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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 μmol/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 stenosis[6–8] 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]

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% CO_2. HASMC were used up to passage 8 for the experiments.

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 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 $AgNO_3$.

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′-GCGTGGCCAAGAGTGTGAAGT-3′; reverse, 5′-CGCCACTCC-TCAACAGAGAT-3′) was amplified by RT-PCR. For Northern blot analysis, harvested RNA (≈5 to 10 μg) 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 ^{32}P-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 μg/mL) in the presence of 2.6 mmol/L Pi after 12 hours of atorvastatin (0.1 μmol/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
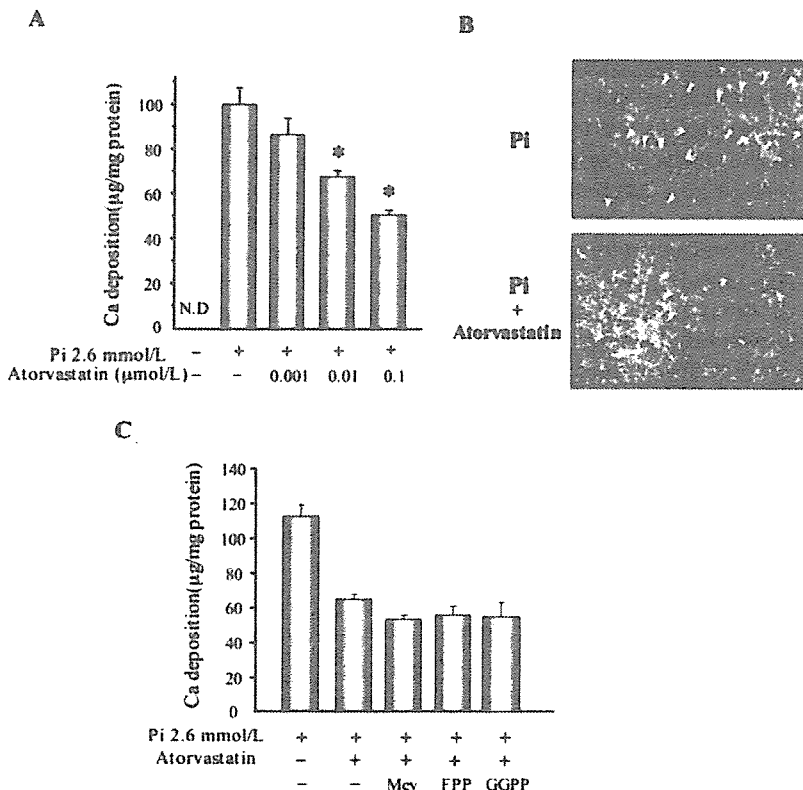
Figure 1. Statins prevent HASMC calcification. A, HASMC were cultured with the indicated concentrations of atorvastatin in the presence of 2.6 mmol/L Pi for 6 days. Ca deposition was measured by o-cresolphthalein complexone method and normalized by cell protein content. All values are presented as mean±SEM (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 μmol/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 μmol/L), farnesylpyrophosphate (1 μmol/L), or geranylgeranylpyrophosphate (1 μmol/L) in the presence of atorvastatin (0.1 μmol/L) and 2.6 mmol/L Pi for 6 days. All values are presented as mean±SEM (n=6).

Figure 2. Pi induces apoptosis, and ZVAD.fmk inhibits Pi-induced calcification. A, After incubation with 1.4 (Pi-) and 3.2 mmol/L (Pi+) Pi for 10 days, apoptotic cells were identified by TUNEL staining (green). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). B, Serum-starved HASMC were cultured with the indicated concentration of Pi for 24 hours. A quantitative index of apoptosis, determined by ELISA, is presented as the relative value to th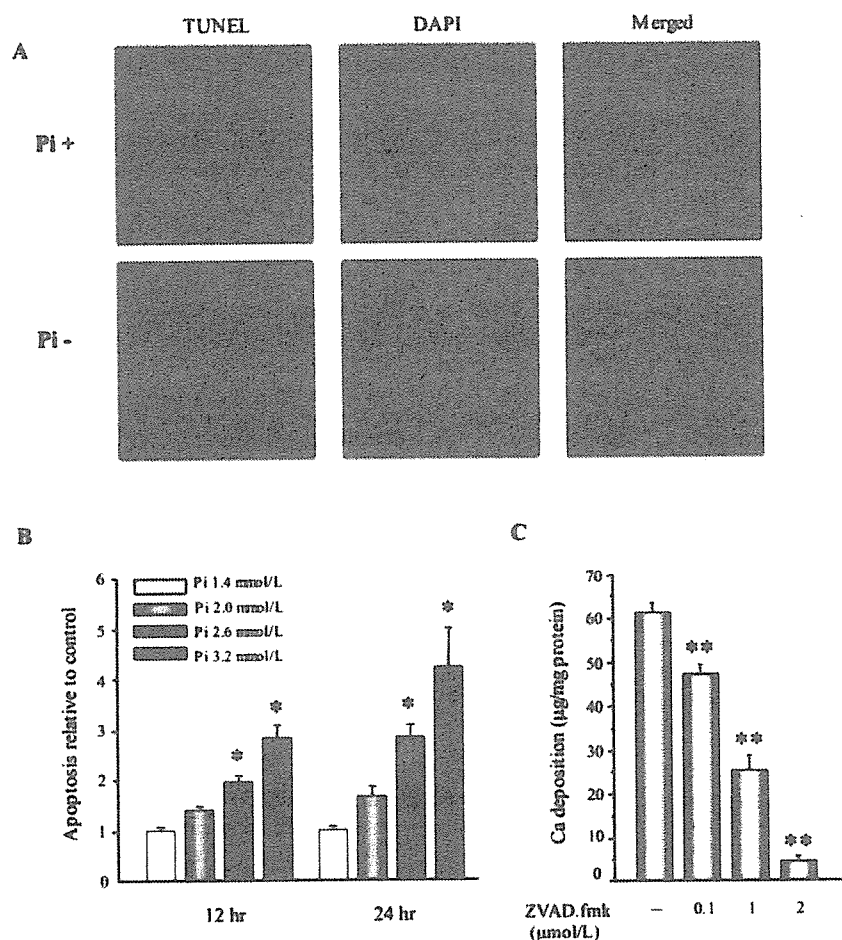at with 1.4 mmol/L Pi. All values are presented as mean±SEM (n=3). *$P<0.05$ vs 1.4 mmol/L Pi by Fisher's test. C, HASMC were incubated with the indicated concentration of ZVAD.fmk in the presence of 2.6 mmol/L Pi for 6 days. Ca content was measured and normalized by cell protein content. All values are presented as mean±SEM (n=6). **$P<0.01$ vs 2.6 mmol/L Pi, ZVAD.fmk(-) by Fisher's test. Experiments were performed with at least 3 different cell populations.

of the 18S RNA at each time point, and the half-life was calculated by linear extrapolation.

Preparation of Small Interfering RNA Targeting Gas6 and Transfection

Two small interfering RNAs (siRNAs) were designed to target human Gas6 (accession no. NM_000820) using siRNA design software (Dharmacon). The sequences for Gas6 were 5′-GGACCTGCCAAGACATAGA-3′ and 5′-ACCTCGTGCAGCCT-ATAAA-3′. Nonspecific control siRNA was synthesized using standard templates (Dharmacon). Twenty-four hours after HASMC seeding onto 12-well plates, cells were cultured in serum-free medium for an additional 24 hours, then transfected with Gas6 (100 nmol/L) and control siRNA using transfection reagent (Upstate). To evaluate the effect of Gas6 siRNA on Ca deposition, siRNA was transfected when HASMC had reached 80% to 90% confluence and then transfected every time the medium was changed (every 2 days) up to 6 days. The loss of Gas6 by transfection of siRNA was validated by immunoblotting for Gas6 protein in the cell lysates 48 hours and 6 days after siRNA transfection.

Statistical Analysis

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

An expanded Materials and Methods section can be found in the online data supplement available at http://circres.ahajournals.org.

Results

Statins Inhibit Pi-Induced HASMC Calcification

To induce HASMC calcification, cells were incubated with calcification medium for 10 days. We confirmed that high phosphate (≥ 2.6 mmol/L) induced Ca deposition in a concentration- and time-dependent manner, whereas 1.4 mmol/L Pi, equivalent to the human physiological serum phosphate level, was not able to induce Ca deposition up to 10 days. To investigate the effect of statins on Pi-induced calcification, HASMC were incubated with atorvastatin in the presence of 2.6 mmol/L Pi. On day 6, Ca deposition was significantly suppressed by atorvastatin in a concentration-dependent manner ($51.1 \pm 1.9\%$ of control at 0.1 μmol/L) (Figure 1A). An inhibitory effect of the statins on Ca deposition was also found by von Kossa's staining (Figure 1B). Atorvastatin was able to be added at as high a concentration as 0.1 μmol/L without cell damage. The inhibitory effect was also observed with fluvastatin (0.001 to 0.1 μmol/L) and pravastatin (0.01 to 50 μmol/L) (data not shown). The inhibitory effect of statins was not blocked by mevalonate (100 μmol/L), farnesylpyrophosphate (1 μmol/L), or geranylgeranylpyrophosphate (1 μmol/L), suggesting that the effect is not dependent on the mevalonate pathway (Figure 1C).

