for 3 days suppressed endothelial apoptosis, as evaluated by chromatin staining of *en face* specimens at 24 h, by approximately 40%. Similarly, edaravone dose-dependently inhibited H₂O₂-induced apoptosis of cultured endothelial cells in parallel with the inhibition of 8-isoprostane formation, 4-hydroxy-2-nonenal (4-HNE) accumulation and VCAM-1 expression. Next, apolipoprotein-E knockout mice were fed a high-cholesterol diet for 4 weeks with edaravone (10 mg/kg i.p.) or vehicle treatment. Edaravone treatment decreased atherosclerotic lesions in the aortic sinus (0.18 ± 0.01 to 0.09 ± 0.01 mm², *P* < 0.001) and descending aorta (5.09 ± 0.86 to 1.75 ± 0.41 mm², *P* < 0.05), as evaluated by oil red O staining without influence on plasma lipid concentrations or blood pressure. Dihydroethidium labeling and cytochrome *c* reduction assay showed that superoxide anions in the aorta were suppressed by edaravone. Also, plasma 8-isoprostane concentrations and aortic nitrotyrosine, 4-HNE and VCAM-1 contents were decreased by edaravone treatment.

Conclusions: These results suggest that edaravone may be a useful therapeutic tool for early atherosclerosis, pending the clinical efficacy.

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Keywords: Atherosclerosis; Reactive oxygen species; Free radical scavenger; Edaravone; 4-HNE; Apolipoprotein E knockout mouse

1. Introduction

Accumulating evidence has shown that stress-induced injury of vascular endothelial cells (ECs) is an initial event in the development of atherosclerosis [1]. In particular, oxidative stress has been implicated in endothelial injury caused by oxidized LDL and smoking as well as hypertension, diabetes and ischemia-reperfusion [1–3]. This notion is supported by the findings that the production of reactive oxygen species (ROS) is upregulated in vascular lesions [4,5], and that lesion formations such as endothelial dysfunction [6]

and atherosclerosis [7] are accelerated by superoxide anion (O₂^{•-}).

Experimental studies have shown the protective effects of antioxidants on atherosclerosis and endothelial injury. Dietary antioxidants were reported to preserve endothelial function [8,9] and inhibit atherosclerosis [10] in cholesterol-fed rabbits. In a well employed animal model of atherosclerosis, apolipoprotein E knockout (ApoE-KO) mouse fed a high fat diet, it has been shown that there was a significant increase in basal superoxide products [11,12], and that both O₂^{•-} levels and aortic lesion areas were attenuated by treatment with Vitamin E [11] or superoxide dismutase [13]. By contrast, it has been reported that elimination of NAD(P)H oxidase [14] or disruption of its subunit p47phox [15] had no effect on lesion size in ApoE-KO mice. Clinical experiments have

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also shown that antioxidants such as Vitamins C and E can ameliorate endothelial dysfunction in patients with hypercholesterolemia or atherosclerosis [16,17], although recent clinical trials have failed to prove the protective effects of Vitamin E on cardiovascular events in patients with risk factors [18] and in healthy subjects [19].

Edaravone is a potent free radical scavenger that has been clinically used to reduce the neuronal damage following ischemic stroke [20]. Edaravone has promising property to quench hydroxyl radical ($\cdot\text{OH}$) and show inhibitory effects on peroxynitrite (ONOO^-) and both water-soluble and lipid-soluble peroxy radical ($\text{LOO}\cdot$) [21,22]. Accordingly, this compound exerts a wide range of antioxidant activity on ROS beyond the effects of water-soluble or lipid-soluble antioxidant vitamins. Based on this idea, we hypothesized that edaravone would inhibit the process of atherosclerosis.

To test this hypothesis, we investigated the effects of edaravone in two experimental models. First, we examined whether edaravone could inhibit hydrogen peroxide (H_2O_2)-induced EC apoptosis in a rat model [23] and cultured ECs. Second, we examined whether edaravone could suppress the atherosclerotic lesion formation in ApoE-KO mice.

2. Methods

2.1. Animals

Male Wistar rats aged 10–12 weeks (Japan Clea), and male C57BL/6 mice and ApoE-KO mice on C57BL/6 background aged 4–6 weeks (Jackson Laboratory) were used in this study. All of the experimental protocols were approved by the Animal Research Committee of the Kyorin University School of Medicine.

2.2. H_2O_2 -induced EC apoptosis in rats and in culture

EC apoptosis was induced by 5-min intra-arterial treatment of a rat carotid artery with 0.01 mmol/L H_2O_2 as previously described [23]. Briefly, edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one; 3 or 10 mg/kg; donated by Mitsubishi Pharma Corporation, Japan) or its vehicle was intraperitoneally injected daily for 3 days before H_2O_2 treatment. A catheter was placed in the common carotid artery via the external carotid artery. The lumen was flushed with saline, replaced with 0.01 mmol/L H_2O_2 diluted with saline for 5 min and recovered. At 24 h after H_2O_2 treatment, EC apoptosis was evaluated by chromatin staining of *en face* specimens of the carotid artery using Hoechst 33342 dye. Apoptotic cells were identified by their typical morphological appearance; chromatin condensation, nuclear fragmentation, or apoptotic bodies. The numbers of apoptotic cells and intact cells were counted in 10 high-power fields for each specimen by an observer blinded to the treatment group.

