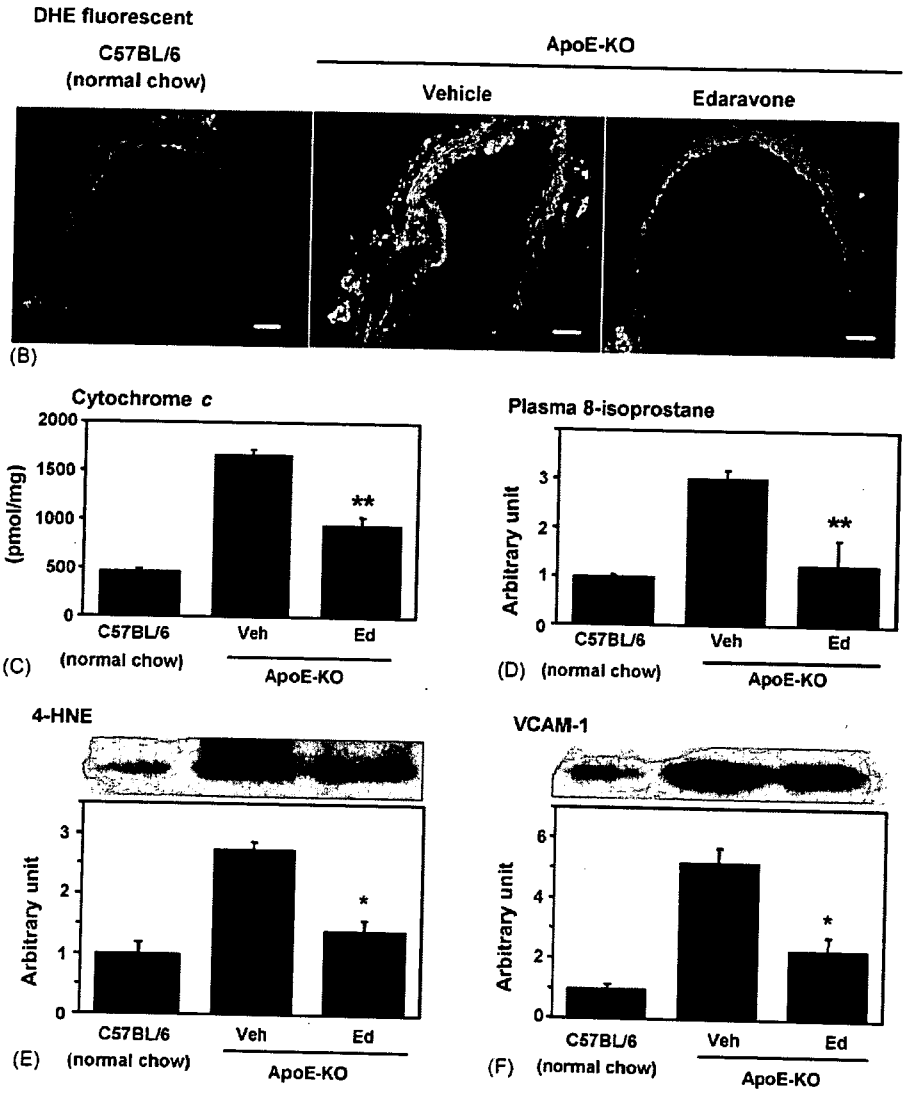

Fig. 4. (*Continued*).

duced via NAD(P)H oxidase [35], •OH as well as LOO• [36] and $ONOO^-$ [37] play a role in atherogenesis. In particular, •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 LOO• [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 •OH and inhibits both •OH-dependent and •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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Gas6/Axl-PI3K/Akt pathway plays a central role in the effect of statins on inorganic phosphate-induced calcification of vascular smooth muscle cells

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Abstract

Apoptosis is essential for the initiation and progression of vascular calcification. Recently, we showed that *3-hydroxy-3-methylglutaryl* (HMG) CoA reductase inhibitors (statins) have a protective effect against vascular smooth muscle cell calcification by inhibiting apoptosis, where growth arrest-specific gene 6 (Gas6) plays a pivotal role. In the present study, we clarified the downstream targets of Gas6-mediated survival signaling in inorganic phosphate (Pi)-induced apoptosis and examined the effect of statins. We found that fluvastatin and pravastatin significantly inhibited Pi-induced apoptosis and calcification in a concentration-dependent manner in human aortic smooth muscle cells (HASMC), as was found with atorvastatin previously. Gas6 and its receptor, Axl, expression were downregulated in the presence of Pi, and recombinant human Gas6 (rhGas6) significantly inhibited apoptosis and calcification in a concentration-dependent manner. During apoptosis, Pi suppressed Akt phosphorylation, which was reversed by rhGas6. Wortmannin, a specific *phosphatidylinositol 3-OH kinase* (PI3K) inhibitor, abolished the increase in Akt phosphorylation by rhGas6 and eliminated the inhibitory effect of rhGas6 on both Pi-induced apoptosis and calcification, suggesting that PI3K-Akt is a downstream signal of the Gas6-mediated survival pathway. Pi reduced phosphorylation of Bcl2 and Bad, and activated caspase 3, all of which were reversed by rhGas6. The inhibitory effect of statins on Pi-induced apoptosis was accompanied by restoration of the Gas6-mediated survival signal pathway: upregulation of Gas6 and Axl expression, increased phosphorylation of Akt and Bcl2, and inhibition of Bad and caspase 3 activation. These findings indicate that the Gas6-mediated survival pathway is the target of statins' effect to prevent vascular calcification.

Keywords: Calcification; Apoptosis; Gas6; Axl; Akt; Bcl2

1. Introduction

Vascular calcification, such as coronary and aortic calcification, is clinically important in the development of cardiovascular disease (Eggen, 1968). Two distinct forms of vascular calcification are well recognized. One is medial calcification, which occurs between the cell layers of smooth muscle cells and is related to aging, diabetes and chronic renal failure (Neubauer, 1971; Goodman et al., 2000). The other is atherosclerotic calcification, which occurs in the intima during the development of atheromatous disease (Wexler et al., 1996). In diabetic patients, medial calcification has been shown to be a strong independent predictor of cardiovascular mortality (Everhart et al., 1988).

We recently demonstrated that atorvastatin prevented inorganic phosphate (Pi)-induced calcification by inhibiting apoptosis, one of the important processes regulating calcification. This was mediated by growth arrest-specific gene 6 (Gas6), a vitamin K-dependent protein (Son et al., 2006). Gas6 binds to Axl, the predominant receptor for Gas6, on the cell surface and transduces the signal by Axl autophosphorylation (Mark et al., 1996). Gas6-Axl interaction has been shown to be implicated in the regulation of multiple cellular functions (Yanagita et al., 2001; Goruppi et al., 1996; Nakano et al., 1997; Fridell et al., 1998). Especially, they are known to protect a range of cell types

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from apoptotic death (Goruppi et al., 1996, 1999; Healy et al., 2001). However, the downstream targets of Gas6-mediated signaling in Pi-induced apoptosis and the effect of statins on this pathway are poorly understood.