Inhibitory Effect of Statins on Calcification Is Caused by Preventing Apoptosis, Not by Inhibiting Sodium-Dependent Phosphate Cotransporter Activity

Two different time courses were tested to examine the effect of Pi on HASMC apoptosis: short-term (up to 24 hours) and long-term (up to 10 days; practical time course of calcifica-

tion process). During calcification, Pi increased the rate of apoptotic cell death detected by terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL) assay (Figure 2A). Furthermore, cytoplasmic histone-associated DNA fragments determined by ELISA, as a quantitative index of apoptosis, were also increased by Pi in a concentration- and time-dependent manner in both short-term (Figure 2B) and long-term conditions (supplemental Figure I). In addition, caspase 3 activation, detected by immunoblotting, by 2.6 mmol/L Pi was observed in short-term and long-term conditions (data not shown). To investigate the relationship between apoptosis and calcification, we used ZVAD.fmk, a general caspase inhibitor. We found that ZVAD.fmk significantly inhibited Pi-induced apoptosis as well as calcification in a concentration-dependent manner (Figure 2C).

It has been reported that sodium-dependent phosphate cotransporter (NPC) activity is an important pathway regulating Pi-induced HASMC calcification.[25] We confirmed that type III NPC (Pit-1) was expressed in the HASMC that we used, and its activity was enhanced by Pi treatment. Furthermore, a specific inhibitor of NPC, phosphonoformic acid (PFA), inhibited Ca deposition (reduced by 90.4% at 0.1 μmol/L), indicating that NPC-mediated Pi uptake is also essential for HASMC calcification.

To investigate the mechanisms of these statins, we examined the effect of atorvastatin on apoptosis and NPC activity. Atorvastatin, at concentrations exerting inhibition of calcification, reduced apoptosis in a concentration-dependent manner (Figure 3A). A beneficial effect of statins was also observed in the long-term condition (supplemental Figure II). On the other hand, statins did not inhibit NPC activity induced by Pi treatment (Figure 3B).

Downregulation of Gas6-Axl Interaction Is Associated With Pi-Induced Apoptosis

Immunoblot analysis showed that the expression of Gas6 and Axl was markedly downregulated by 2.6 mmol/L Pi in both short-term (Figure 4A) and long-term (supplemental Figure III) conditions. To further examine whether Pi affects the secretion of Gas6 by HASMC, conditioned medium was collected after Pi treatment. Gas6 production in the medium was reduced by 2.6 mmol/L Pi, along with a reduction in its intracellular expression (Figure 4B). Gas6 production was also reduced in an immunoprecipitation-immunoblotting study on day 10 (Figure 4C). Next, to investigate the role of Gas6-Axl interaction in the process of apoptosis and calcification, rhGas6 and Axl-ECD were supplemented in Pi-treated HASMC. The addition of rhGas6 significantly inhibited both Pi-induced apoptosis and calcification. Addition of Axl-ECD to block the binding of Gas6 to Axl clearly abrogated the inhibitory effect of rhGas6 (Figure 4D and 4E). These results indicate that Pi-induced apoptosis and calcification are associated with downregulation of the Gas6-Axl interaction.

Statin-Mediated Induction of Gas6 Expression Is Dependent on mRNA Stabilization, Not on Transcription

To investigate whether the antiapoptotic effect of statins is dependent on restoration of the Gas6-Axl interaction, we first

assessed the effect of statins on Gas6 expression. As shown in Figure 5A, atorvastatin increased Gas6 expression, which was downregulated by Pi at both the mRNA and protein levels. Upregulation of Gas6 expression was also observed in the long-term condition (supplemental Figure IV). Furthermore, to elucidate the mechanism of statins on restoration of Gas6 mRNA, a promoter study was undertaken. Reporter assay using the -1.9 kb Gas6-luciferase DNA construct revealed that atorvastatin did not have a significant effect on Gas6 promoter activity (supplemental Figure V), as well as mRNA expression under the condition in which it was significantly inhibited by PDGF-BB (data not shown). Next, we investigated the effect of atorvastatin on mRNA stabilization using an RNA polymerase inhibitor, actinomycin D (ActD). As shown in Figure 5B, Gas6 mRNA expression was more stable in the presence of atorvastatin than in its absence under Pi and ActD treatment. The half-life was 15.9 hours with atorvastatin and 5 hours without atorvastatin, suggesting the capacity of statins to improve Gas6 mRNA stabilization (Figure 5C). Taken together, these findings suggest that the restoration of Gas6 mRNA by statins appears to be mediated by decreasing the mRNA degradation rate, and not by stimulating transcriptional activity.

Furthermore, to determine whether Gas6 is required for statin-mediated effects, we tried to knock down the action of
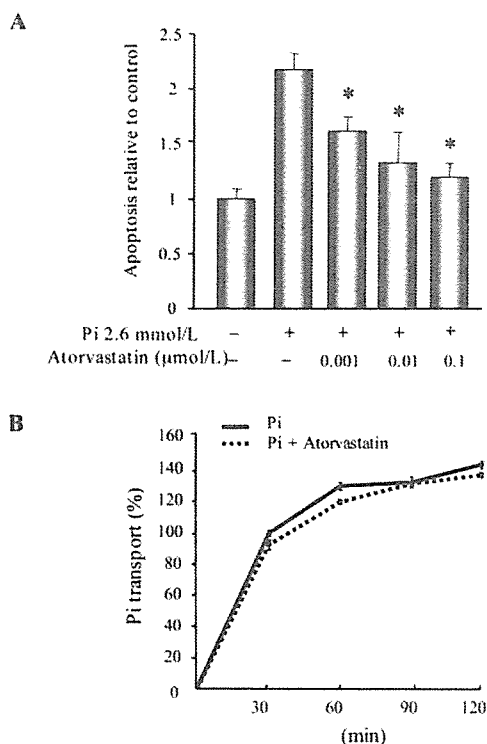
Figure 3. Effect of atorvastatin on Pi-induced apoptosis and NPC activity. A, HASMC were cultured with the indicated concentration of atorvastatin for 12 hours and then incubated with 2.6 mmol/L Pi for an additional 24 hours. All values are presented as mean±SEM (n=3). *P<0.05 vs 2.6 mmol/L Pi, statin (-) by Fisher's test. B, HASMC were treated with (dotted line) or without (solid line) 0.1 μmol/L atorvastatin in the presence of 2.6 mmol/L Pi. On day 6, NPC activity was determined in Earl's balanced salt solution containing 0.1 mmol/L $H_3{}^{32}PO_4$ (1 μCi/mL) with 143 mmol/L sodium chloride for the indicated period. All values are presented as mean±SEM (n=6).