Apoptosis of ECs isolated from a bovine carotid artery was induced as previously described [24]. Briefly, subconfluent ECs were pretreated for 24 h with culture medium containing edaravone or vehicle. After washing twice with Hank's balanced salt solution, the cells were exposed to H_2O_2 (0.2 mmol/L) diluted in Hank's balanced salt solution for 1.5 h at 37 °C to induce apoptosis. Then ECs were cultured in culture medium containing edaravone or vehicle until assay. Apoptosis was evaluated at 24 h after H_2O_2 treatment as histone-associated DNA fragments using a photometric enzyme immunoassay (Cell Death Detection ELISA, Roche), according to the manufacturer's instructions.

2.3. Atherosclerosis in ApoE-KO mice

ApoE-KO mice received a high-cholesterol diet (1% cholesterol, 10% fat in CE-2 standard diet; Japan Clea) for 4 weeks. Simultaneously, edaravone (10 mg/kg) or its vehicle was intra-peritoneally injected daily throughout the experiments. Body weight and systolic blood pressure were recorded every week in a conscious state by the tail cuff method (BP-98A; Softron, Tokyo).

At 4 weeks of treatment, mice were sacrificed with an overdose of diethyl ether and perfusion-fixed. Atherosclerotic lesions in the aortic sinus were quantified according to the method described previously [25]. We also measured the surface area of atherosclerotic lesions in the whole descending aorta including the abdominal aorta just proximal to the iliac bifurcation. *En face* specimens of the descending aorta were stained with oil red O, photographed and analyzed using the NIH image software. Total cholesterol, high-density lipoprotein cholesterol and low-density lipoprotein cholesterol in mice plasma were determined by a commercial laboratory (SRL, Japan).

2.4. Measurement of ROS

Aortic samples for ROS measurements were prepared separately from those for atherosclerosis evaluation. At 4 weeks of treatment, ApoE-KO mice were sacrificed with CO_2 inhalation. Descending aortas were rapidly removed and placed into chilled modified Krebs/HEPES buffer. C57BL/6 mice fed a standard diet were also used as the control. To determine superoxide production *in situ*, frozen cross-sections of the aorta were stained with 10 $\mu\text{mol/L}$ dihydroethidium (DHE; Molecular Probes), followed by fluorescent microscopy [26]. Also, superoxide production in aortic rings was quantified using the superoxide dismutase-inhibitable cytochrome *c* reduction assay as previously described [27]. Immunohistochemical detection of 3-nitrotyrosine in the aorta was visualized by diaminobenzidine as reported previously [28].

Intracellular production of superoxide anions was measured using DHE as described previously [29], and the intensity values were calculated using the Metamorph software [24]. Concentrations of 8-isoprostane (8-iso prostaglandin

F_{2a}) in the culture supernatants and mouse plasma were measured using a commercially available EIA kit (Cayman Chemical). Culture supernatants were directly applied to ELA, while plasma was applied to ELA after solid phase extraction purification according to the manufacturer's instructions.

2.5. Western blotting

Western blotting was performed as previously described [30], to detect the expression of VCAM-1 and 4-HNE in cultured ECs and mouse aortas. Descending aortas were prepared as described in ROS measurements. The antibodies used in this study were anti-4-HNE monoclonal antibody (JaICA, Shizuoka, Japan), anti-VCAM-1 polyclonal antibody (Santa Cruz Biotechnology) and anti-3-nitrotyrosine monoclonal antibody (Upstate). Densitometric analysis was performed using an image scanner and the NIH software.

2.6. Data analysis

All values are expressed as mean \pm S.E.M. Data were analyzed using one-factor ANOVA. If a statistically significant effect was found, Newman–Keuls' test was performed to isolate the difference between the groups. Differences with a value of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of edaravone on H₂O₂-induced EC apoptosis and ROS

As shown in Fig. 1A, edaravone dose-dependently inhibited EC apoptosis in culture, which was induced 24 h after H₂O₂ treatment. Edaravone was then employed in a rat model of H₂O₂-induced EC apoptosis. Consistent with the *in vitro* experiment, edaravone of 10 mg/kg/day decreased EC apoptosis of the rat carotid artery by approximately 40% (Fig. 1B).

We next examined whether edaravone decreased ROS production in the process of H₂O₂-induced EC apoptosis. For this purpose, DHE fluorescent, a marker of intracellular production of superoxide anions, release of 8-isoprostane into the culture supernatants and accumulation of 4-HNE, a pivotal end-product of lipid peroxidation [31], were measured using cultured ECs. We also examined the expression of VCAM-1 as a marker of endothelial injury or activation [32]. Edaravone decreased DHE fluorescent, 8-isoprostane formation and VCAM-1 expression at 3 h after H₂O₂ treatment in a dose-dependent manner (Fig. 2A–C). As shown in Fig. 2D, multiple bands showing 4-HNE-Michael protein adducts [33,34] were accumulated after H₂O₂ treatment in a time-dependent manner. Consequently, the effect of edaravone on 4-HNE expression was examined at 3 h after H₂O₂ treatment (4.5 h after H₂O₂ was initially added). Edaravone decreased 4-HNE expression in a dose dependent manner.