With respect to the targets of Gas6-Axl interaction, Lee et al. (2002) showed that activation of Akt is necessary for Gas6-dependent cell survival. Akt is an important mediator of metabolic and survival responses after growth factor stimulation. Akt is activated by phosphorylation, which is performed by phosphatidylinositol 3-OH kinase (PI3K), a kinase that is activated by Gas6-Axl interaction (Lee et al., 2002; Ming Cao et al., 2001). Activation of Akt leads to downstream signaling events including those associated with mitochondrial regulators of apoptosis such as Bcl2 and Bad.

In the present study, we examined the effect of statins using two different types: lipophilic fluvastatin and hydrophilic pravastatin. We investigated the effect of statins on Pi-induced apoptosis and calcification as well as on signaling components in this process. Consequently, we found that both statins restored the Gas6-mediated survival pathway, with upregulation of the expression of Gas6 and Axl, increased phosphorylation of Akt, Bcl2 and Bad; and finally inhibition of caspase 3 activation, resulting in the prevention of apoptosis and subsequent calcification in human aortic smooth muscle cells (HASMC).

2. Materials and methods

2.1. Materials

Pravastatin and fluvastatin were supplied by Sankyo Co. Ltd. and Tanabe Seiyaku Co., Ltd., respectively. Recombinant human Gas6 (rhGas6) was prepared as described previously (Ming et al., 2001). Wortmannin was purchased from Calbiochem. All other reagents were of analytical grade.

2.2. Cell culture

HASMC were obtained from Clonetics. They were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% *fetal bovine serum* (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. HASMC were used up to passage 8 for the experiments.

2.3. Induction and quantification of calcification

For Pi-induced calcification, Pi (a mixed solution of Na_2HPO_4 and NaH_2PO_4 whose pH was adjusted to 7.4) was added to serum-supplemented DMEM to a final concentration of 2.6 mM. After the indicated incubation period, cells were decalcified with 0.6 M 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 M NaOH/0.1% *sodium dodecyl sulfate* (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 $AgNO_3$.

2.4. Induction and determination of apoptosis

Two different time courses were tested to investigate Pi-induced apoptosis and examine the effect of statins, under short-term (within 24 h) and long-term (up to 10 days) conditions (Son et al., 2006).

2.4.1. TdT-mediated dUTP nick end-labeling (TUNEL) assay

TUNEL assay to detect DNA fragmentation was performed using a commercially available kit (ApopTag Plus, Chemicon). Briefly, the samples were preincubated with equilibration buffer for 10 min, and subsequently incubated with terminal deoxyribonucleotidyl transferase in the presence of digoxigenin-conjugated dUTP for 1 h at 37 °C. The reaction was terminated by incubating the samples in stopping buffer for 30 min. After 3 rinses with *phosphate-buffered saline* (PBS), a fluorescein-labeled anti-digoxigenin antibody was applied for 30 min, and the samples were rinsed 4 times with PBS. The samples were then stained, mounted with DAPI (*4′,6-diamino-2-phenylindole*)/antifade, and examined by fluorescence microscopy.

2.4.2. Detection of DNA fragmentation by ELISA

Cytoplasmic histone-associated DNA fragments were determined with a cell-death detection ELISA^{plus} kit (Roche) as a quantitative index of apoptosis. Briefly, after the cells were incubated in lysis buffer for 30 min, 20 μl of the cell lysates was used for the assay. Following addition of substrate, colorimetric change was determined as the absorbance value measured at 405 nm.

2.5. Immunoblotting

The effect of Pi and statins on the expression of Gas6 and Axl, phosphorylation of Akt, Bcl2 and Bad, and activation of caspase 3 was examined at 12 h. The collected cell lysates were applied to SDS-polyacrylamide gels under reducing conditions, and transferred to a polyvinylidene difluoride (PVDF) membrane. Immunoblot analysis was performed using specific primary antibodies: anti-Axl, anti-Gas6 (Santa Cruz Biotechnology), anti-caspase 3, anti-Akt, anti-Bcl2, anti-phospho-Akt, anti-phospho-Bcl2, anti-phospho-Bad (Cell Signaling Technology), and anti-Bad (Transduction Laboratories). After incubation with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia), blots were visualized by enhanced chemiluminescence and autoradiography (ECL Plus, Amersham Pharmacia). Experiments were performed with at least three different cell populations.

2.6. Statistical analysis

All results are presented as mean±S.E.M. Statistical comparisons were made by ANOVA, unless otherwise stated. A value of $P<0.05$ was considered to be significant.

3. Results

3.1. Statins inhibit Pi-induced apoptosis and calcification in HASMC

In HASMC, a high Pi level (≥2.6 mM), comparable to that of hyperphosphatemia in end-stage renal disease, significantly induced calcification. Fluvastatin showed an inhibitory effect on Pi-induced calcification at as high a concentration as 0.1 μM (26.1±2.3% of control), while pravastatin showed the degree of effect at 50 μM (27.4±3.1% of control) (Fig. 1A). An inhibitory effect on Ca deposition was also found by von Kossa's staining (Fig. 1B). Both statins prevented Pi-induced apoptosis at the same concentrations as those at which they prevented calcification (Fig. 1C). An antiapoptotic effect of statins was also observed by TUNEL assay on day 6 (Fig. 1D).

3.2. Gas6 plays an important role in Pi-induced apoptosis

In the presence of 2.6 mM Pi, the expression of Gas6 and Axl was markedly downregulated (Fig. 2A). To investigate the role of Gas6 in Pi-induced apoptosis and calcification, first, we tested whether supplementation of rhGas6 could prevent Pi-induced apoptosis. In HASMC, rhGas6 significantly inhibited Pi-induced apoptosis in a concentration-dependent manner (Fig. 2B). Furthermore, during apoptosis, activated products of caspase 3 (17 and 19 kDa) were significantly increased by 2.6 mM Pi, which was reversed by rhGas6 (Fig. 2C). Next, we examined the effect of rhGas6 on calcification. Recombinant human Gas6 significantly inhibited Pi-induced calcification on day 6 in a concentration-dependent manner (Fig. 2D), suggesting that Gas6 plays an important role in Pi-induced apoptosis and calcification.