Figure 4. Pi reduces production of Gas6 and Axl, and rhGas6 inhibits Pi-induced apoptosis and calcification via Axl. A, HASMC were cultured in the presence of 2.6 mmol/L Pi for 12 hours. Cell lysates were subjected to SDS-PAGE followed by immunoblotting with antibodies to Gas6, Axl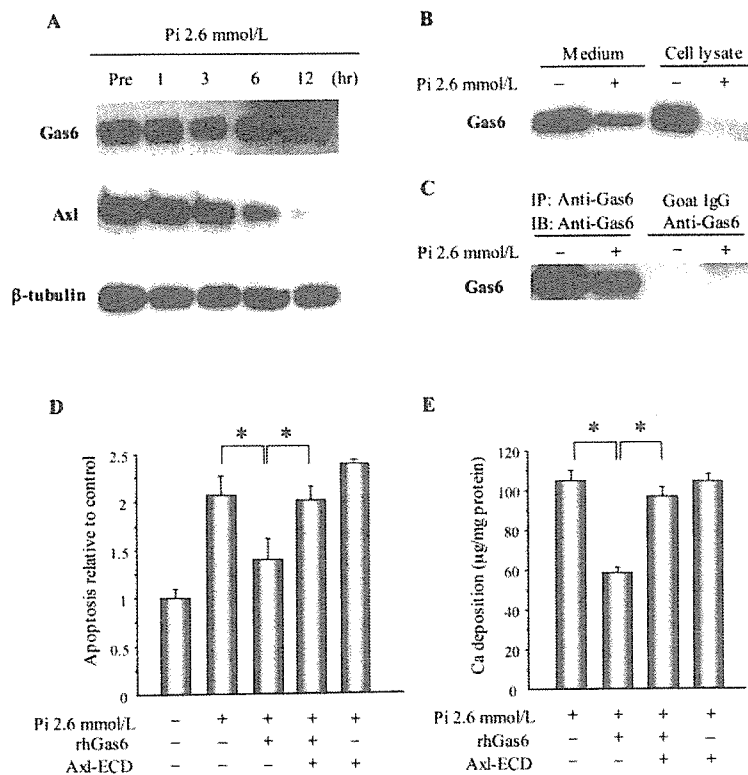, or β-tubulin. B, Conditioned medium of HASMC in the absence (lane 1) or presence (lane 2) of 2.6 mmol/L Pi at 12 hours was concentrated and separated by SDS-PAGE along with cell lysates. C, Conditioned medium of HASMC on day 10 in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 2.6 mmol/L Pi was subjected to immunoprecipitation with anti-Gas6 antibody (lanes 1 and 2) or control goat IgG (lanes 3 and 4). Precipitates were immunoblotted with anti-Gas6 antibody. D, After pretreatment with rhGas6 (400 ng/mL) with or without Axl-ECD (1 μg/mL), apoptosis was induced by 2.6 mmol/L Pi. All values are presented as mean±SEM (n=3). *$P<0.05$ by Fisher's test. E, For measurement of Ca deposition, HASMC were cultured with rhGas6 (400 ng/mL) with or without Axl-ECD (1 μg/mL) in the presence of 2.6 mmol/L Pi for 6 days. All values are presented as mean±SEM (n=6). *$P<0.05$ by Fisher's test. Experiments were performed with at least 3 different cell populations.

Gas6 and examined the effect of atorvastatin on Pi-induced apoptosis and calcification. Transfection of Gas6 siRNA markedly decreased Gas6 expression in the short-term and long-term conditions (Figure 6A). The inhibitory effect of atorvastatin on Pi-induced apoptosis and calcification was reversed by Gas6 siRNA (Figure 6B and 6C). Similarly, the beneficial effect of atorvastatin was also abolished by blocking the binding of Gas6 to Axl using Axl-ECD (Figure 6D and 6E). These data support a critical role of Gas6 in the preventive effect of statins on apoptosis and calcification.

Discussion

The present study demonstrated that statins protected HASMC from Pi-induced calcification. The clinical effect of statins on vascular calcification is controversial. Many retrospective clinical studies[6,7,9] and a prospective study[8] have shown beneficial effects, whereas recent prospective studies were unable to show such effects.[12,13] The reason is not yet clear, and the time window of statin use has been raised as an important matter. The discrepancy may also derive from the complex in vivo effects of statins. In this regard, it is important to analyze the detailed regulatory mechanism of statins in a simple model.

In Pi-induced calcification, HASMC undergo apoptosis. A causal link between apoptosis and calcification was evident from the finding that both apoptosis and calcification were inhibited by the general caspase inhibitor, ZVAD.fmk. As reported previously,[25] we confirmed that NPC-mediated Pi uptake is another essential mechanism for HASMC calcification. Given that apoptosis does not always lead to calcification, Pi-induced HASMC calcification is presumably dependent on both an NPC-mediated phenotypic transition from SMC to an osteoblastic phenotype and apoptotic cell death.

With respect to the mechanism of action of statins, they clearly inhibited Pi-induced apoptosis, although they did not have an effect on Pi-induced NPC activity or osteoblastic differentiation; Pi-induced upregulation of matrix Gla protein (MGP) mRNA was not inhibited by atorvastatin (supplemental Figure VI). These results suggest that apoptosis is the target of statins in inhibiting HASMC calcification.

Another important signal in Pi-induced calcification is an increase in intracellular Ca ($[Ca^{2+}]_i$). Statins have been shown to inhibit VSMC proliferation[5] and reduce the acute increase of $[Ca^{2+}]_i$ in a mevalonate and isoprenoid pathway–independent manner.[26] On the other hand, $[Ca^{2+}]_i$ is reported to modulate Pi-induced apoptosis of terminally differentiated chondrocytes.[27] Therefore, modulation of $[Ca^{2+}]_i$ is another possible mechanism of the inhibition of apoptosis by statins. In this study, we investigated the association of proliferation with Pi-induced apoptosis and calcification. We found that Pi did not affect proliferation, measured by the incorporation of 5-bromo-2'-deoxyuridine (BrdU) during calcification (data not shown). We also found that the inhibitory effect of statins on calcification was not affected by an inhibitor of Rho kinase (Y-27632), an important modulator of the mevalonate and isoprenoid pathway affecting proliferation and apoptosis (supplemental Figure VII). These results suggest that proliferation is not associated with Pi-induced calcification. The inhibitory effect of statins on calcification was not blocked by mevalonate, farnesylpyrophosphate, geranylgeranylpyrophosphate, or Rho kinase inhibitor, suggesting that the effect of statins is not dependent on the mevalonate and isoprenoid pathways. Indeed, a mevalonate pathway–independent effect of statins has been reported previously,[26,28–30] although the precise mechanism has not been shown. The pleiotropism of statins is of continuing interest.
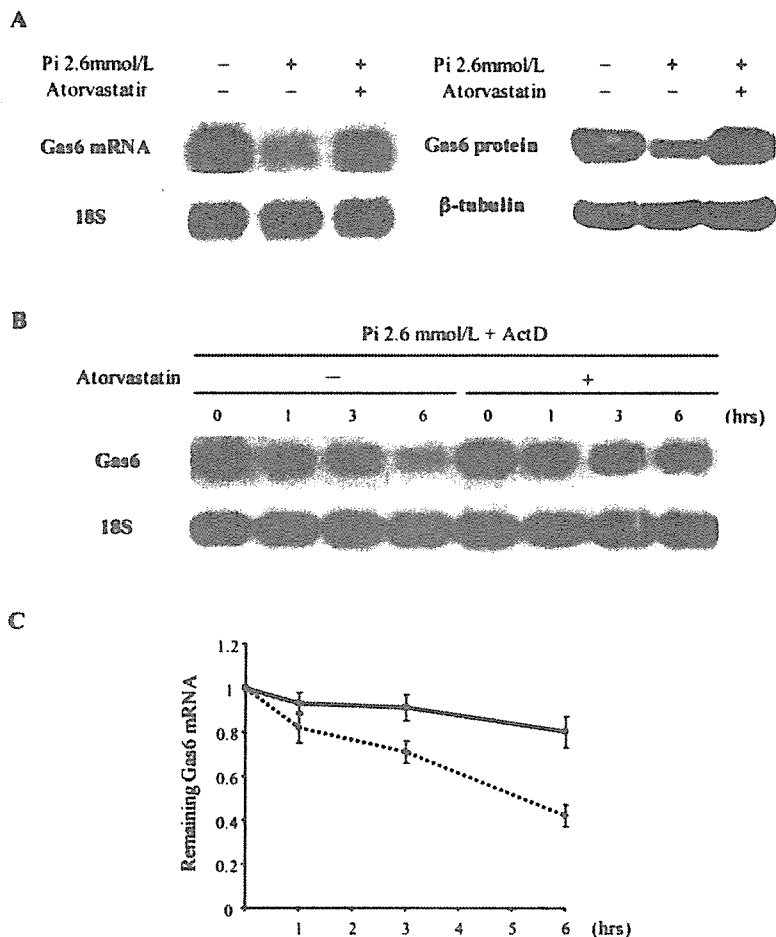
A

B

C

Figure 5. Atorvastatin enhances Gas6 mRNA stabilization, but not transcription. A, After pretreatment with atorvastatin (0.1 μmol/L) for 12 hours, apoptosis was induced by 2.6 mmol/L Pi. At 12 hours, mRNA was isolated and Northern blot analysis for Gas6 and 18S was performed. Simultaneously, cell lysates were collected and subjected to SDS-PAGE followed by immunoblotting with antibodies to Gas6 and β-tubulin. B, Serum-starved HASMC were incubated with actinomycin D (Act D) (5 μg/mL) in the presence of 2.6 mmol/L Pi after 12 hours of atorvastatin (0.1 μmol/L) treatment. Total RNA was harvested at 0, 1, 3, and 6 hours for Northern blot analysis. C, Signal density of Gas6 mRNA with (solid line) or without (dotted line) atorvastatin (0.1 μmol/L) in the presence of 2.6 mmol/L Pi and Act D (5 μg/mL) was normalized to that of 18S RNA at each time point. Gas6 mRNA level at time 0 was given the value 1. Each experiment was performed in triplicate for each condition.