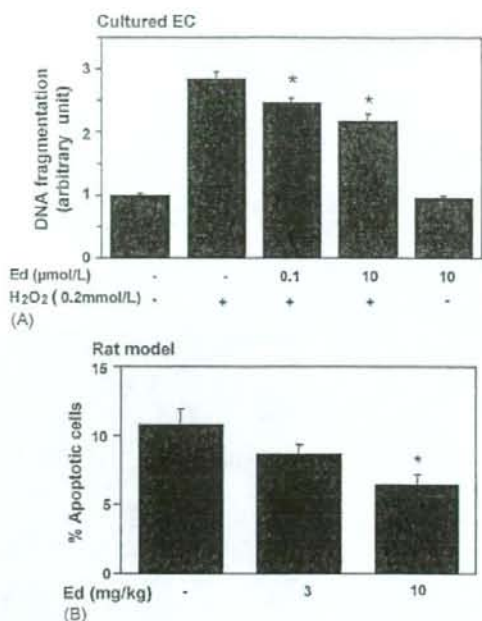


Fig. 1. Effects of edaravone (Ed) on H₂O₂-induced EC apoptosis in culture (A) and in a rat model (B). (A) Ed or its vehicle was added to the culture medium 24 h before H₂O₂ treatment until assay. EC apoptosis was evaluated 24 h after H₂O₂ treatment (0.2 mmol/L) by means of DNA fragmentation. Values are expressed as mean \pm S.E.M. ($n = 3$). * $P < 0.05$ vs. H₂O₂ (+) + Ed (-). (B) Ed or its vehicle was intraperitoneally injected once a day for 3 days before H₂O₂ treatment. At 24 h after H₂O₂ treatment, apoptotic ECs were counted per high power field and the ratio of the apoptotic cell number to the intact cells was calculated using *en face* specimens of the carotid artery stained with Hoechst 33342. Values are expressed as mean \pm S.E.M. ($n = 7$). * $P < 0.05$ vs. vehicle.

3.2. Effects of edaravone on atherosclerotic lesions and ROS in ApoE-KO mice

In the next set of experiments, we examined whether edaravone could suppress the atherosclerotic lesions in ApoE-KO mice fed a high cholesterol diet for 4 weeks. As shown in Fig. 3A and B, atheromatous lesions both in the aortic sinus and the descending aorta were smaller in mice treated with 10 mg/kg/day edaravone than in those with vehicle. This dose of edaravone did not influence body weight, blood pressure or plasma LDL and HDL cholesterol levels (Table 1).

Then, we examined whether the anti-atherogenic effects of edaravone were associated with the decrease in ROS production. Peroxynitrite formation was assessed as 3-nitrotyrosine accumulation in the aorta [28]. Both immunohistochemistry and Western blotting showed that edaravone inhibited nitrotyrosine accumulation in the aorta of ApoE-KO mice (Fig. 4A(a) and A(b)). Superoxide production *in situ* was examined using DHE staining of the descend-

Table 1

Body weight, blood pressure and plasma lipid levels in ApoE-KO mice treated with edaravone or vehicle

	Vehicle	Edaravone
Body weight (g)	21.4 ± 0.5	21.0 ± 0.5
Systolic blood pressure (mmHg)	106 ± 2	103 ± 3
Total cholesterol (mg/dL)	1967 ± 38	1872 ± 66
HDL cholesterol (mg/dL)	66 ± 6	82 ± 9
LDL cholesterol (mg/dL)	602 ± 24	602 ± 12

The values are shown as mean ± S.E. ($n = 14$). There were no significant differences in the values between the two groups.

ing aorta. As shown in Fig. 4B, ethidium fluorescence, which was amplified in ApoE-KO mice, was decreased by edaravone treatment. A quantitative analysis by the superoxide dismutase-inhibitable cytochrome *c* reduction assay revealed that $O_2^{\bullet-}$ levels in aortic rings of ApoE-KO mice were decreased by 43% in edaravone-treated ApoE-KO mice compared to those in vehicle-treated mice (Fig. 4C). Consistent with these results, plasma 8-isoprostane levels and 4-HNE expression in the descending aorta, both of which were elevated in ApoE-KO mice compared to