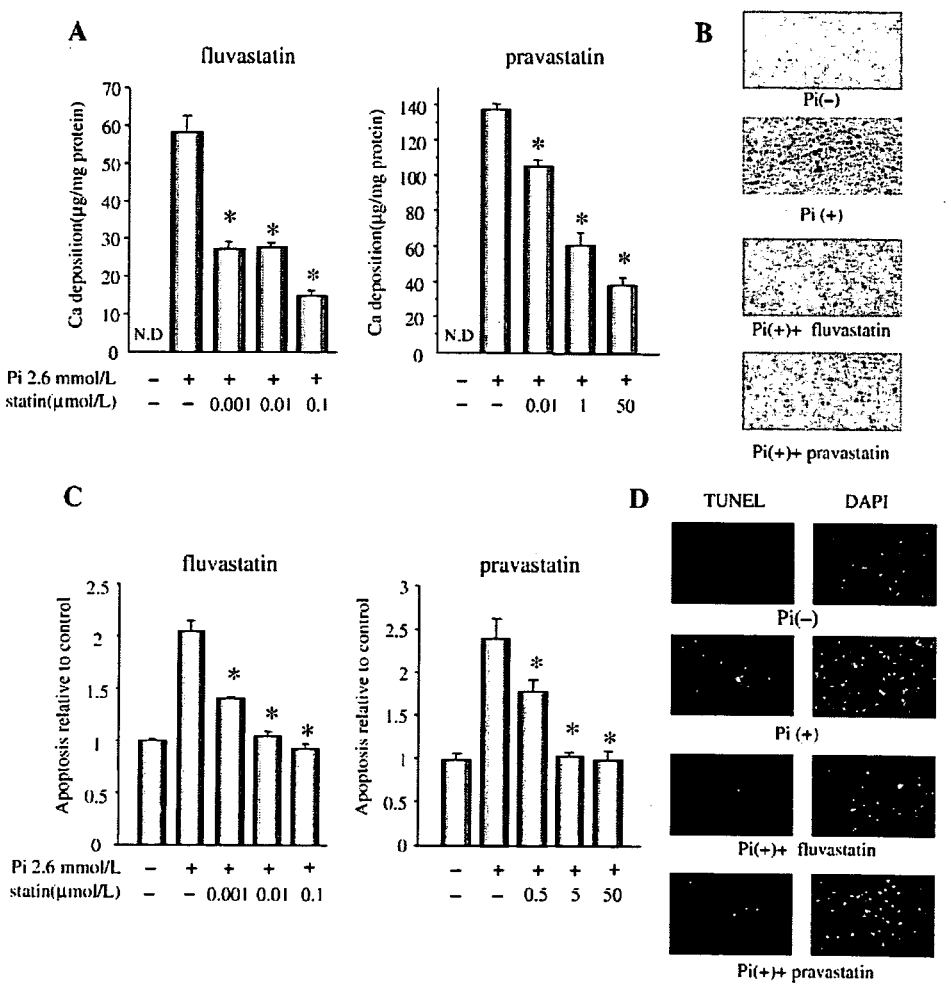

Fig. 1. Statins prevent Pi-induced apoptosis and calcification. HASMC were cultured with the indicated concentrations of fluvastatin and pravastatin in the presence of 2.6 mM Pi for 6 days. Ca deposition was measured by *o*-cresolphthalein complexone method, and normalized by cell protein content. All values are presented as mean± S.E.M. ($n=6$). *$P<0.05$ vs. statin (−) by Fisher's test. N.D. stands for "not detected" (A). On day 6, the inhibitory effect of fluvastatin (0.1 μM) and pravastatin (50 μM) on 2.6 mM Pi [Pi(+)]-induced Ca deposition was evaluated at the light microscopic level with von Kossa's staining (B). Serum-starved HASMC were cultured with the indicated concentrations of fluvastatin and pravastatin for 12 h and then incubated with 2.6 mM Pi for an additional 24 h. A quantitative index of apoptosis, determined by ELISA, is presented as the relative value to that without statins and 2.6 mM Pi. All values are presented as mean±S.E.M. ($n=3$). *$P<0.05$ vs. 2.6 mM Pi, statin (−) by Fisher's test (C). The antiapoptotic effect of fluvastatin (0.1 μM) and pravastatin (50 μM) was evaluated by TUNEL staining (green) on day 6. Nuclei were counterstained with DAPI (4′,6-diamino-2-phenylindole, blue) (D).

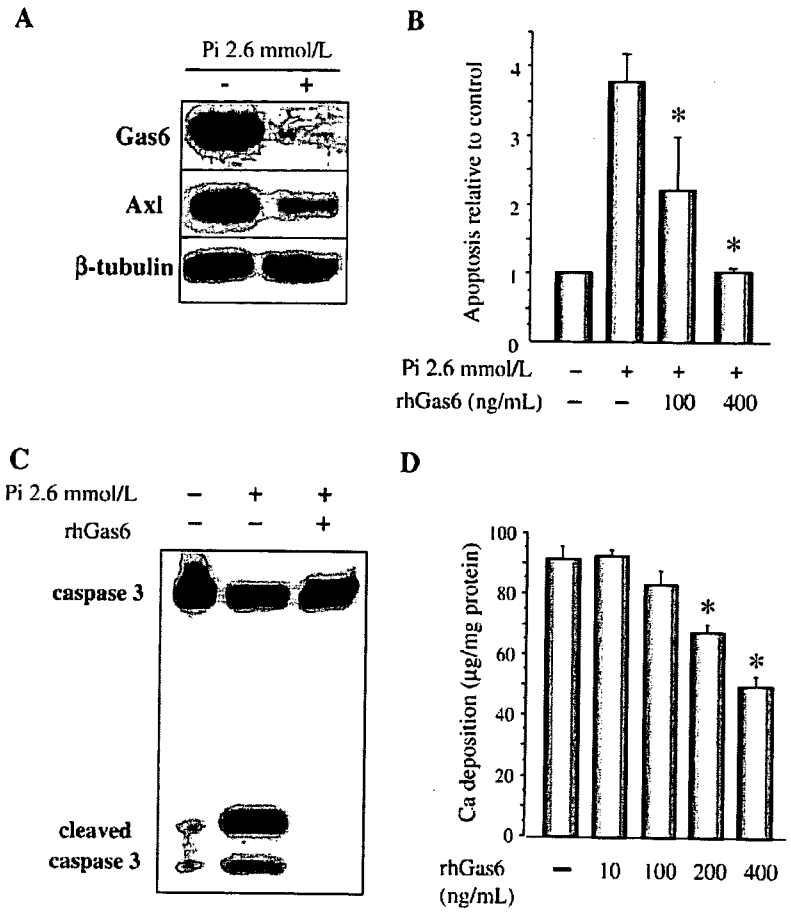

Fig. 2. Pi suppresses Gas6 and Axl expression, and rhGas6 inhibits caspase-dependent apoptosis and calcification. HASMC were cultured in the presence or absence of 2.6 mM Pi for 12 h. Cell lysates were collected and subjected to SDS-PAGE followed by immunoblotting with antibodies to Gas6, Axl or β-tubulin (A). After pretreatment with the indicated concentrations of rhGas6, apoptosis was induced by 2.6 mM Pi. All values are presented as mean±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±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.