An antiapoptotic effect of statins has been shown in various cell types.[31-34] In cardiomyocytes, apoptosis induced by hypoxia or protein kinase C (PKC) inhibitors was inhibited by 10 μmol/L pravastatin or 0.1 μg/mL atorvastatin, respectively.[31,32] Simvastatin (1 μmol/L) promoted endothelial cell survival.[33] In VSMC, 7-ketocholesterol–induced apoptosis was inhibited by 10 μmol/L pravastatin.[34] However, in contrast to the results of the present and other studies, a proapoptotic effect of statins has also been reported in VSMC,[35] endothelial cells,[36] and cardiac myocytes.[37] Although the precise mechanism is not understood, it can be postulated that statins have biphasic effects on cell survival (an antiapoptotic effect at low concentrations and a proapoptotic effect at high concentrations) depending on the type of cell, statins, and apoptotic stimulus. Indeed, Weis et al showed dose-dependent biphasic effects of statins on apoptotic activity in microvascular endothelial cells.[30] Consistent with these data, we found that 3 different statins displayed an antiapoptotic effect at low concentrations and a proapoptotic effect at high concentrations (>1 μmol/L for atorvastatin and fluvastatin; >100 μmol/L for pravastatin) (data not shown).

During Pi-induced apoptosis, we have shown that Pi downregulates the Gas6-Axl interaction, resulting in blockade of a survival signal, thereby promoting apoptosis and calcification. We previously proposed that Gas6 may allow Axl-expressing phagocytic cells, eg, macrophages and

VSMC, to recognize cells exposing phosphatidylserine (PS) on the outer cell membrane, the initial step of the apoptotic process.[38] Proudfoot et al also showed that in vascular calcification, several PS-exposing cells are observed within and on the periphery of the nodules.[16] PS exposure by apoptotic bodies generates a potential Ca-binding site and membrane surface suitable for hydroxyapatite deposition.[39,40] Based on these observations, Gas6-Axl downregulation is presumably involved in decreased cell survival and clearance, both directing cells to apoptosis-mediated mineral deposition.

With regard to the molecular pathway of the restoration of Gas6 by statins, we have shown that statins retarded degradation of Gas6 mRNA, not increasing the transcriptional rate. Indeed, it was reported that statins improve mRNA stability as well as transcription.[41,42] In addition, the result that suppression of the action of Gas6 by siRNA and Axl-ECD abrogated the inhibitory effect of statins on apoptosis and inhibition clearly indicates a pivotal role of Gas6 in the effect of statins.

We conclude that statins inhibit Pi-induced HASMC calcification by preventing apoptosis via restoration of the Gas6-Axl pathway. The regulation of Gas6 by statins occurs at the posttranscriptional level. The present study provides evidence of a preventive role of statins in vascular calcification and further indicates the pleiotropic effects of statins, which could potentially contribute to the treatment of cardiovascular disease.
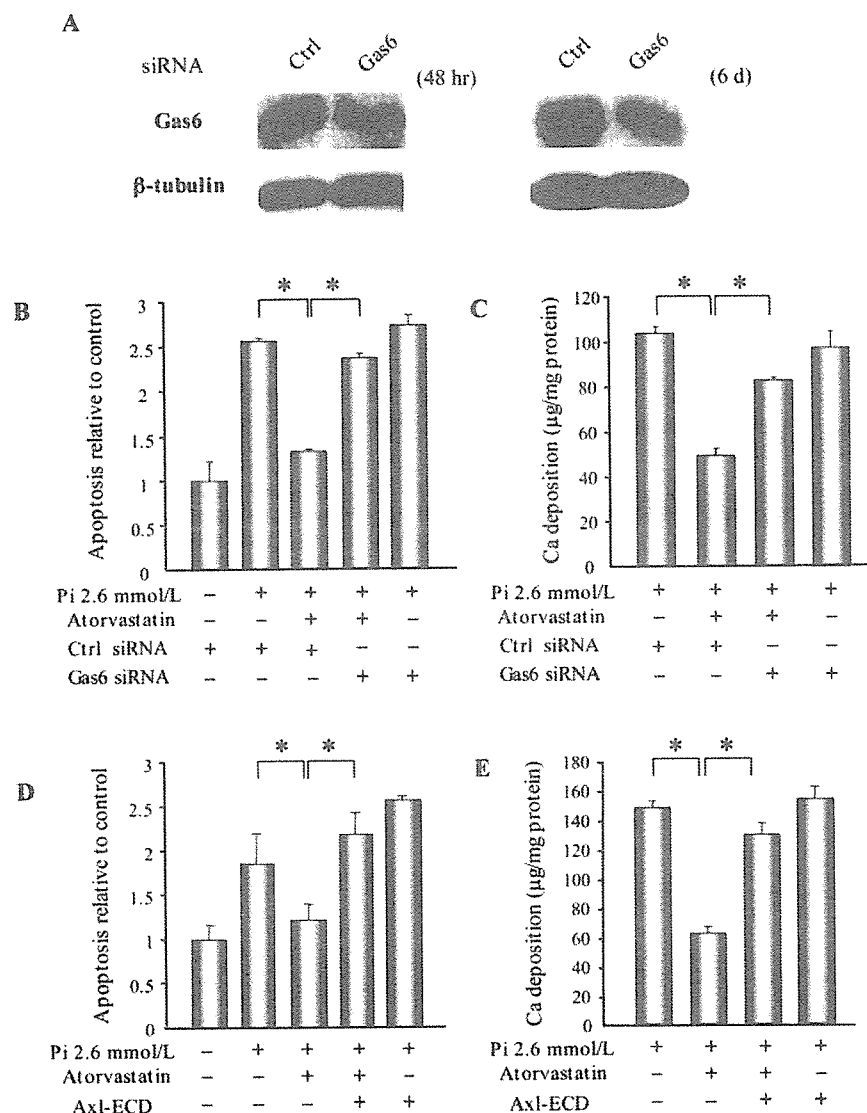
Figure 6. Gas6 knockdown abolishes inhibition of Pi-induced apoptosis and calcification by atorvastatin. A, Gas6-specific siRNA (100 nmol/L) and nonspecific siRNA (Ctrl siRNA) were transfected into HASMC, and immunoblotting was performed at 48 hours and 6 days after transfection. B, Serum-starved HASMC were transfected with 100 nmol/L Gas6 siRNA and control (Ctrl) siRNA. After transfection, cells were treated with atorvastatin (0.1 μmol/L) for 12 hours, then with 2.6 mmol/L Pi for an additional 24 hours before measurement of apoptosis (n=3). C, For measurement of Ca deposition, HASMC were transfected with 100 nmol/L Gas6 siRNA and control siRNA and incubated with atorvastatin (0.1 μmol/L) and 2.6 mmol/L Pi for 6 days (n=3). D, In the case of Axl-ECD, HASMC were pretreated with atorvastatin (0.1 μmol/L) and Axl-ECD (1 μg/mL) for 12 hours, then incubated with 2.6 mmol/L Pi for an additional 24 hours. Thereafter, a quantitative index of apoptosis was determined by ELISA (n=3). E, HASMC were cultured with atorvastatin (0.1 μmol/L) and Axl-ECD (1 μg/mL) in the presence of 2.6 mmol/L Pi for 6 days. Ca content was measured and normalized by cell protein content. All values are presented as mean±SEM (n=6). *$P<0.05$ by Fisher's test. Each panel shows a representative example of 3 independent experiments.

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ORIGINAL ARTICLE

Simple screening test for risk of falls in the elderly

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Gerontology, Tokyo, and* [7]*Department of Geriatric Medicine, Tohoku University, Sendai, Japan*

Background: The aim of this study is to construct a simple screening test for the risk of falls in community-dwelling elder persons.

Methods: A total of 1378 community-dwelling people aged 65 years and older in five different communities in Japan were asked to answer a self rated questionnaire including 22 items covering physical, cognitive, emotional and social aspects of functioning and environmental factors. At a six-month follow-up, the outcome of fall occurrence and the number of falls was ascertained by social workers, health visitors or nurses.

Results: Five out of 22 items were selected using a logistic regression model. Using this five-item version, a screening test was constructed, and at the best cut-off point, the sensitivity and specificity were 68% and 70%, respectively. The validity of this scale was tested on persons with cognitive dysfunction.

Conclusion: The simplicity and the predictive validity of the screening test support the use of this test in health check ups or general outpatient facilities.

Keywords: accidental fall, aged, mass screening, reliability and validity, risk factor.

Introduction

Falls are rated as the third leading cause of a bed-ridden state and are among the principal causes of morbidity in the elderly in Japan.[1] Previous studies evaluating the risk factors for falls have used history of falls, results of physical performance tests,[2] activity of daily living (ADL)[2,3] and balance and gait[4] as predictors.