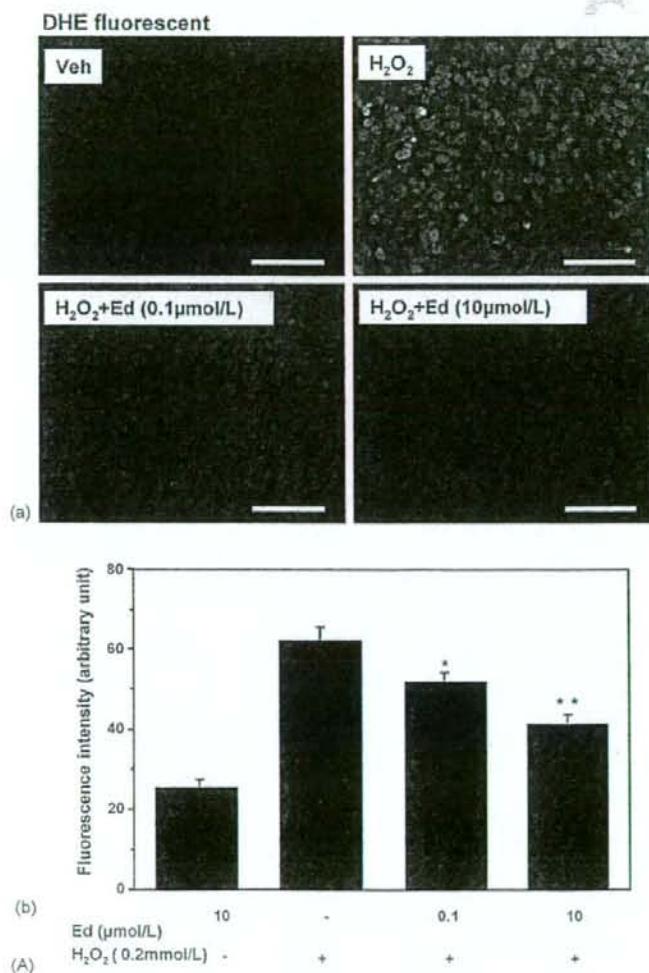


Fig. 2. Effects of edaravone (Ed) on DHE fluorescent (A) and 8-isoprostane formation (B), VCAM-1 expression (C) and 4-HNE expression (D) in cultured EC. Ed or its vehicle was added to the culture medium 24 h before H_2O_2 treatment until assay. DHE fluorescent ($n = 6$), 8-isoprostane concentration ($n = 3$) and VCAM-1 expression ($n = 3$) in the cell lysate were measured 3 h after H_2O_2 treatment. Values are expressed as mean ± S.E.M. Time dependent changes of 4-HNE expression after H_2O_2 treatment was detected by Western blotting. Representative image showed that 4-HNE-Michael protein adducts were accumulated after treatment (D(a)). The major 97 kDa band was measured 4.5 h after H_2O_2 treatment in the presence or absence of edaravone (D(b)). Values are expressed as mean ± S.E.M. ($n = 3$). * $P < 0.05$, ** $P < 0.01$ vs. H_2O_2 (+) + Ed (-).

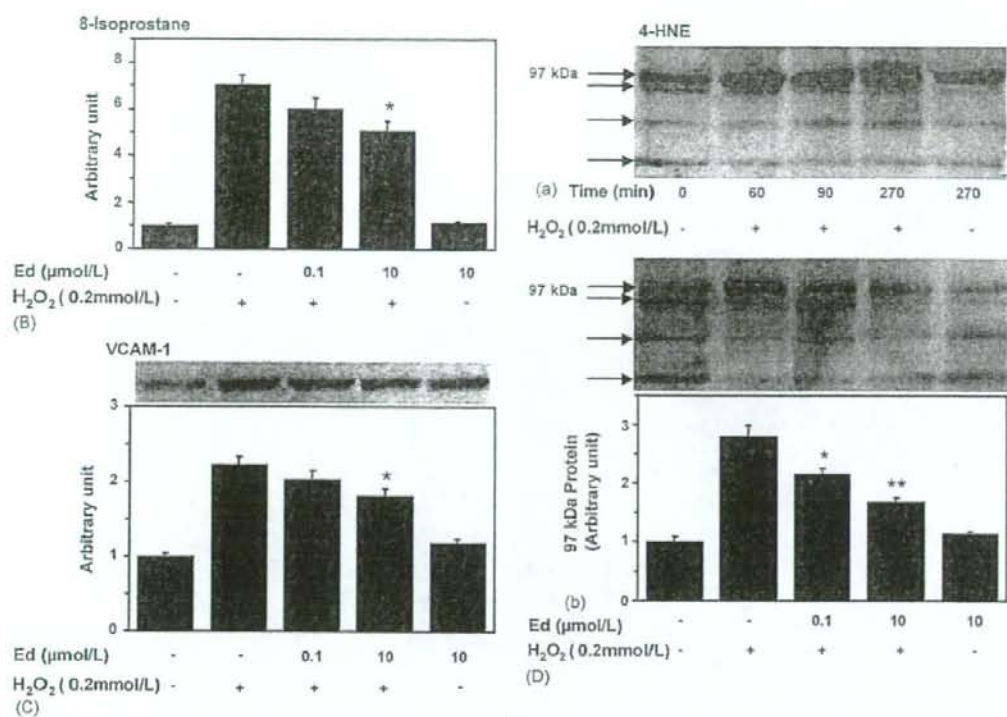


Fig. 2. (Continued).

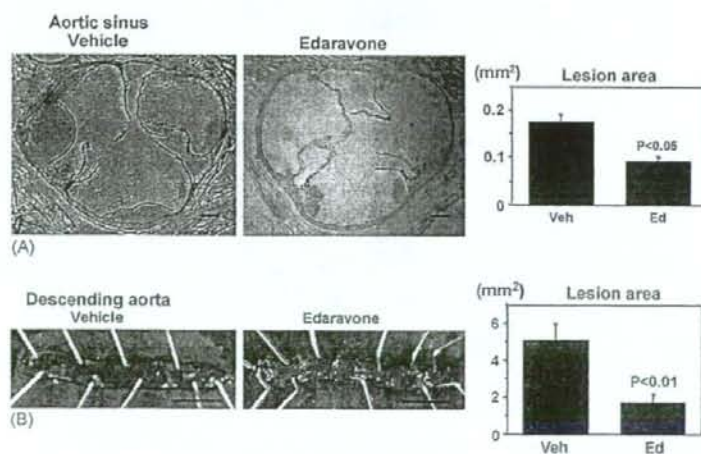


Fig. 3. Effects of edaravone on atherosclerotic lesion in ApoE-KO mice. ApoE-KO mice were fed a high-cholesterol diet for 4 weeks with the administration of edaravone (10 mg/kg daily) or its vehicle by i.p. injection. (A) Oil red O-stained cross-sections of the aortic sinus (bar = 100 μm) and morphometric analysis of the lesions are shown. (B) Oil red O-stained *en face* specimens of the descending aorta (bar = 5 mm) and morphometric analysis of the lesions are shown. Values are expressed as mean \pm S.E.M. ($n = 14$).

those in wild-type C57BL/6 mice fed a normal chow, were decreased by edaravone treatment (Fig. 4D and E). Finally, the increase in VCAM-1 expression in the aorta of ApoE-KO mice was attenuated by edaravone as well (Fig. 4F).