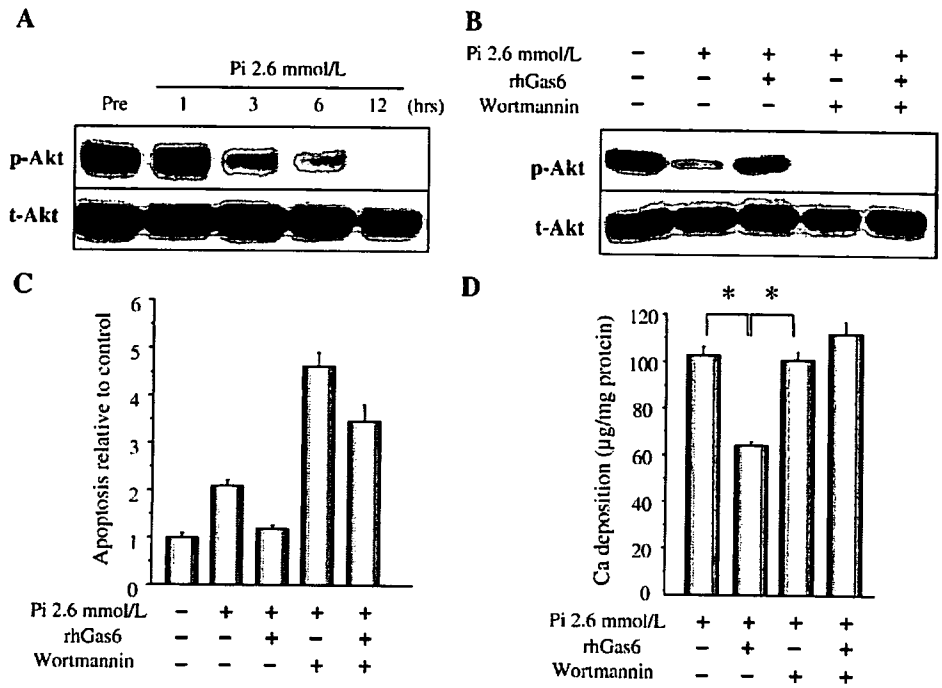

Fig. 3. Pi decreases Akt phosphorylation, and wortmannin abrogates the inhibitory effect of rhGas6 on Akt phosphorylation, apoptosis and calcification. HASMC were cultured in the presence of 2.6 mM Pi for the indicated periods. Cell lysates were immunoblotted with anti-phospho-Akt (p-Akt) antibody and 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

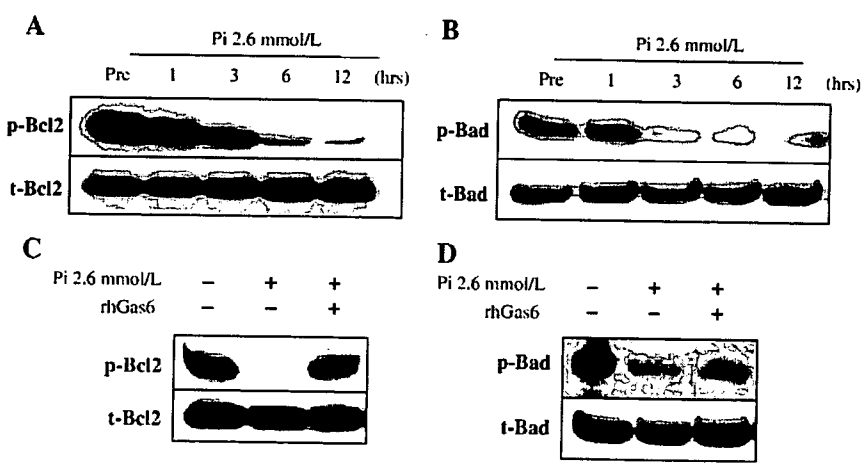

Fig. 4. RhGas6 restores Pi-induced decrease in phosphorylation of Bcl2 and Bad. HASMC were exposed to 2.6 mM Pi for the indicated periods, and cell lysates were subjected to immunoblotting with anti-phospho-Bcl2 (p-Bcl2) antibody and total Bcl2 (t-Bcl2) antibody (A), or with anti-phospho-Bad (p-Bad) 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).

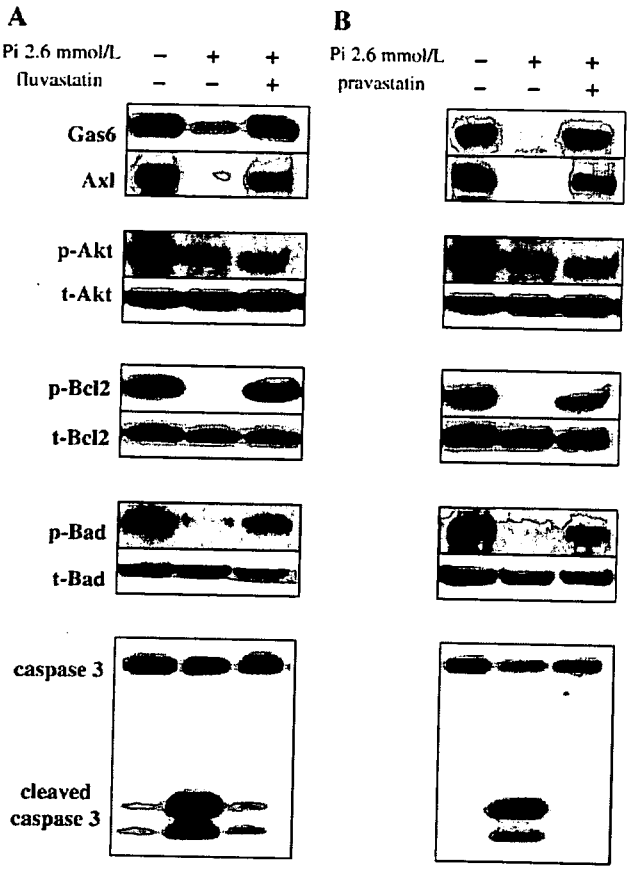

Fig. 5. Antiapoptotic effect of statins is associated with upregulation of Gas6-Axl survival pathway. After pretreatment with 0.1 μM fluvastatin (A) and 50 μM pravastatin (B) for 12 h, apoptosis was induced by 2.6 mM Pi. After 12 h, cell lysates were collected and subjected to SDS-PAGE followed by immunoblotting 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 anti-apoptotic 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 anti-apoptotic 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.

Acknowledgements

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CASE REPORT

Improved cognitive function, mood and brain blood flow in single photon emission computed tomography following individual reminiscence therapy in an elderly patient with Alzheimer's disease

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An 88-year-old man who was suffering from chronic renal failure and hypertension visited our memory clinic because of recent cognitive decline and a gradual decrease in his vitality and volition. His Mini-Mental State Examination (MMSE) score was 22, his 15-item Geriatric Depression Scale (GDS-15) score was 10, and his Vitality Index (VI; full score, 10) was 6. We diagnosed Alzheimer's disease with depressive mood, and this was supported by findings of global brain atrophy by magnetic resonance imaging and decrease in brain blood flow in the posterior cingulated gyrus and frontal association area by single photon emission computed tomography (SPECT). After completion of a life review of the patient, individual reminiscence therapy was performed once a week for 2 months. After the therapy, a comprehensive geriatric assessment showed that cognitive function, depressive mood and decreased vitality had all markedly improved (MMSE, 29; GDS, 7; VI, 9). Moreover, SPECT showed improved brain blood flow, especially in the frontal lobe. We believe that this is the first case in which reminiscence therapy alone not only improved cognitive function and mood but also reduced neuroimaging abnormalities.