Early identification of falls risk is likely to result in earlier implementation of interventions and to minimize development of unwanted sequels such as reduced confidence and activity levels.[5]

In Japan, the Ministry of Health, Labour and Welfare has put roughly 6000 local home care support centers around Japan. The task of these centers, according to Long-Term Care Insurance for the elderly, includes screening of the elders at risk of developing disabilities, including risk for falls. In this context, it is critical to develop a simple screening test to adequately evaluate the risk of falls for each elderly person.

The aim of this study is to evaluate predictive validity of a simple questionnaire composed of 22 items, with the intention of constructing a shortened version that would be simple, but effective to assess the future risk of falls during periodic health check-up or outpatient visits.

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All elderly persons who participated in this research gave written informed consent.

Methods

The initial 22-item questionnaire was constructed by the Working Group of Fall Prevention commissioned by the Japanese Ministry of Health, Labour and Welfare. Known risk factors are transformed into comprehensible text for the elderly, as shown in Table 1. These items were selected by studying both international and Japanese research articles on fall risk factors.[6]

The interclass coefficient (ICC) of the one month test-retest reproducibility study of the 22-item questionnaire score was satisfactory (ICC 0.74, 95% CI 0.46–0.89, $n = 21$).[6]

Individuals chosen for this study lived in five different urban and rural communities and they were over 65 years old.

In cases where subjects had cognitive impairment or difficulty answering, a family member acted as a proxy to help answer the questionnaire.

The outcome of fall occurrence and the number of falls were confirmed by social workers, health visitors or nurses six months after baseline measurement. A fall was defined as an unintentional change in position resulting in coming to rest on the ground or other lower positions.[3]

Statistical analysis was performed on subjects who completed the questionnaire both at baseline and at six month follow-up. One half of the subjects were randomly selected, and the relationship between falls and potential predictors was examined by χ^2 test for each predictor separately (developing samples). Items that achieved statistical significance of $P < 0.05$ were incorporated in the logistic regression analysis to identify predictors. Then, the questionnaire items considered to be associated with falls were selected using any falls as an outcome variable, by forward stepwise selection by the logistic regression model ($P < 0.05$).

The predictive power of the set of selected items, adjusted by the odds ratio, was determined using the area under the Receiver-Operating Characteristic (ROC) curve (AUC) on the other half of the subjects as the validating sample. Finally, the sensitivity and specificity of the model were calculated to obtain the cut-off point.

To test the validity of the scale on persons with cognitive dysfunction, different item functioning (DIF) analysis was performed on subgroups with and without cognitive dysfunction using the Rasch measurement

Table 1 The initial 22-item questionnaire constructed by the Working Group of Fall Prevention and commissioned by the Japanese Ministry of Health, Labour and Welfare

Questionnaire items	Answer (%)[†]	Incidence of fall (%)[‡]	P
Q1. History of fall within one year = yes	107 (16%)	54 (50%)	$P < 0.0001$
Q2. History of stumbling within one year = yes	288 (42%)	75 (42%)	$P < 0.0001$
Q3. Can you climb stairs without help? = no	261 (38%)	65 (25%)	$P = 0.0001$
Q4. Do you feel your walking speed declined recently? = yes	353 (51%)	76 (22%)	$P = 0.0025$
Q5. Can you cross the road within the green signal interval? = no	74 (11%)	25 (11%)	$P = 0.0019$
Q6. Can you walk 1 km continuously? = no	172 (25%)	46 (27%)	$P = 0.0011$
Q7. Can you stand on one foot for about five seconds? = no	180 (26%)	55 (31%)	$P < 0.0001$
Q8. Do you use cane when you walk? = yes	123 (18%)	43 (35%)	$P < 0.0001$
Q9. Can you squeeze the towel tightly? = no	80 (12%)	26 (33%)	$P = 0.0026$
Q10. Do you feel dizzy? = yes	151 (22%)	39 (26%)	$P = 0.0076$
Q11. Is your back bended? = yes	213 (31%)	62 (29%)	$P < 0.0001$
Q12. Do you have knee pain? = yes	264 (38%)	64 (24%)	$P = 0.0005$
Q13. Do you have a vision problem? = yes	292 (42%)	56 (19%)	$P = 0.2794$
Q14. Do you have a hearing problem? = yes	227 (33%)	48 (21%)	$P = 0.0781$
Q15. Do you think you are forgetful? = yes	332 (48%)	73 (22%)	$P = 0.0020$
Q16. Do you feel anxious to fall when you walk? = yes	226 (33%)	60 (27%)	$P = 0.0001$
Q17. Do you take more than five kinds of prescribed medicines? = yes	161 (23%)	39 (24%)	$P = 0.0231$
Q18. Do you feel dark walking within your home? = yes	54 (8%)	18 (33%)	$P = 0.0124$
Q19. Are there any obstacles within the house? = yes	87 (13%)	25 (29%)	$P = 0.0181$
Q20. Is there any level difference within your home? = yes	426 (62%)	79 (19%)	$P = 0.1799$
Q21. Do you have to use stairs in daily living? = yes	129 (19%)	23 (18%)	$P = 0.7951$
Q22. Do you walk steep slope around the house? = yes	202 (29%)	28 (14%)	$P = 0.2517$

[†]The answers as indicated in the question raw. [‡]The incidence of fall among the relevant answer.

technique.[7-9] Three hundred persons were randomly selected to obtain adequate sample size for this analysis.[10]

In addition, results of the ROC curve were stratified by the presence and absence of memory problem using Q15 of the questionnaire to test the validity of the short version on those with cognitive function problems.

Results

Of 1734 elderly, 1378 (79%) completed the questionnaire both at the baseline study and its six month follow-up. The mean age of the subjects was 75.8 (SD 6.8) years. The number of elders by five research centers was, 1050, 104, 82, 81 and 61, respectively. At least one fall had occurred in 208 elderly (15.1%) during the six month follow-up period. Of these, 103 (50%) suffered from multiple falls, ranging in number from 2 to 20.

Of eligible samples, 1026 elders provided information regarding mobility, cognitive status and ADL regarding eating and toileting. In mobility, no disability was seen in 69.8% of them, while mild difficulty in climbing stairs was present in 18.1%, and moderate or severe difficulty required cane or wheel chair for moving around outside in 12.1%.

In cognitive status, no memory disturbance was seen in 62.8%, while mild and severe memory dysfunctions were in 26.0% and 8.0%, respectively.

Regarding eating ADL, 93.4% showed no problem, while 4.6% complained they had a mild problem, and 2.0% required assistance. Toileting related ADL was intact in 89.0% of the elders while mild difficulty and dependent status on toileting were seen in 6.0% and 5.0%, respectively. Although 8.3% of them were living alone, 23.0% were with their spouse, and the rest were with their children.

The samples were then divided into the developing samples ($n = 689$) and validating samples ($n = 689$). There was no statistical significance between these two samples, in distribution of living areas, gender and response pattern to the questionnaire items examined by χ^2 test (data not shown). The average age of the validating samples (75.8) was not significantly different from developing samples (75.7) by t-test.

Table 1 shows the predictors in relation to falls in developing samples. The incidence of at least a single

fall and multiple falls were 108 (15.7%) and 55 (8.0%), respectively. Gender did not achieve the statistical significance to single fall ($P = 0.05$) and multiple falls ($P = 0.15$), respectively. Fallers were elder than non-fallers ($P < 0.01$) with average age of 79.1 versus 75.8, respectively.

Questionnaire items, except for Q13, Q14, Q20, Q21 and Q22, achieved statistical significance and were entered into the regression model. Table 2 shows the item selected by the stepwise logistic regression model.

Using the odds ratio at integer level as the weight of these five items, we constructed a screening test whose score ranged from 0 to 14, and the AUC was 74% (95% CI 69–79%) in the validating samples, as shown in Figure 1. This was at the same level as the AUC of initial 22 items score (72%:95% CI 67–79%)

The maximum sum of sensitivity and specificity reached <6 (sensitivity 0.68, specificity 0.70) and <7 (sensitivity 0.67, specificity 0.71). If a cut-off score of <6 was applied, subjects identified as positive had a 27.9% rate of falls (positive predictive value) compared with a
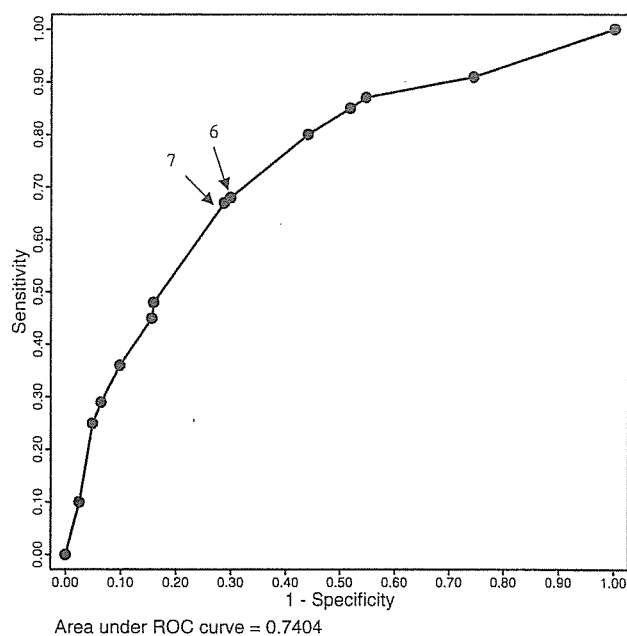

Area under ROC curve = 0.7404

Figure 1 The Receiver-Operating Characteristic (ROC) of the five-item screening test to detect elderly persons at risk of falling.