4. Discussion

A number of studies have shown that ROS contribute to the pathogenesis of endothelial dysfunction and atherosclerosis formation. In addition to $O_2^{\bullet-}$ that is predominantly pro-

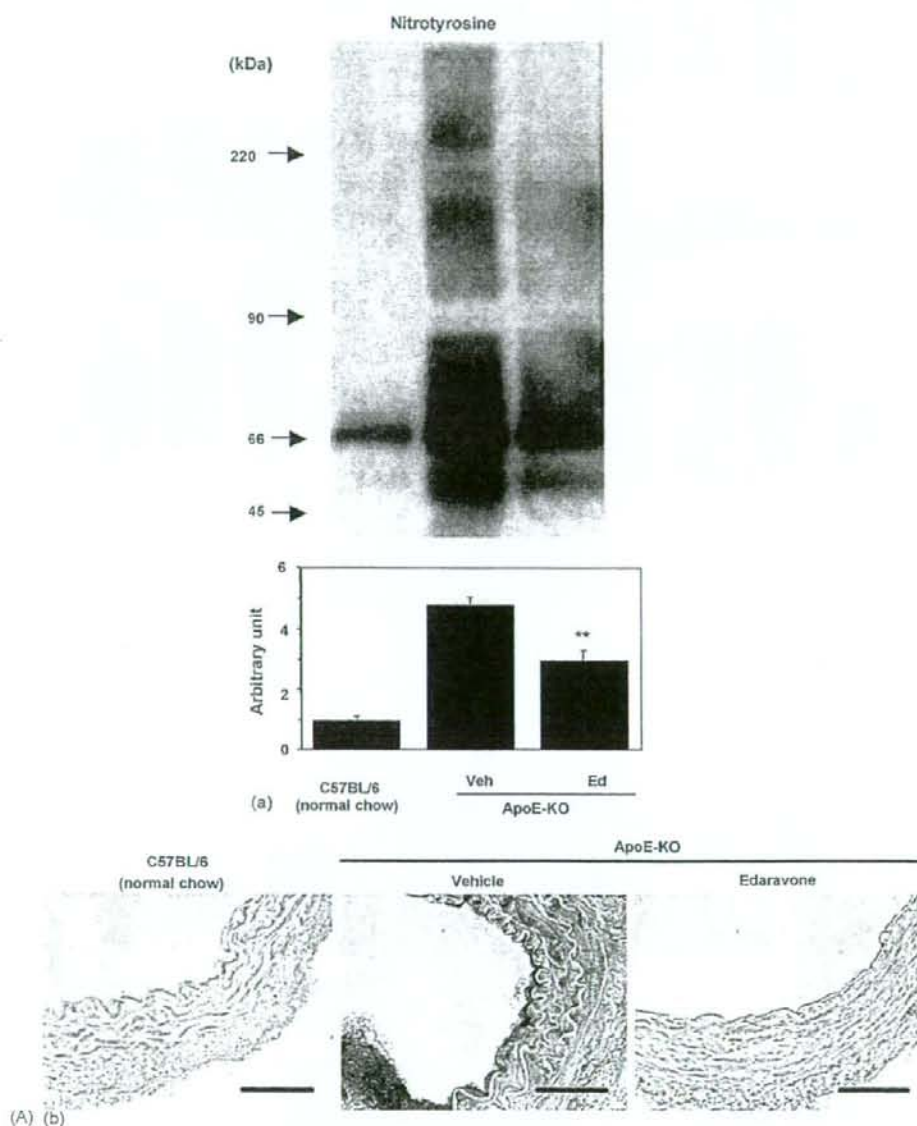


Fig. 4. Effects of edaravone (Ed) on ROS production (A–E) and VCAM-1 expression (F) in ApoE-KO mice. (A) Nitrotyrosine contents in the aorta was examined by Western blot analysis (A(a), $n=6$) and immunohistochemistry (A(b)). Bar = 50 μm. (B) Fresh-frozen cross-sections of the aorta were stained with DHE, and representative fluorescent micrographs are shown (bar = 100 μm). (C) Superoxide anion in aortic rings was determined using SOD-inhibitable-cytochrome *c* reduction assay ($n=6$). (D) 8-Isoprostane level in mouse plasma was measured with EIA ($n=6$). (E and F) Representative Western blotting for 4-HNE (97 kDa band) and VCAM-1 expression in the aorta and densitometric analysis are shown ($n=3$). Values are expressed as mean ± S.E.M. * $P < 0.05$, ** $P < 0.01$ vs. vehicle (Veh). C57/BL6 mice fed a normal chow serve as the control.