Keywords: Alzheimer's disease, cognitive function, life review, reminiscence, single photon emission computed tomography easy Z-score imaging system (SPECT eZIS).

Introduction

Reminiscence is a psychophysiological therapy proposed by an American geriatric psychiatrist, Robert N. Butler, in 1963,[1] and recommended as a grade D' psychological approach to the management of neuropsychiatric symptoms of dementia by Livingstone *et al.*[2] There are two methods of reminiscence therapy: group reminiscence and individual reminiscence. The former is very common and widely performed in public welfare facilities;[3] in contrast, very few facilities perform individual reminiscence[4] and related documents and references are limited in Japan.[5,6]

Herein, we report the case of an 88-year-old man who was treated by individual reminiscence therapy at our outpatient memory clinic. We show that cognitive function, depressive condition and volition were all

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improved in a comprehensive geriatric assessment performed after therapy. Objective changes supporting these outcomes were noted in imaging after completion of the individual reminiscence program.

Case report

The patient was an 88-year-old man suffering from chronic renal failure, hypertension and hyperuricemia. He was taking Nifedipine CR tablet 20 mg/day and allopurinol tablet 50 mg/day. He had been born in Asakusa, Tokyo, and had lived at his mother's home in Gifu Prefecture while he was a primary schoolboy. His surviving family members were his wife, a second son and his wife, two grandsons and one granddaughter. His occupation had been as a private primary school teacher, and after retirement he was engaged in editing and publishing biographies of great persons at an educational book publishing company.

Based on a family interview, the patient had shown temporal disorientation, derangement of the capacity to register and decreased activities of daily living (ADL) for several years; for these reasons he visited our outpatient memory clinic. His present illness was not specific, his neurological findings were normal, and no other psychological or psychiatric symptoms were noted. In radiological imaging, global cerebral atrophy was noted in brain magnetic resonance imaging (MRI), and relative decreases in blood flow in the posterior cingulated gyrus and right median plane of the frontal lobe were noted on single photon emission computed tomography (SPECT; ethylene cystine dimer [ECD]).

Although his Geriatric Depression Scale score suggested depression, his symptoms did not satisfy major depression criteria of the *Diagnostic and Statistical Manual of Mental Disorders*, 4th edition (DSM-IV). Dementia with Lewy bodies (DLB) was suspected based on decreased blood flow in the occipital lobe on SPECT; however, because neurological symptoms such as parkinsonism, hallucination and visual hallucination were absent, and reduction of cognitive function was mild, Alzheimer's-type dementia was diagnosed. Based on this diagnosis, administration of donepezil hydrochloride (Aricept) was considered, but the patient had chronic renal failure and we were concerned about the risk of donepezil-induced rhabdomyolysis. Considering the risk, the family requested that the patient should not receive this drug, so individual reminiscence therapy was initiated instead.

The individual reminiscence procedure was performed as follows:

1 The person with the main responsibility for taking care of the patient (the wife of his second son) first visited the hospital alone, and were interviewed about the patient's profile (shown below) before therapy. This allowed discussion of episodes that she could not mention in the presence of the patient, and allowed a prior understanding of things that should be avoided when talking to the patient. The interview clarified: (i) details of the interviewee and her relationship with the patient; (ii) the patient's diagnosis and medications; (iii) the patient's past medical history, lifestyle and other diseases; (iv) the patient's family members (i.e. marital status and presence or absence of spouse), nickname, parents, siblings, children, grandchildren, close relatives and friends, and other people; (v) native town, places of importance to the patient other than his native town, history of moving; (vi) final level of school education; (vii) professional career (i.e. thoughts on work and feelings of accomplishment); (viii) hobbies (including interests and matters of concern); (ix) likes and dislikes; (x) religion and beliefs; and (xi) particulars regarding medical care and mental state.
2 The above items were discussed in the interview, and the first session of reminiscence therapy was performed 1 week later. Subsequently, sessions of approximately 1 h were performed weekly for 8 weeks (one set of therapy).
3 The person taking care of the patient attended all the sessions to correct paramnesia, and to insert words related to the date and weather to correct temporal disorientation.
4 In the first session, the patient was allowed to talk freely about the times that he had held strong feelings. Later sessions progressed through a chronological life review from boyhood to late middle age, including background, life and manners, and social conditions of the times.
5 The following items were used to jog the memory: a cup and ball, ohajiki (small discs of glass), menko (cardboard), a five-bead abacus, an antique watch, an empty lemonade bottle, primary and junior high school text books from approximately 1910 until the mid-1920s, and a collection of old photographs from the mid-1920s until approximately 1960.
6 Before the fifth session, the effect of the first four sessions was evaluated using a Comprehensive Geriatric Assessment (CGA). The CGA was also performed after completion of all sessions before initiation of the next outpatient treatment, and the final outcome of the individual reminiscence therapy was determined. Only the Mini-Mental State Examination (MMSE), Hasegawa's Dementia Scale – Revised (HDS-R), 15-item Geriatric Depression Scale (GDS-15) and Vitality Index (VI) were evaluated after completion of the fourth session. The CGA was comprised of: (i) Barthel Index (BI) as an index of ADL (full score, 100 points); (ii) MMSE and HDS-R for evaluation of cognitive function; (iii) Dementia Behavior Disturbance Scale (DBD) for behavioral and psychological symptoms of dementia; (iv) GDS-15 as

an index of depression (full score, 15 points); (v) VI for evaluation of vitality and volition (full score, 10 points); and (vi) Zarit's Burden Interview (ZBI) for evaluation of carer's load (full score, 88 points).

There are two approaches to reminiscence therapy: one in which the patient recalls memories from historical news and events, old articles regarding every-day issues, old toys, and printed material such as old books; and a second in which a life review is held, in which the patient looks back on their own history and life and compares this with their current condition. In our case, individual reminiscence therapy was performed using the latter procedure, but the former procedure was employed concomitantly as needed for introductory purposes in the first session and as idle talk when the conversation halted during a session.

On MRI, the entire brain was seen to be markedly atrophied, and some ischemic lesions, such as periventricular high intensity lesions, were also noted. Almost no changes were noted on MRI performed 6 months after completion of the reminiscence program.

On SPECT (^{99m}Tc-ECD) Relative reduction of blood flow was noted in the frontal and occipital lobes, posterior cingulated gyrus and precuneus in easy Z-score imaging system (eZIS) analysis. In eZIS images after completion of the program, improvements were noted in regions that had previously shown reduced blood flow, with a particularly marked increase in blood flow in the frontal lobe, compared to that before therapy (Fig. 1).