Table 2 Questionnaire items selected by the stepwise logistic regression model

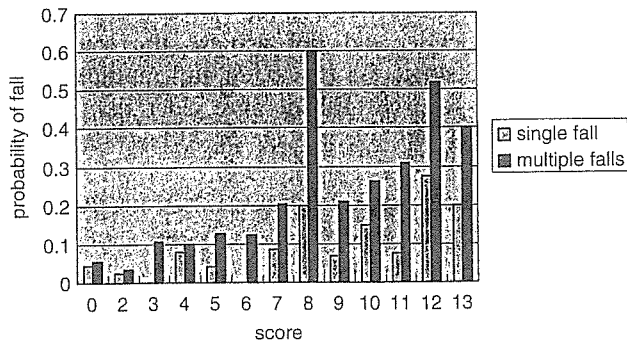| Questionnaire item selected by step wise logistic regression model	Odds ratio	95%CI	P
Q1. History of fall within one year = yes	4.5	(2.8–7.2)	0.00
Q4. Do you feel your walking speed declined recently? = yes	1.9	(1.0–3.6)	0.04
Q8. Do you use cane when you walk? = yes	1.8	(1.1–2.8)	0.02
Q11. Is your back bended? = yes	1.8	(1.1–2.8)	0.02
Q17. Do you take more than five kinds of prescribed medicines? = yes	1.7	(1.0–2.7)	0.03

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Figure 2 The probability of single and multiple falls by score.

7.2% rate in negative individuals (negative predictive power: 93%), with an odds ratio of 3.88 (95% CI 3.16–4.75).

The sensitivity and specificity was 0.63 and 0.67, respectively, for multiple falls. The positive and negative predictive value at this cut off score for multiple falls was 0.12 and 0.96, respectively, with the odds ratio of 3.04. Figure 2 illustrates the probability of fall by score levels.

On Rasch analysis of each item, some items did not fit the Rasch Model (Q16, Q20, Q21 and Q22) and these items were deleted for subsequent DIF analysis. Then no item showed DIF on cognitive functioning after Bonferroni adjustment (data not shown). After stratifying the sample with Q15, the area under ROC curve was 0.74 (95% CI 0.66–0.82) and 0.74 (0.69–0.78) for with and without cognitive dysfunction, respectively.

Discussion

Falls are considered as having multiple risk factors.[11] Previous epidemiological studies have identified the risk for falls, for example, history of falls,[2,3,12–15] activity of daily living (ADL),[2,3,15] cognitive and sensory function,[2,3,12,15] chronic conditions,[12,16,17] and medication use.[3,16–19]

Many studies tried to convert these risk factors for fall risk screening.[3,4,20] These screening tools for elders have been developed for various care settings, including residential,[14,21] intermediate[22] and inpatient care[23–25] as well as for community.[26–28]

Initially, the authors selected a comprehensive questionnaire composed of 22 items that can be answered by yes or no, and then selected several items that can be applied for mass screening or in general practice settings[6] because of the requirement of Japanese long-term care insurance (LTCI) law.

The items selected by the logistic model in this study were history of falls, walking speed, cane use, back deformation and medication use. All of these items were in concordance with the previous reports.

We also included environmental factors as part of the questionnaire. On comparison between fallers and non-

fallers, environmental barriers such as level difference, stair and slope were not identified as risk factors, indicating the barrier recognized by the elders may not be associated with falls. All other items, except for vision problems were associated with incidence of falls.

The use of large prospective validating samples adds strength to this study. In most similar studies, the predictive validity is tested only on the developmental sample of the tools, and thus the predictive performance in a new sample is expected to be optimistic.[29] Although the predictive power on the development sample is usually high, the predictive power is usually lower in the validating samples.[30] In addition, the sensitivity of the scale is lower in the validating sample[31] and only a few studies use a large scale validating sample as was used in this study.[26]

Finally, the AUC of the initial 22 items were at the same level of the shortened five-item version. Therefore, the shortened version is preferred for its simplicity. In addition, the five-item scale was validated on the elderly with and without problems of cognitive function.

In the process of item selection using the logistic regression, inclusion criteria were $P < 0.05$, and exclusion criteria were $P > 0.10$. This procedure resulted in inclusion of items with weak association, such as Q4 and Q17. However, the adequacy of including these two items was proved on the validating sample.

In validating samples, the negative predictive value was 0.92 for single falls and 0.96 for multiple falls indicating that those with negative result have very low risk of falling in the next six months. This property of the high negative predictive validity makes the use of the screening test useful in mass screening.

History of fall was one of the most frequently reported risk factor of falls.[32,33] Decline of walking speed was captured with other questionnaire studies, as well as by physiological measurement.[6,18,34] Cane users and kolioskiphosis might have relation to bone abnormalities such as osteoporosis or arthritis.[13] These Q4, Q8 and Q11 compose a spectrum of physiologic decline referred to as frailty.[35,36] The relationship between medication use and falls can be explained by the effects of a drug itself that might cause sensory and balance disturbance, and also decreased metabolism, which relates to the loss of physiologic and metabolic function. Medication review is a possible intervention to prevent falls.[37]

In conclusion, a simple screening tool for falls is constructed using a large scale developing and validating sample. The scale constructed in this study is simple and valid. Therefore, it can be used as a screening tool of falls for community-dwelling elders.

Acknowledgment

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CASE REPORT

Elderly patient presenting with severe thyrotoxic hypercalcemia

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An 81-year-old woman with Graves' disease and osteoporosis was referred to the hospital because of anorexia over one month and impaired consciousness. She also presented with low-grade fever and emaciation. Laboratory tests revealed marked hypercalcemia (corrected serum calcium level of 12.4 mg/dL), which was initially suspected to result from vitamin D toxicity, because she had been taking vitamin D3 (alphacalcidol of 0.5 µg/day) for the treatment of osteoporosis. However, discontinuation of vitamin D3 and fluid infusion did not ameliorate hypercalcemia one week later. After excluding hyperparathyroidism and malignancy-related hypercalcemia, hypercalcemia was considered to be attributable to the exacerbation of hyperthyroidism (free T4 of 6.69 ng/dL, free T3 of 13.27 pg/mL and thyroid stimulating hormone (TSH) <0.015 µIU/mL) with increased bone resorption. Finally, the increased dose of thiamazole (30 mg/day) normalized serum calcium level and thyroid function three months later. Laboratory tests suggested that normal bone formation in spite of increased bone resorption contributed to hypercalcemia in hyperthyroid state.

Keywords: deoxypyridinoline, hypercalcemia, hyperthyroidism, osteoporosis, p-N-telopeptides of collagen cross-links.

Introduction

Hypercalcemia has been associated in approximately 20% of the patients with hyperthyroidism, but is mild in most cases, ranging from the upper normal limit to the slightly elevated level.[1-3] Consequently, we rarely see hyperthyroidism with symptomatic hypercalcemia. Many genotypes have been associated with Graves' disease.[4] Also, a small number of studies have shown that polymorphisms in calcium-regulating genes such as calcium-sensing receptor[5] and vitamin D receptor[6] may influence calcium metabolism in adults. However, no study has reported the association of those polymorphisms with thyrotoxic hypercalcemia. More studies as well as more polymorphisms including haplotype

analysis should be performed to clarify the underlying mechanism.

Here, we report an elderly patient presenting with severe symptomatic hypercalcemia resulting from hyperthyroidism.

Case report

An 81-year-old woman was admitted to the Department of Geriatric Medicine, Kyorin University Hospital because of hypercalcemia on February 14 2004. She had Basedow's disease and osteoporosis, and had been taking thiamazole 5 mg/day and alphacalcidol 0.5 µg/day. In January 2004, anorexia had gradually developed followed by gait disturbance. When she was referred to the hospital on February 14, she also presented with confusion and low-grade fever of 37.2°C. Her blood pressure was 122/62 mmHg with a pulse rate of 98 bpm. Physical examination showed a soft diffuse goiter and a systolic ejection murmur of Levine II/VI at the apex, while abdominal and neurological findings were normal.