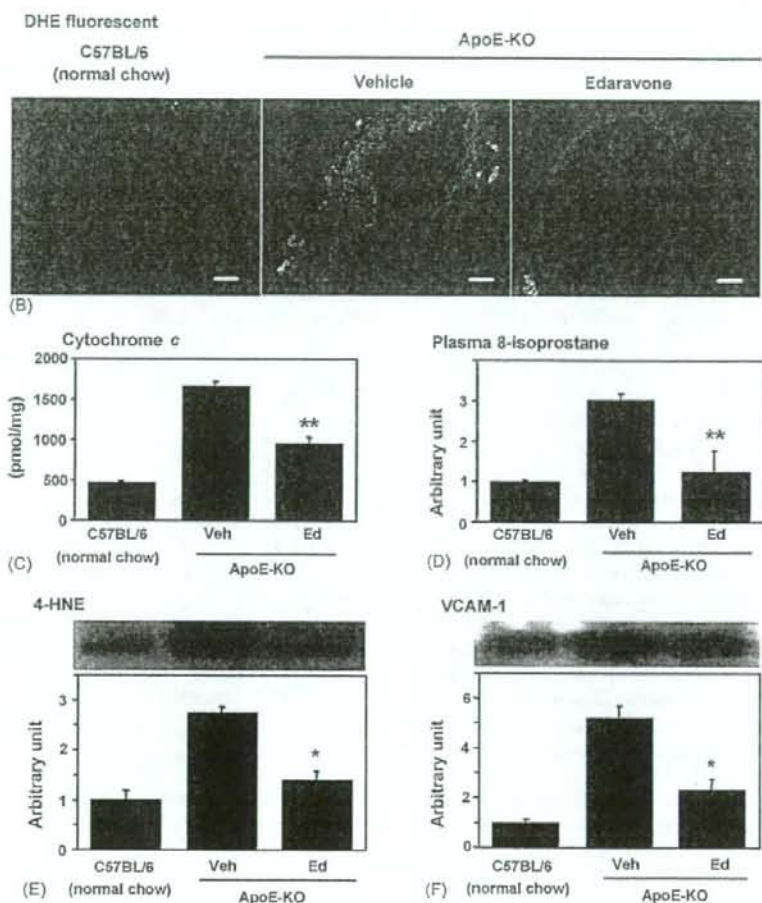


Fig. 4. (Continued).

duced via NAD(P)H oxidase [35], $\bullet\text{OH}$ as well as $\text{LOO}\bullet$ [36] and ONOO^- [37] play a role in atherogenesis. In particular, $\bullet\text{OH}$ is extremely strong in terms of oxidative activity and cellular damage [38]. Therefore, it might be essential to scavenge the wide range of ROS for the prevention of atherosclerosis. As a matter of fact, recent clinical trials have denied the protective effects of Vitamin E, which predominantly reacts with $\text{LOO}\bullet$ [39], on cardiovascular events [18,19].

Edaravone, a potent free radical scavenger with unique properties, works by donating an electron from edaravone anion to free radicals [22]. Edaravone quenches $\bullet\text{OH}$ and inhibits both $\bullet\text{OH}$ -dependent and $\bullet\text{OH}$ -independent lipid peroxidation [22]. Edaravone shows inhibitory effects on both water-soluble and lipid-soluble LOO -induced peroxidation systems [22]. Edaravone also inhibits ONOO^- -induced tyrosine nitration [22]. These properties are different from those of water-soluble Vitamin C and lipid-soluble Vitamin E.

In the present study, we demonstrated that edaravone suppressed endothelial apoptosis and fatty streak formation. Reduced expression of VCAM-1, a marker of vascular injury and activation [32], were corroborated with these results. In cultured ECs, protein expression of VCAM-1 was induced as early as 3 h after H_2O_2 treatment (actually 4.5 h after addition of H_2O_2 , Fig. 2C). This is reasonable based on our time course experiments (data not shown), and is consistent with the previous reports that VCAM-1 protein has been induced 4–6 h after cytokine stimulation through an antioxidant-sensitive mechanism [40,41]. Although the experimental conditions were different between the cell culture and animal studies, edaravone inhibited both the rapid induction of VCAM-1 in cultured ECs and the chronic upregulation of VCAM-1 in the aorta of ApoE-KO mice, further supporting the vasoprotective effects of edaravone.

Edaravone has been clinically used as a neuroprotectant in the treatment of ischemic stroke in Japan from 2001. The dose of edaravone used in this study (intraperitoneal injection of 10 mg/kg) has been reported to be comparable to that of intravenous injection in clinical use in terms of plasma concentration [42]. This compound has been reported to preserve endothelial function in ischemic brain [43] and ameliorate ischemia-reperfusion injury in various organs such as kidney [44] and heart [45]. Also, edaravone has been shown to inhibit pressure overload-induced cardiac hypertrophy [42]. To our knowledge, however, the effect of edaravone on atherosclerosis has never been reported till now.

The effects of edaravone on endothelial injury and atherosclerosis were associated with the decrease in ROS production including peroxynitrite, superoxide anion and 8-isoprostane, suggesting the mechanistic role of antioxidation in vascular protection. Edaravone also inhibited the expression of 4-HNE in vascular tissues, further indicating the antioxidant activity and suggesting the signaling cascade leading to endothelial injury, because 4-HNE triggers cellular damages through the MAP kinase pathway as an end-product of ROS [34]. Antioxidant effects of edaravone on lipoproteins were not determined in the present study because of the methodological limitation in mice. It has been reported, however, that edaravone can inhibit oxidative modification of low-density lipoprotein *in vitro* and in rats [46]. Consequently, it is likely that reduced lipoprotein oxidation would have played a role in the anti-atherosclerotic effects of edaravone in ApoE-KO mice. Furthermore, edaravone has been reported to stimulate the expression of endothelial nitric oxide synthase in cultured ECs [46] and the artery [47], leading to the increased production of nitric oxide. Taken together with the effects on peroxynitrite formation, edaravone might synergistically increase the availability of nitric oxide, which exerts vasoprotective and anti-atherosclerotic action.