Results of the CGA are given in Table 1 and as follows. At the first examination, the MMSE and HDS-R scores were 22 and 14, respectively. These scores increased to 25 and 24, respectively, after completion of four sessions of reminiscence therapy, and to 29 and 21, respectively, after completion of the program, showing a marked improvement of cognitive function compared to that before therapy. The patient showed temporal disorientation and delayed recall of three words, and was unable to enumerate a list of 10 vegetables before therapy, and these characteristics were also markedly improved by the therapy. The MMSE score was still 29 on re-evaluation 6 months after completion of the program.

The GDS-15 score was 10 on the first examination and showed no change after four sessions; however, the score decreased to 7 at completion of all sessions, indicating a slight improvement of depression, and we saw the patient smile more often than before treatment.

The VI and BI scores were 6 and 85, respectively, at the first examination, and increased to 10 and 90, respectively, after four sessions. After completion of all sessions, these scores were 9 and 95, respectively. The patient started to do things that he previously left to others, and started to read ancient documents again. The VI and BI scores remained at 9 and 95, respectively, in tests 6 months after completion of all sessions.

For the DBD and ZBI, the patient had no behavioral or psychological symptoms of dementia, and no numerical changes were noted after the therapy.

Discussion

Treatment with individual reminiscence therapy alone markedly improved attention, volition and depression in the patient. According to Butler, the pioneer of reminiscence therapy, life review is a healthy psychological behavior in which past events are re-evaluated, and this process brings about improvements in physical as well as mental and social activities,[7] thereby showing an effect on volition. Bohlmeijer *et al.* also reported that reminiscence was effective for senile depression;[8] however, there have been no reports of an objective effect on cognitive function. Several Japanese studies have suggested that reminiscence is effective mainly for psychological depressive tendency and decreased volition, but a marked effect on cognitive function has only been noted in a few reports. Kurokawa *et al.* reported that reminiscence was more effective for vascular dementia than for Alzheimer's-type dementia with regard to improvement of cognitive function,[9] and Urabe *et al.* showed that individual reminiscence improved cognitive function only in a few patients with Alzheimer's-type dementia.[5]

What was the cause of the marked improvement in cognitive function in our patient? One characteristic of the patient was an interest in ancient documents, history, education, politics and economics. He remembered some details of interesting events in his childhood and adolescence, and his memories became clearer as the sessions progressed. Furthermore, his family very cooperatively[10] attended all sessions, understood his condition in detail at each time point, and provided information to the physician, as reflected by the abundant information in the personal chart (life review) obtained before therapy. This background suggests that the following factors contributed to the effectiveness of individual reminiscence therapy for this patient: (i) a personal chart that provided extensive information prior to therapy; (ii) cooperation of the patient's family, not only in taking care of the patient but also in visiting the hospital and attending the therapy sessions; (iii) the patient's retention of memories of childhood and adolescence; (iv) the patient's interest in certain fields, although not very active; and (v) the patient had been solitary, and the sessions provided company and the chance for conversation. The effectiveness of individual reminiscence therapy may be greater in cases that follow this pattern.

An important characteristic of this case was the increased blood flow in the frontal association area, which is considered to be the center of volition, in

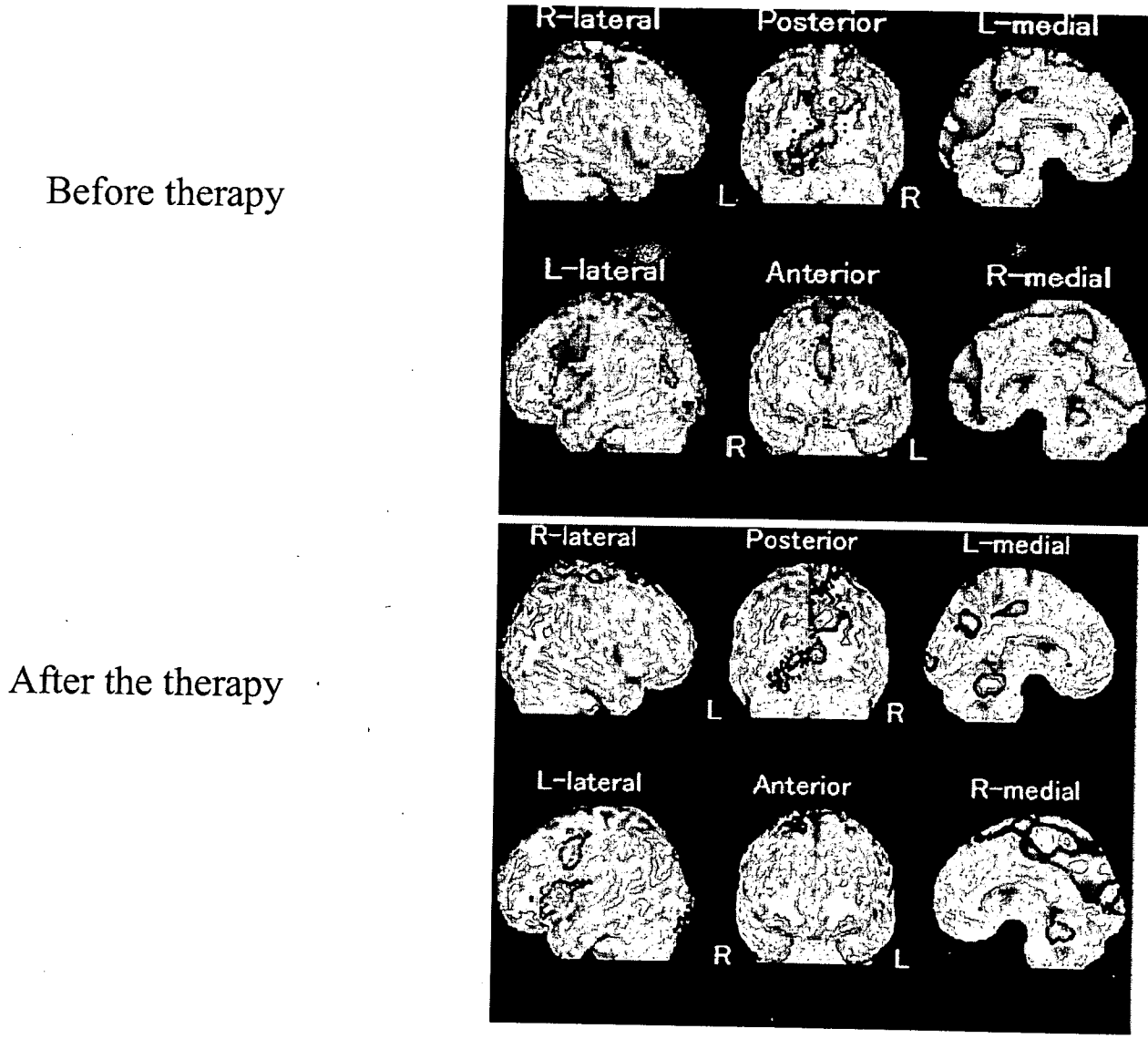

Figure 1 Easy Z-score imaging system (eZIS) image of single photon emission computed tomography (SPECT) before and after individual reminiscence therapy. Reduction of blood flow in the right frontal, and both left temporal, median-parietal and occipital lobes, posterior cingulated gyrus, and precuneus (upper panel). Comparing the SPECT findings before therapy, marked increase in the frontal lobe is noted (bottom panel).