Table 1 Laboratory tests on admission

Test	Result
Hb	10.5 g/dL
Ht	32.6%
RBC	$367 \times 10^4/\mu L$
PLT	$22.2 \times 10^4/\mu L$
WBC	3200/μL
Na	144 mEq/L
K	3.1 mEq/L
Cl	100 mEq/L
Ca	11.7 mg/dL
IP	3.4 mg/dL
BUN	19.3 mg/dL
Cr	0.7 mg/dL
TP	6.4 g/dL
Alb	3.3 g/dL
ALP	226 IU/L
AST	37 IU/L
ALT	35 IU/L
LDH	333 U/L
CK	25 IU/L
Glu	126 mg/dL
CRP	0.2 mg/dL

1 Alb, ...; ALP, ...; ALT, ...; AST, ...; BUN, ...; CK, ...; CRP, ...; LDH, ...; PLT, ...; RBC, ...; TP, ...; WBC, ...

Table 2 Results of thyroid function test

Test	Result (normal range)
FreeT4	6.69 ng/dL (0.73–1.53)
FreeT3	13.27 pg/mL (1.63–3.20)
Thyroid stimulating hormone (THS)	0.015 IU/mL (0.41–5.27)
TSH receptor antibody	51.2% (15<)
TSAb (thyroid stimulatory antibody)	540% (180<)
Antithyroid peroxydase antibody	43.8 U/mL (0.3<)
Serum thyroglobulin autoantibodies	0.3 < U/mL (0.3<)

On laboratory tests (Table 1), she showed blood hemoglobin of 10.5 g/dL, white blood cell counts of 3200/μL and serum calcium of 11.7 mg/dL (corrected calcium of 12.4 mg/dL). Other electrolytes as well as liver and kidney function were normal. Thyroid function tests (Table 2) revealed marked hyperthyroidism; free T4 of 6.69 (reference, 0.90–1.70) ng/dL, free T3 of 13.27 (2.3–4.3) pg/mL and thyroid stimulating hormone (TSH) of <0.01S (0.5–5.0) μIU/mL. Plasma levels of TSH receptor antibody, thyroid stimulating antibody and anti-TPO antibody were elevated, compatible with the findings in Graves' disease. Plasma intact PTH was

Table 3 Results of markers of bone metabolism

Marker	Result (normal range)
Osteocalcin	9.5 ng/mL (2.5–13)
Bone-specific alkaline phosphatase	24.2 U/L (9.6–35.4)
p-N-telopeptides	43.3 nMBCE/L (10.7–24.0)
Deoxypyridinoline/Cr	43.8 nmol/L/nMCr (2.8–7.6)
calcitonin	33 pg/mL
1-25(OH)VitD₃	6 pg/mL (20–60)
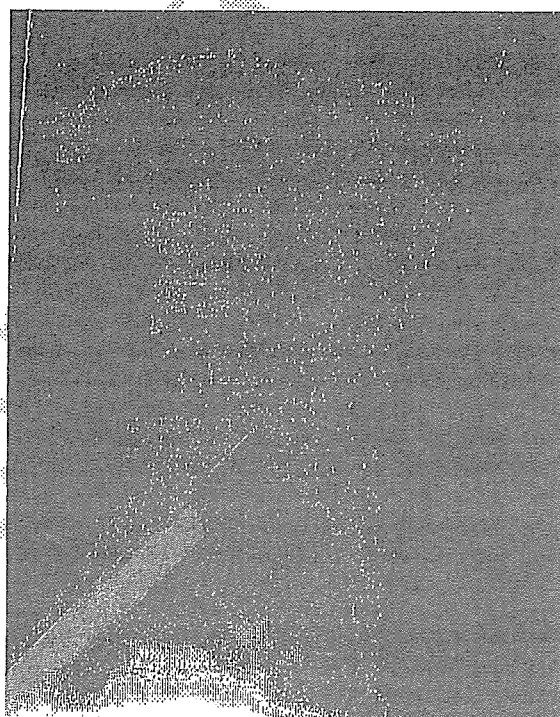
Figure 1 X-ray of lumbar vertebrae.

13 (10–65) pg/mL and PTH-related protein was not detected.

As shown in Table 3, markers of bone resorption such as deoxypyridinoline (DPD) and N-telopeptides of collagen cross-links (NTx) were elevated, whereas those of bone formation such as osteocalcin and bone-type alkaline phosphatase were not. Bone mineral density of lumbar vertebrae was −3.29 (T score), and that of femur was −3.72 (T score). Multiple compression fractures and remarkable reduction in bone mineral density were found on spinal lateral X-rays and dual energy X-ray absorptiometry, respectively (Fig. 1).

Initially, vitamin D toxicity was suspected as a cause of hypercalcemia; thus, alphacalcidol was ceased with fluid infusion to wash out calcium. However, the
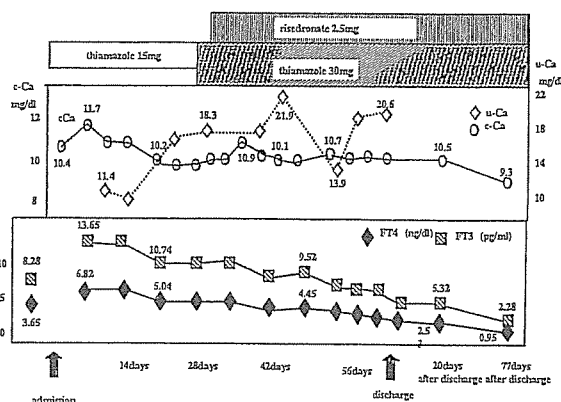
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Figure 2 Clinical course of the patient. Thyroid stimulating hormone (TSH) was below the detection limit throughout the clinical course. c-Ca, collected serum calcium; u-Ca, urinary calcium; FT4, free thyroxine; FT3, free triiodothyronine.

hypercalcemia had not improved one week later. Laboratory and imaging tests were carried out to exclude hyperparathyroidism, humoral hypercalcemia of malignancy, osteolytic bone metastases and multiple myeloma. Finally, hypercalcemia was considered to be attributable to the exacerbation of hyperthyroidism with high bone turnover. Consequently, the dose of thiamazole was increased to 30 mg/day to normalize thyroid function. As shown in Figure 2, free T4 and free T3, as well as serum calcium were gradually decreased, and the patient was discharged on May 14 2004. In August 2004, her thyroid function returned to normal (free T4 of 0.95 ng/dL and free T3 of 2.28 pg/ mL) with corrected serum calcium concentration of 9.2 mg/dL.

Discussion

Hypercalcemia associated with hyperthyroidism has been reported to occur more frequently in elderly patients than in younger patients; the incidence of hypercalcemia was 2.3% in hyperthyroid patients under 60 years of age and was 18.8% in those over 60 years of age.[2] The severity of hypercalcemia, however, is generally mild, ranging from the upper normal limit to the slightly elevated level,[3] and other complications should be suspected when serum calcium concentration is over 12 mg/dL[7] Actually, case reports have shown that hyperparathyroidism is uncommonly associated with hypercalcemia in thyrotoxicosis.[8] Only several cases have been reported that hyperthyroidism was considered the only cause of hypercalcemia over 12.0 mg/ dL.[9-11] In our case, laboratory tests and diagnostic imaging excluded hyperparathyroidism as well as malignant neoplasms. Furthermore, hypercalcemia was ameliorated in parallel with the improvement of hyperthy-

roidism, indicating that hypercalcemia resulted from hyperthyroidism.

Thyroid hormones play a critical role in bone development because hypothyroidism in childhood results in the impaired skeletal development.[12] In adults, thyroid hormones are important in the maintenance of bone mass. Thyroid hormone receptors are expressed in bone cells such as osteoblasts and osteoclasts.[12] In adult hyperthyroidism, there is increased bone remodelling, characterized by an increase in both bone resorption and formation, and an imbalance between bone resorption and formation, which results in bone loss and an increased risk for osteoporotic fracture.[12] In our case, however, the markers of bone resorption were elevated but those of bone formation were not. This pattern is consistent with the changes of bone metabolism in older osteoporotic patients,[13] but is different from that in hyperthyroidism as mentioned above. This might be due to the age-related decline in thyroid hormone signaling that leads to bone formation. However, no reports including animal experiments to support this hypothesis can be found so far. This should be investigated in the future.

Anti-thyroid drugs restore not only serum calcium levels[14] but also bone mineral density[15] in patients with thyrotoxic hypercalcemia. It has been also reported that a β blocker, propranolol,[16,17] and radioiodine therapy[10] may ameliorate thyrotoxic hypercalcemia. In our case, an increased dose of thiamazole normalized both thyroid function and serum calcium levels several months later, but bone mineral density was not increased. Longer time periods would be necessary to see the recovery of bone mass if possible.