The effects of edaravone on advanced and complicated lesions of atherosclerosis were not investigated in this study. Neither, the effects on plaque ruptures nor consequent cardiovascular events are known. This study demonstrated that edaravone might be a potential new therapeutic agent for the prevention and treatment of early atherosclerosis. For the purpose of chronic use, however, the innovation of drug preparation for oral administration is necessary. Another application of edaravone might be the prevention of restenosis after percutaneous coronary interventions, since ROS plays an important role in neointimal formation after angioplasty [48]. Intravenous injection of edaravone for several days might inhibit neointimal formation in addition to ischemia reperfusion injury of cardiomyocytes [45]. Taken together, edaravone is expected to show protective effect on ROS-related vascular diseases beyond cerebral infarction.

In summary, edaravone, a free radical scavenger with unique properties, attenuated oxidative stress-induced endothelial damage in rats and early atherosclerosis in ApoE-KO mice in association with the inhibition of ROS formation.

These findings provide new information on the role of ROS in atherogenesis and the therapeutic strategy for atherosclerosis.

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Statins Protect Human Aortic Smooth Muscle Cells From Inorganic Phosphate-Induced Calcification by Restoring Gas6-Axl Survival Pathway

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Abstract—Vascular calcification is clinically important in the development of cardiovascular disease. It is reported that hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors (statins) inhibited vascular calcification in several clinical trials. However, the mechanism is poorly understood. Recently, it has been suggested that apoptosis is one of the important processes regulating vascular smooth muscle cell (VSMC) calcification. In this study, we investigated the effect of statins on VSMC calcification by testing their effect on apoptosis, focusing in particular on regulation of the survival pathway mediated by growth arrest-specific gene 6 (Gas6), a member of the vitamin K-dependent protein family, and its receptor, Axl. In human aortic smooth muscle cells (HASMC), statins significantly inhibited inorganic phosphate (Pi)-induced calcification in a concentration-dependent manner (reduced by 49% at 0.1 $\mu\text{mol/L}$ atorvastatin). The inhibitory effect of statins was mediated by preventing apoptosis, which was increased by Pi in a concentration-dependent manner, and not by inhibiting sodium-dependent phosphate cotransporter (NPC) activity, another mechanism regulating HASMC calcification. Furthermore, the antiapoptotic effect of statins was dependent on restoration of Gas6, whose expression was downregulated by Pi. Restoration of Gas6 mRNA by statins was mediated by mRNA stabilization, and not by an increase in transcriptional activity. Suppression of Gas6 using small interfering RNA and the Axl-extracellular domain abolished the preventive effect of statins on Pi-induced apoptosis and calcification. These data demonstrate that statins protected HASMC from Pi-induced calcification by inhibiting apoptosis via restoration of the Gas6-Axl pathway. (*Circ Res.* 2006;98:1024-1031.)

Key Words: calcification ■ statins ■ apoptosis ■ Gas6 ■ Axl

Vascular calcification, such as coronary and aortic calcification, is a significant feature of vascular pathology, because this lesion is associated with cardiovascular disease.^{1,2} It has been recognized that statins exhibit various protective effects against atherosclerosis, including modification of endothelial function,³ decreased inflammation,⁴ and inhibition of vascular smooth muscle cell (VSMC) proliferation and migration,⁵ all of which cannot be accounted for by lipid reduction. One of the interesting pleiotropic effects of statins is the inhibition of vascular calcification. Results from clinical trials suggest an association of statin use with slowed progression of calcific aortic stenosis⁶⁻⁸ and coronary artery calcification.⁹ Statins also inhibited calcification of atherosclerotic plaques in experimental hyperlipidemic animals.^{10,11} On the other hand, some recent clinical trials were not able to find such an inhibitory effect.^{12,13} To clarify these discrepancies, it is important to identify the detailed regulatory mechanism of vascular calcification and the target of effect of statins.

Based on clinical findings,¹⁴ inorganic phosphate (Pi) has been shown to be an important inducer of VSMC calcification, which is morphologically similar to that observed in calcified human heart valves and the aortic media. Transport of Pi into VSMC has been suggested to play an important role in the initiation of extracellular matrix calcification.¹⁵ Recently, it has been shown that similar structures to matrix vesicles, derived from apoptotic VSMC, have been identified in human calcified arteries.¹⁶ These vesicles have the capacity to concentrate and crystallize Ca, initiating calcification. Pi has been shown to induce apoptosis of hypertrophic chondrocytes, which is associated with cell maturation and extracellular matrix mineralization.¹⁷ However, it is not clear whether or not apoptosis plays a regulatory role in the occurrence of VSMC calcification induced by Pi.

Recently, it was shown that growth arrest-specific gene 6 (Gas6), a member of the vitamin K-dependent protein family, and its receptor, Axl, a membrane receptor tyrosine kinase, are decreased on calcification of vascular pericytes.¹⁸

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Gas6 is a secreted protein that harbors a γ -carboxylglutamic acid-rich domain and 4 epidermal growth factor-like repeats.¹⁹ Gas6-Axl interaction has been shown to be implicated in the regulation of multiple cellular functions, including growth, survival, adhesion, and chemotaxis.²⁰⁻²³ In particular, they are known to protect a range of cell types from apoptotic death. However, there is no evidence that Gas6-Axl interaction is involved in Pi-induced apoptosis and calcification of VSMC.

In the present study, we found that statins inhibited Pi-induced calcification by preventing apoptosis in human aortic smooth muscle cells (HASMC). The effect of statins was dependent on restoration of the Gas6-Axl pathway. Furthermore, this beneficial effect was mediated by Gas6 mRNA stabilization, and not by increasing the transcription rate. Our results reveal a novel pathway by which statins regulate Pi-induced calcification in HASMC.