SPECT (eZIS) performed after reminiscence therapy. Ushijima *et al.* performed SPECT and MMSE in 59 patients with Alzheimer's-type dementia and in 12 normal volunteers to find areas of reduced blood flow, and investigated the relationship between cognitive function and blood flow;[11] the results showed that attentiveness and calculation ability were associated with reduced blood flow in the frontal cortex. Migneco *et al.* reported that decreased volition (apathy) in Alzheimer's disease is related to reduced blood flow in the anterior cingulated gyrus in SPECT,[12] and Holthoff *et al.* also investigated decreased volition in early stage Alzheimer's disease by positron emission tomography, and found that decreased blood flow in the left orbitofrontal region had an influence.[13] Although association of decreased cognitive function and volition with a reduction of regional blood flow in the brain has been reported,[14] there has been no previous report of a marked increase in cerebral blood flow caused by individual reminiscence therapy. Therefore, this case provides an important demonstration of the relationship between blood flow in the frontal lobe and volition and cognitive function.

Table 1 Effect of reminiscence therapy on the score of Comprehensive Geriatric Assessment

	Before 13 May	After 2 months 5 August	End of session 16 September
Barthel Index (0–100)	85	90	95
MMSE (0–30)	22	25	29
HDS-R (0–30)	14	24	21
GDS (0–15)	10	10	7
Vitality Index (0–10)	6	10	9
Zarit Burden Interview (0–88)	21		19

GDS-15, 15-item Geriatric Depression Scale; HDS-R, Hasegawa's Dementia Scale – Revised; Mini-Mental State Examination (MMSE).

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I. 老化と老年病

A. 老化と老年病(生物学的側面)

1 老化とは

老化 senescence とは，時間経過にともなって起こる体細胞の減少と機能変化に基づく，臓器の縮小，組織の退行変性，組織成分の変化とされている．ヒトの生命は，卵子と精子から受精卵が生まれることから始まり，胎児として発生し，出産によって母体から誕生し，成長し，さらに成熟と進むなかで，この老化は成熟期以降に生じる変化である．一方，加齢(かれい) aging とは，生後から時間経過とともに個体に生じるすべての現象をいう．老化の特徴は，普遍性，進行性，内在性，有害性をもつことがあげられる．老化すなわち身体機能の退行変性が成熟期から始まることと，臨床老年医学で老人を「高齢者」として，65 歳から 74 歳を「前期高齢者 young-old」，75 ～ 84 歳を「後期高齢者 old-old」，85 歳以降を「超高齢者 oldest-old」と名付ける立場とは必ずしも一致していない．

学生に，高齢者と自分たちとの違いを訊ねると，「顔に皺がある．頭髪が少ないかあるいは白髪が多い．皮膚のみずみずしさが喪失している．シミが多い．立位が前傾している．歩き方が機敏でない」などを指摘する．これらの多くは皮膚の老化を示している．確かに老化した表皮では，角質細胞内のグルタミン由来保湿因子(プロリンなど)が減少することで皮膚の乾燥や粗造(そぞう)化が生じる．表皮細胞の小型化や数の減少により表皮の厚みや表皮突起が減少し皮膚の萎縮をもたらす．真皮の膠原線維(こうげんせんい)の減少や弾力線維の変性は皮膚の皺やたるみをもたらす．白髪は毛母メラノサイトの機能低下や脱落によるものである．毛の生長速度が遅延し，成長期間が短縮するために脱毛が起こる．このような皮膚細胞の細胞数減少および機能低下と同じような変化が身体の中のほかの臓器でも普遍的に生じているではないかと考えることは正しい．これらの老化現象がどのようにして生じるのかを研究してきたのが老化研究の歴史である．

表 A-1　早老症と遺伝子異常

症候群	発症頻度	平均寿命	遺伝子異常	主症状
ハッチンソン-ギルフォード症候群	＜1/100 万人	～12 年	Lamin A	動脈硬化，脂肪萎縮症 糖尿病，がん
コケイン症候群	＜1/10 万人	～20 年	DNA ヘリカーゼ	難聴，網膜変性，脱髄
ウェルナー症候群	＜1/10 万人	～46 年	DNA ヘリカーゼ	白髪，脱毛，しわがれ声，動脈硬化，がん
毛細血管拡張性運動失調症	＜1/6 万人	～20 年	DNA 損傷シグナル	皮膚硬化，免疫不全 白髪，がん
ダウン症候群	＜1/1,000 人	～60 年	不明	白内障，白髪，脱毛，知能低下，低皮下脂肪

(Kipling, D et al : What can progeroid syndromes tell us about human aging ? Science 305 : 1426-1431, 2004 より一部引用)

a. 老化の研究

老化についての研究は，従来より細胞レベルでの研究が主であった．すなわち細胞老化 cellular senescence の機構を解明する過程で，細胞の分裂寿命を制御するテロメアの意義や，細胞の形質変化や分化増殖，さらにはアポトーシスを制御する仕組みが明らかにされ，その結果老化関連遺伝子が早老症 progeria の原因となることもわかり，老化研究の発展に大きく寄与してきている．しかしながら，老化は身体全体に起こる現象であることから，個体老化 individual senescence の研究が細胞老化研究の成果を踏まえて大きく発展することが望まれる．現在は，老化関連遺伝子のノックアウトあるいは遺伝子導入マウスや老化促進マウスなどを用いた研究が進展している．

b. 早老症

早老症の遺伝子異常を表 A-1 に示した．早老症の原因遺伝子探索で明らかになったことは，①早老症の遺伝子異常の多くが核 DNA の修復に関与するものであることと，②体細胞と組織の老化が通常の「老化」プロセスに大きく関与していること，である．とくに，最近ハッチンソン-ギルフォード Hutchinson-Gilford 症候群の原因遺伝子が，核の形態を維持するうえで重要なラミナの主要成分，Lamin A であることが明らかにされたことからも，核の機能低下と老化が関連することが示唆される．一方，アポトーシスのシグナルを引き起こすことが知られているミトコンドリアも老化との関連が注目されている．ミトコンドリア脳筋症(レーバー遺伝性視神経萎縮症 Leber's hereditary optic neuropathy やリー脳症 Leigh disease など)でミトコンドリア DNA の変異が報告されている．実験動物で，ミトコンドリアにある電子伝達系の呼吸鎖複合体の異常がパーキンソン Parkinson 病様の症状を示すことも知られている．さらに，分泌タンパク質の品質管理を行っている小胞体の異常も老化に結びつく

と考えられている.

c. 生理的老化と病的老化

老化の特徴に内在性をもつことを前述したが，老化には生理的老化 physiological aging と病的老化 pathological aging がある．前者は成熟期から始まる各臓器の生理的な機能変化をさし，普遍的かつ不可逆的な現象である．一方病的老化は，生活習慣病などに認められる加速された老化現象によって，病的状態に陥る状態である．この病的老化にはしばしば血管の老化が基盤に認められる．そのほか，骨粗鬆症や神経の変性疾患など臓器特異的病的老化もある．病的老化と生理的老化の区別は必ずしも明確ではないが，病的老化は基礎疾患によって老化現象が加速されたものである．生活習慣病の糖尿病，高血圧症，高脂血症，肥満などは，それぞれが老化を促進するほかに，重複したものほど老化に加速度が加わるのが特徴である．この病的老化は原理的には予防と治療で阻止することが可能である.