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〈原　著〉

超高齢者におけるクレアチニンクリアランス推定式の比較検討

平山　俊一[1]　　菊池　令子[2]　　井上慎一郎[2]　　塚原　大輔[2]　　末光　有美[2]
小林　義雄[2]　　杉山　陽一[2]　　長谷川　浩[2]　　神崎　恒一[2]　　井上　剛輔[3]
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　要　約　目的：高齢患者は外来では 24 時間クレアチニンクリアランスの測定が困難であり，服用薬物数も多いため，クレアチニンクリアランス実測値をできるだけ正確に反映する推定式を利用することは臨床上重要である．対象：各種基礎疾患を有する 85 歳以上の超高齢者 67 名を含む入院高齢者 143 名（男性 73 名　女性 70 名　平均年齢 82.9±8.6 歳）．方法：4 種のクレアチニンクリアランス推定式から得られた推定値と 24 時間クレアチニンクリアランスの実測値との相関を比較検討した．結果と結論：全体として今回の検討では超高齢者においても Cockcroft and Gault の式による推定値が最もよい相関を示した．85 歳以上の女性超高齢者において実測値と推定式の相関が低く，推定式の改定についても今後の検討課題と思われる．
　Key words：超高齢者，クレアチニンクリアランス，推定式，Cockcroft and Gault の式，安田の式

緒　　言

　高齢社会の到来により，外来入院を問わず，高齢患者が増加の一途をたどっている．厚生労働省の推計によると，2004 年度において 85 歳以上の超高齢者は 273.4 万人と報告されている[1]．高齢者に腎排泄型薬剤を投与する際，適正な用量を設定するため腎機能を正確に評価する必要がある．腎機能を表す指標として，糸球体濾過量には一般的に内因性クレアチニンクリアランス（以下 Ccr と略す）が使われている．クリアランス試験には 24 時間蓄尿が必要であるが，時間を要することや被験者に排尿，蓄尿という負担があり繁雑であることから外来で測定することは容易ではない．このため血清クレアチニン値（以下 Scr と略す）から Ccr を推定するいくつかの数式が提案されている．しかしこれらの数式は実際に投薬の必要な諸疾患を有する高齢者に当てはめる際，筋肉量の減少などのため Scr による Ccr 推定値と実測した Ccr がかけ離れた値を取ることがある．外来の超高齢患者においても適切な薬物療法を行うためには腎機能

を正確に評価する必要がある．このため種々の推定式による相関を調べどの推定式が最もよく超高齢者に適合するか検討を行った．

対象及び方法

　杏林大学病院高齢医学科に 2004 年 9 月から 2006 年 1 月の間に入院した 60 歳以上の症例のうち，短期入院や，蓄尿不可能症例を除外し，尿道留置カテーテルを使用している患者や蓄尿が可能と判断された症例全例を対象にした．疾患や治療による除外は設けず，脳血管障害，感染症，経口摂取不良，利尿剤，補液などの様々な基礎疾患，治療を有する高齢者（平均年齢 82.9±8.6 歳（男性 82.0±8.8 歳　女性 83.8±8.3 歳））例を対象に行った．男女比及び 84 歳以下と 85 歳以上の症例数に偏りはなかった（表 1）．対象高齢者全体の平均 Scr は 1.31±0.87mg/dl であった．身体測定，血液検査，尿検査などを測定し 24 時間蓄尿による Ccr を計算した．なお，Ccr は未補正のものを使用した．安田の式[2]，Cockcroft and Gault の式[3]（以下 C&G 式と略す），折田の式[4]，Walser の式[5]の推定値を算出し，それぞれ推定値と実測値の相関を回帰分析，相関係数の差の検定により解析し比較検討した．さらに，層別解析として，84 歳までの前期及び後期高齢者群 76 名と，85 歳以上の超高齢者 67 名について男女別に層別解析を行った．
　また実測値と推定式からの値との一致を箱ヒゲ図で求

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表1　対象年齢分布

Age（歳）	n		
	男性	女性	全体
〜84	42	34	76
85〜	31	36	67
全体	73	70	143

め，値が外れ値となった症例については，患者の疾患や治療の背景，測定時の問題点について調査した．

本研究は，杏林大学高齢医学の入院に際して，CCr測定値を臨床研究に使用することを口頭で説明し同意を得て試行した．

(1) 安田の式

男性：Ccr (mℓ/min) = (176－年齢)×体重 (kg) ÷ (100×Scr (mg/100mℓ))

女性：Ccr (mℓ/min) = (158－年齢)×体重 (kg) ÷ (100×Scr (mg/100mℓ))

(2) Cockcroft and Gault の式

男性：Ccr (mℓ/min) = (140－年齢)×体重 (kg) ÷ (72×Scr (mg/100mℓ))

女性：Ccr (mℓ/min) = {(140－年齢)×体重 (kg) ÷ (72×Scr (mg/100mℓ))}×0.85

(3) 折田の式

男性：Ccr (mℓ/min) = (－0.065×年齢－0.493× BMI＋33) ÷ (体重 (kg)×Scr (mg/100mℓ))×14.4

女性：Ccr (mℓ/min) = (－0.052×年齢－0.202× BMI＋21) ÷ (体重 (kg)×Scr (mg/100mℓ))×14.4

(4) Walser の式

男性：Ccr (mℓ/min) = 7.57÷Scr (mM) －0.103×年齢＋0.096×体重 (kg) －6.66

女性：Ccr (mℓ/min) = 6.06÷Scr (mM) －0.08×年齢＋0.08×体重 (kg) －4.81

成　　績

85歳未満の前期及び後期高齢者群において，安田，C&G，折田，Walser の推定値と24時間蓄尿による実測値の相関係数 (r) は安田 r＝0.761，C&G　r＝0.761，折田 r＝0.693，Walser　r＝0.553と安田の式，C&G 式で強い傾向があった．超高齢者群において，各々の推定式による推定値と実測値の相関係数は安田 r＝0.718，C&G r＝0.739，折田 r＝0.697，Walser r＝0.645と，安田の式，C&G 式で相関が強い傾向があった（図1，図2）．超高齢者を男女に分け両群で各々の推定値と実測値の相関係数 r を比較したところ，男性で安田 r＝0.840，C&G r＝0.841，折田 r＝0.791，Walser　r＝0.736，女性で安田

r＝0.678，C&G　r＝0.690，折田 r＝0.667，Walser　r＝0.582となり，男性に強い相関傾向があり，女性の相関係数は低かった（図3，図4）．また，超高齢者群において回帰係数を比較したところ，男性で安田＝0.796，C&G＝0.988，折田＝0.577，Walser＝0.375　女性で安田＝1.088，C&G＝1.262，折田＝0.776，Walser＝0.395となった．

図5は超高齢者を男女で比較したものである．縦軸は実測値と推定値のずれの割合を示したもの（（実測値－推定値）×100/実測値）である．折田，Walser の式では，男女共に推定値が高く評価される傾向がある．

85歳以上の超高齢者での箱ひげ図における外れ値を検討し，実測値が高値となる6例の患者背景を調べた．輸液4例，利尿剤や Ca 拮抗薬など腎血流量を増加させる薬剤4例，腎不全2例，Scr 高値2例，心不全2例，CRP 高値2例であった．また，推定値が高値となる7例の患者背景を調べた．輸液5例，蓄尿不全または蓄尿少量4例，腎不全4例，癌3例，コントロール不良の糖尿病1例，胸水貯留，腹水貯留1例，肥満1例であった．

考　　察

服用薬物数が多いほど薬剤有害作用の発現率は増加する傾向にある．また，加齢によってもその傾向は増加する[6]．その原因には加齢に伴う薬物動態学的・薬力学的な変化，多剤併用による相互作用，日常生活活動度（ADL）・認知機能の低下などが考えられるが，特に重大な原因として，腎機能の低下による相対的過量投与が挙げられる．Scr による腎機能の推定にはいくつか方法があるが高齢者，特に超高齢者になると筋肉量の低下により Scr が腎機能の低下と不相応な低値を示すことがしばしば見られる．Ccr 測定上の更なる問題点として正確な蓄尿の可否がある．加齢に伴う残尿，失禁の増加や患者自身による蓄尿もれなどにより，正確な24時間蓄尿が困難なことがある．1日尿量が少ないとき，Ccr 実測値と推定値のばらつきが大きいとの報告もある．今回は尿道留置カテーテルを使用している患者や蓄尿が可能と判断された患者の症例を対象とし，努めて正確な採尿を試みた．しかしながら，本来行うべきクリアランス法の実施には正確な蓄尿と安静を要し，判定に時間がかかるため実際の外来診療では実施困難なことが多い．従って Scr より Ccr を推定する種々の方法が提案されてきた．今回検討した安田の式，Cockcroft and Gault の式，折田の式，Walser の式は代表的な推定式であり Scr 値，性別，年齢，体重より Ccr を推定できる．C&G 式は欧米で最も広く用いられており欧米人によい相関を示して