Materials and Methods

Materials

Pravastatin, atorvastatin, and fluvastatin were supplied by Sankyo Co Ltd, Pfizer Inc (New York), and Tanabe Seiyaku Co Ltd, respectively. Recombinant human Gas6 (rhGas6) and Axl-ECD were prepared as described previously.^{22,24} All other reagents were of analytical grade.

Cell Culture

HASMC were obtained from Clonetics. They were cultured in DMEM supplemented with 20% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO₂. HASMC were used up to passage 8 for the experiments.

Induction and Quantification of Calcification

For Pi-induced calcification, Pi (a mixed solution of Na₂HPO₄ and NaH₂PO₄ whose pH was adjusted to 7.4) was added to serum-

supplemented DMEM to final concentrations of 2.0, 2.6, and 3.2 mmol/L ("calcification medium"). After the indicated incubation period, cells were decalcified with 0.6 mol/L HCl, and Ca content in the supernatant was determined by the *o*-cresolphthalein complexone method (C-Test, WAKO). The remaining cells were solubilized in 0.1 mol/L NaOH/0.1% SDS, and cell protein content was measured by Bio-Rad protein assay. Calcification was visualized by von Kossa's method. Briefly, the cells were fixed with 4% formaldehyde and exposed to 5% aqueous AgNO₃.

Induction of Apoptosis

Two different time courses were tested to investigate Pi-induced apoptosis and examine the effect of statins. (1) Short-term condition: Pi was added at final concentrations of 2.0, 2.6, and 3.2 mmol/L for 24 hours at confluence, after the cells were incubated with serum-free DMEM for 48 hours. To test the effect of statins on apoptosis, they were added 24 hours after incubating the cells with serum-free DMEM (12 hours before adding Pi). (2) Long-term condition: at confluence, the medium was switched to calcification medium and cells were cultured for up to 10 days. The medium was changed every 2 days. To test the effect of statins, each was added simultaneously when the medium was switched to the calcification medium.

RNA Extraction, Northern Blot, and mRNA Stability Analysis

The 304-bp product of the Gas6 cDNA probe (forward, 5'-GCGTGGCCCAAGAGTGTGAAGT-3'; reverse, 5'-CGCCACTCC-TCAACAGAGAT-3') was amplified by RT-PCR. For Northern blot analysis, harvested RNA (~5 to 10 μ g) was fractionated on 1.4% formaldehyde-agarose gel and transferred to a nylon filter. The filter was hybridized at 68°C for 2 hours with ³²P-labeled Gas6 cDNA and 18S probe in QuickHyb solution (Stratagene) and autoradiographed. To examine Gas6 mRNA stability, serum-starved HASMC were incubated with actinomycin D (Act D, 5 μ g/mL) in the presence of 2.6 mmol/L Pi after 12 hours of atorvastatin (0.1 μ mol/L) treatment. Total RNA was harvested at 0, 1, 3, and 6 hours for Northern blot analysis. Signal density of the Gas6 mRNA was normalized to that

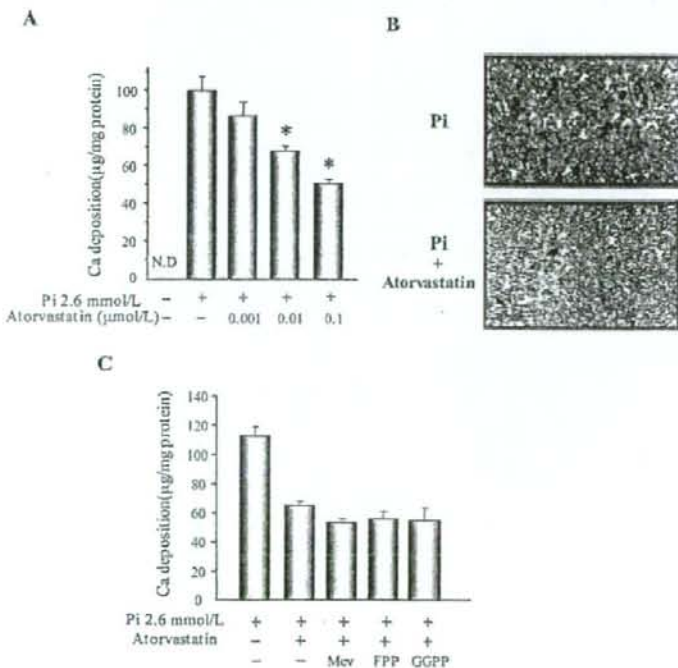


Figure 1. Statins prevent HASMC calcification. A, HASMC were cultured with the indicated concentrations of atorvastatin in the presence of 2.6 mmol/L Pi for 6 days. Ca deposition was measured by *o*-cresolphthalein complexone method and normalized by cell protein content. All values are presented as mean \pm SEM (n=6). **P*<0.05 vs statin (-) by Fisher's test. N.D. indicates not detected. B, On day 6, the inhibitory effect of atorvastatin (0.1 μ mol/L) on 2.6 mmol/L Pi-induced Ca deposition was evaluated at the light microscopic level with von Kossa's staining. The arrow points to an area of Ca deposition. C, HASMC were cultured with mevalonate (100 μ mol/L), farnesylpyrophosphate (1 μ mol/L), or geranylgeranylpyrophosphate (1 μ mol/L) in the presence of atorvastatin (0.1 μ mol/L) and 2.6 mmol/L Pi for 6 days. All values are presented as mean \pm SEM (n=6).