2 老化学説

老化の本体については，従来多くの学説があった．その考え方は大きく内因説と外因説に分けられる．内因説は，プログラム説に代表されるように，老化が遺伝子で制御されているというものである．外因説は，酸化ストレスや外部環境(環境汚染物質，運動，栄養など)によって細胞傷害や遺伝子変異が引き起こされて老化が促進されるという考えである．寿命を制御する遺伝子が明らかにされる一方，遺伝子異常だけでは老化促進は説明できず，外因説に述べられている種々のストレスを避けることや外部環境を整えることで老化促進を予防することが重要であるという考えも多い．以下に，おもな老化学説を紹介する.

a. 酸化ストレス説

酸化ストレスが老化を促進させるという考えは，ハルマン D. Harman によって The Free Radical Theory of Aging(老化の活性酸素学説)として提唱された．これは，酸素呼吸を行うなかで発生する活性酸素(O_2^-)による連続した組織細胞傷害が老化と死を生じるとする考えである．近年活性酸素と関連物質を活性酸素種 reactive oxygen species(ROS)と称し，その産生とその消去機構の研究が進展している．ROS には，O_2^-のほかに，過酸化水素(H_2O_2)，ヒドロキシラジカル(.OH)などがある．O_2^-の産生場所は主としてミトコンドリアの電子伝達系と赤血球である．細胞によっては積極的にO_2^-を産生する．たとえば炎症細胞は，細胞膜に局在する NADH-オキシダーゼの働きでO_2^-を産生して殺菌や貪食を行う．ROS による細胞傷害を防ぐ目的で，細胞には消去酵素が存在している．O_2^-はスーパーオキシドジスムターゼ superoxide dismutase(SOD)によってH_2O_2に変えられる．H_2O_2はグルタチオン過酸化酵素の働

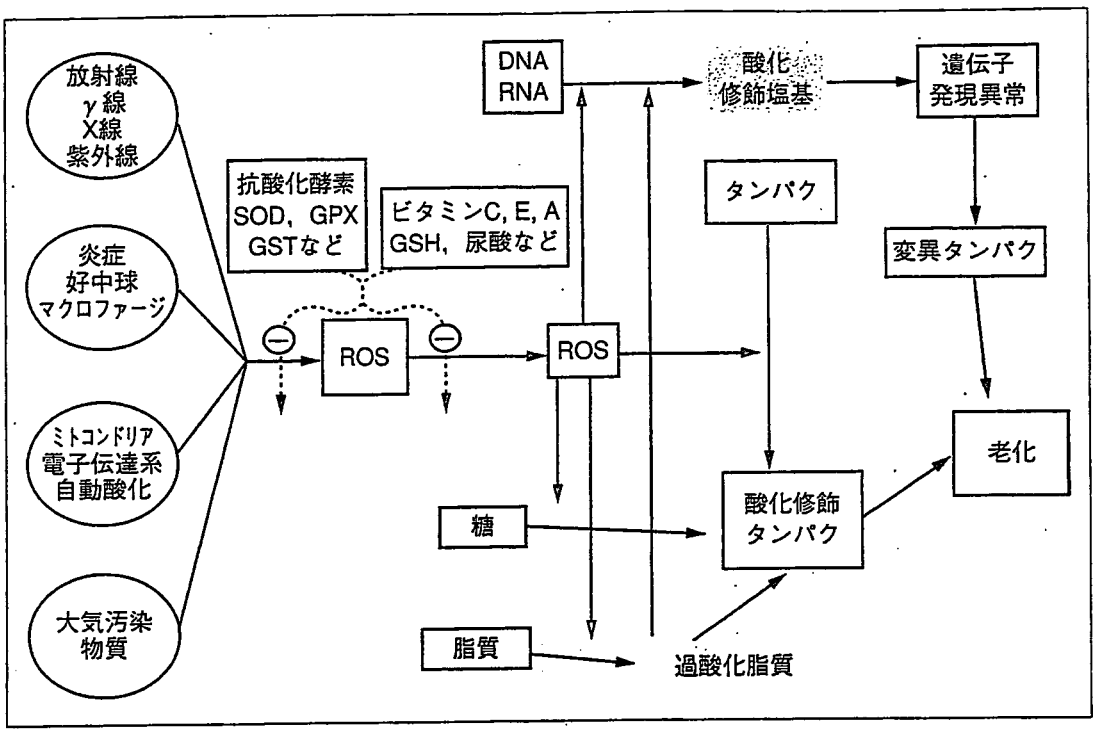

図 A-1　酸化ストレスと老化

GPX：グルタチオン過酸化酵素，GST：グルタチオン S-トランスフェラーゼ，GSH：還元型グルタチオン．

(Stadtman ER：Importance of individuality in oxidative stress and aging. Free Rad Biol Med, 33：597-604, 2002 より一部引用，一部改変)

きで消去される．ROS と消去酵素のバランスが保たれていることは生体にとって重要である．

そのほか，ROS は種々の条件下で生体内に発生し，酸化ストレスとしてタンパク質，脂質，核酸を酸化修飾する．酸化修飾された生体構成成分が蓄積することが老化の促進につながる．図 A-1 に示すように，どのような酸化ストレスでも，過剰になれば酸化ストレスで修飾されたタンパク質が増加して蓄積してくる．修飾されたタンパク質はクロスリンクしやすいために，プロテアソームでのタンパク分解を受けづらくなっているのも一因である．

酸化ストレスの原因として必ずあげられる喫煙は，最近その分子機構がしだいに明らかにされてきている．①タバコの煙自体に含まれる ROS が内皮細胞を傷害する，②ニコチンが NO 合成酵素を不活性化させて NO 合成の低下とこの酵素による ROS の産生をもたらす，③ニコチンがキサンチンオキシダーゼ活性を上昇させて ROS を産生する，④喫煙がマクロファージや好中球による炎症反応を刺激するなどである．

ROS は量が多い場合は，細胞傷害を引き起こす．ところが，少量の ROS は細胞内の情報伝達シグナルを活性化する働きもある．さらに重要なことは，ROS の濃度によって細胞内の酸化還元(レドックス)状態が変動することである．このレドックスは細胞内情報伝達を含めたほとんどの細胞機能の制御に関連することが明らかにされてきており，長期間の破綻が老化促進に結びつくと考えられている．